

**UNIVERSITAT POLITÈCNICA DE VALÈNCIA**

**DEPARTAMENTO DE QUÍMICA**

Instituto Interuniversitario de Reconocimiento Molecular y Desarrollo  
Tecnológico



**Synthesis and evaluation of antigenic determinants for  $\beta$ -lactam  
allergy diagnosis**

**Ph.D. DISSERTATION**

Submitted by

**Edurne Peña Mendizabal**

Ph.D. Supervisors:

Ángel Maquieira Catalá  
Sergi Morais Ezquerro

Valencia, April 2022





UNIVERSITAT  
POLITÈCNICA  
DE VALÈNCIA



Instituto Interuniversitario de Reconocimiento  
Molecular y Desarrollo Tecnológico

D. Ángel Maquieira Catalá, Full Professor in Chemistry at the Universitat Politècnica de València.

D. Sergi Morais Ezquerro, Associate Professor in Chemistry at the Universitat Politècnica de València.

DECLARE:

That the dissertation entitled “*Synthesis and evaluation of antigenic determinants for  $\beta$ -lactam allergy diagnosis*” has been carried out by Edurne Peña Mendizabal under our supervision in the Instituto de Reconocimiento Molecular y Desarrollo Tecnológico at the Universitat Politècnica de València.

In recognition whereof, we sign the present declaration in Valencia, April 2022

Dr. Ángel Maquieira Catalá

Dr. Sergi Morais Ezquerro





**STUART L. SCHREIBER, PH.D.**

MORRIS LOEB PROFESSOR, HARVARD UNIVERSITY  
DEPARTMENT OF CHEMISTRY AND CHEMICAL BIOLOGY  
CO-FOUNDER, BROAD INSTITUTE OF HARVARD AND MIT  
CHEMICAL BIOLOGY AND THERAPEUTICS SCIENCE PROGRAM

January 20, 2021

**Re: Edurne Peña Mendizábal Certificate of Stay**

To Whom It May Concern,

This letter is to verify that Edurne Peña Mendizábal was a member of my lab at the Broad Institute of MIT and Harvard from May 7, 2018 through August 6, 2018.

If you require any additional information regarding Edurne, please feel free to contact me at 716-714-7845.

Sincerely,

Stuart L. Schreiber, Ph.D.





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MORRIS LOEB PROFESSOR, HARVARD UNIVERSITY  
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This letter is to verify that Edurne Peña Mendizábal was a member of my lab at the Broad Institute of MIT and Harvard from May 6, 2019 through November 4, 2019.

If you require any additional information regarding Edurne, please feel free to contact me at 716-714-7845.

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Stuart L. Schreiber, Ph.D.





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## ABSTRACT

About 10 % of all adverse drug reactions are due to allergies, with  $\beta$ -lactam antibiotics causing the majority of the episodes. Although the actual incidence remains unknown, individuals suspected of being allergic to a drug end up being prescribed with other medications that are less effective, more expensive or harmful. Consequently, a correct diagnosis is key to reduce the derived economic costs and proceed to an adequate ‘delabeling’ of the population. At present, clinical approaches to diagnose allergies to  $\beta$ -lactam antibiotics are based on *in vivo* and *in vitro* tests. These tests present limited clinical performances since they are invasive, dangerous, and provide false positives and/or negatives. Moreover, the diagnostic sensitivity is far from what is expected, possibly because the epitopes that cause the allergic episodes are still not well detected. In this respect, the preparation of antigens has commonly been determined by the direct attachment of antibiotics to carrier molecules through the formation of an amide bond between amino lysine groups of the carrier molecule and the carboxylate group of the antibiotic. Even so, specific IgE are barely detected with such antigens.

This dissertation addresses the synthesis of haptens and the generation of antigens to  $\beta$ -lactam antibiotics, which develop a more reliable *in vitro* diagnosis of allergies to these drugs. The evaluation of the antigens has been carried out by means of multiplex *in vitro* tests based on compact disc technology.

This research begins by focusing on the synthesis and preparation of penicillin antigens. To this end, first, the effect of the incorporation of aliphatic spacer arms in the chemical structure of penicillin has been approached, considering the possibility that a better molecular recognition is obtained by moving the hapten away from the carrier protein. Thirteen haptens derived from benzylpenicillin and amoxicillin were synthesized in order to prepare antigens with human serum albumin. The evaluation of the antigens revealed that even though they were immunogenic and were detected by the raised IgG antibodies, they were not detected by specific IgE from allergic patients. Additionally, the next approach considered the cationization of the carrier proteins, human serum albumin and histone. The modification of carboxylate groups of the protein to amino groups allow for an increase of the molar hapten/protein ratio. This strategy led to the

generation of five antigens, four of which (only those histone-based antigens), did increase the sensitivity of the assay. Concretely, specific IgE has been determined in sera from allergic patients at low concentrations (LOD = 0.07 IU/mL) with a diagnostic specificity of 100 % and a sensitivity of 60 and 31 % for benzylpenicillin and amoxicillin, respectively. That means a 60 % improvement in the diagnostic sensitivity when compared to the *in vitro* reference test.

Subsequently, the idea of preparing minor antigens based on penicillin metabolites was approached. Penicilloic, penilloic, penicillic, and 6-aminopenicillanic acids, together with penicillamine, were therefore conjugated to the carrier proteins human serum albumin and histone. Except penilloic acid, the rest of antigens were selectively detected when testing a set of serum samples from allergic patients. The diagnostic specificity obtained was 100 %, 94 % in the case of penicillic acid, and the sensitivity was between 67 and 100 %.

Another approach was focused on the production of antigens for other families of  $\beta$ -lactam antibiotics. The generation of antigens for cephalosporins, carbapenems, monobactams or  $\beta$ -lactam inhibitors is essential, since *in vitro* tests for the detection of allergies to these antibiotics are not commercially available. Therefore, the results obtained after the preparation of major and minor antigens for the antibiotics cefuroxime, cefotaxime, ceftriaxone, meropenem, and aztreonam were evaluated. While major antigens did not detect samples from allergic patients, minor antigens did. The application of these minor antigens, prepared after acidification of their corresponding salts, improved the analytical performances of the developed microimmunoassay. Thus, high levels of analytical sensitivity (LOD < 0.01 IU/mL) and diagnostic specificity (100 %) were obtained. Furthermore, diagnostic sensitivity values of 35, 49, and 51 % were obtained for the antibiotics cefuroxime, cefotaxime, and meropenem.

Afterwards, the research was oriented to the preparation of clavulanic acid antigens since its combination with amoxicillin, commonly known as Augmentin, is today the most prescribed drug. As far as is known, there is no *in vitro* assay for the determination of specific IgE to clavulanic acid, which highlights the importance of addressing the synthesis of haptens. In this case, three clavulanic acid-derived haptens were synthesized and conjugated to human serum albumin, histone, and keyhole limpet hemocyanin. The results obtained revealed the importance of the chemical structure on the immunological response since two of these haptens generated IgG-type antibodies

with high selectivity to clavulanic acid. Specifically, the selectivity was three times higher (the cross-reactivity decreases from 30 to 10 %) than for other families of  $\beta$ -lactam antibiotics. Their evaluation with human sera revealed that only the antigen conjugated to human serum albumin and prepared after the esterification of potassium clavulanate was able to detect specific IgE in allergic patients. Therefore, the diagnostic sensitivity of the assay was 50 % and the diagnostic specificity was 100 %.

Despite the improvements achieved in the diagnostic performances with the former strategies, other non-classical pathways for the synthesis of novel haptens with greater chemical diversity were studied. This approach focused on the generation of antigens in chemical libraries of compounds, with structural diversity, to find new biologically active haptens. This strategy, to the best of our knowledge, has not been employed for synthesis of  $\beta$ -lactam derived haptens for drug allergy diagnosis. In order to incorporate structural diversity, 22 compounds of the precursors of penicillins and cephalosporins, 6-aminopenicillanic acid and 7-amino-deacetoxycephalosporanic acid, respectively, and the antibiotics amoxicillin and ampicillin were synthesized by diversity-oriented synthesis. Their evaluation with the *in vitro* immunoassay showed that the incorporation of diversity allows for the recognition of epitopes causing allergic episodes. Specially, it was observed that these antigens were capable of detecting specific IgG and IgE antibodies from sera of immunized rabbits and samples from allergic patients, with amoxicillin and ampicillin-based antigens being especially selective. In particular, excellent diagnostic sensitivity (79 %) and specificity (100 %) were achieved.

## RESUMEN

Alrededor del 10 % de las reacciones adversas a medicamentos son debidas a alergias, siendo los antibióticos  $\beta$ -lactámicos los que más episodios alérgicos ocasionan. Aunque la incidencia real sigue siendo desconocida, los individuos sospechosos de presentar alergia a algún medicamento acaban siendo prescritos con otros medicamentos, menos efectivos, más caros o perjudiciales. Así pues, un correcto diagnóstico resulta clave para disminuir los costes económicos derivados y proceder a un adecuado ‘desetiquetado’ de la población. En la actualidad, las pruebas de diagnóstico de alergias a antibióticos  $\beta$ -lactámicos se basan en ensayos *in vivo* e *in vitro*. Estos ensayos muestran bajas prestaciones, ya que son invasivos y peligrosos y proporcionan falsos positivos y/o negativos. Además, la sensibilidad diagnóstica está lejos de ser la esperada, posiblemente porque aún no se ha conseguido reconocer todos los epítomos causantes de los episodios alérgicos. En este sentido, la preparación de antígenos se ha basado hasta el momento, en mayor medida, en la unión directa de los antibióticos a las moléculas portadoras mediante la formación de un enlace amida entre los grupos amino de las lisinas de la molécula portadora y el grupo carboxilato del antibiótico. Aun así, las IgE específicas son vagamente detectadas con estos antígenos.

En esta tesis se ha abordado la síntesis de haptenos y la generación de determinantes antigénicos a antibióticos  $\beta$ -lactámicos con los que poder realizar un diagnóstico *in vitro* más fiable de alergias a estos fármacos. La evaluación de los mismos se ha llevado a cabo mediante ensayos *in vitro* multiplex basados en tecnología de disco compacto.

Esta investigación comienza centrándose en la síntesis y preparación de antígenos de penicilina. Para ello, en una primera fase se ha estudiado el efecto de la incorporación de brazos espaciadores alifáticos en la generación de antígenos, considerando la posibilidad de que se obtenga un mejor reconocimiento molecular al alejar el hapteno de la proteína portadora. Se sintetizaron trece haptenos derivados de bencilpenicilina y amoxicilina con los que se prepararon antígenos con la proteína albumina de suero humano. La evaluación de los antígenos reveló que a pesar de ser suficientemente inmunogénicos y ser reconocidos por anticuerpos IgG de conejo, éstos

no fueron reconocidos por IgE específicas de muestras de pacientes alérgicos. Así bien, por otro lado, la estrategia de cationización de las proteínas albumina de suero humano e histona fue abordada teniendo en cuenta que la modificación de grupos carboxilatos de la proteína a grupos amino aumenta la relación molar hapteno/proteína. Esta estrategia permitió la generación de 5 antígenos, 4 de los cuales (los antígenos de histona), esta vez sí, incrementaron la especificidad de la respuesta inmunológica obtenida, reconociendo IgE específicas. Concretamente, se han determinado IgE específicas en suero de pacientes alérgicos a bajas concentraciones (LOD = 0.07 IU/mL) con una especificidad diagnóstica del 100 % y una sensibilidad del 60 y 31 % para bencilpenicilina y amoxicilina, respectivamente, mejorando la sensibilidad un 60 % en comparación con el ensayo *in vitro* de referencia.

A continuación, se abordó la idea de preparar antígenos menores basados en metabolitos de penicilina. Así pues, los ácidos peniciloico, peniloico, penicílico y 6-aminopenicilánico, junto con la penicilamina fueron conjugados a las proteínas albumina de suero humano e histona. Su evaluación mediante el ensayo *in vitro* mostró que todos, excepto el ácido peniloico, fueron reconocidos específicamente y selectivamente al analizar un conjunto de muestras de suero de pacientes positivos. La especificidad diagnóstica obtenida fue del 100 %, 94 % en el caso del ácido penicílico, y la sensibilidad se situó entre el 67 y 100 %.

Evaluada la preparación de antígenos de penicilina, la tesis se centró en la producción de antígenos para otras familias de antibióticos  $\beta$ -lactámicos. La generación de antígenos para las cefalosporinas, carbapenemas, monobactamas o los inhibidores  $\beta$ -lactámicos resulta imprescindible, ya que no se encuentran actualmente en el mercado ensayos *in vitro* para la detección de alergias a estos antibióticos. Así bien, en primer lugar, se evaluaron los resultados obtenidos tras la preparación de antígenos mayoritarios y minoritarios para los antibióticos cefuroxima, cefotaxima, ceftriaxona, meropenem y aztreonam. Mientras los antígenos mayores no fueron reconocidos al analizar muestras de suero de pacientes, los menores sí. La utilización de estos antígenos menores, preparados tras la acidificación de sus correspondientes sales, mejoró las prestaciones analíticas del microinmunoensayo desarrollado. Así, se obtuvieron altos niveles de sensibilidad analítica (LOD < 0.01 IU/mL) y especificidad diagnóstica (100 %). Finalmente, se obtuvieron valores de sensibilidad diagnóstica del 35, 49 y 51 % para los antibióticos cefuroxima, cefotaxima y meropenem.

A continuación, el trabajo se orientó en la preparación de antígenos de ácido clavulánico, ya que la combinación del mismo con amoxicilina, comúnmente conocido como Augmentin, es hoy en día el medicamento más prescrito. Hasta lo que se conoce, no existe un ensayo *in vitro* para la determinación de IgE específica de ácido clavulánico, por lo que resulta muy interesante abordar la síntesis de haptenos. En este caso, se sintetizaron tres haptenos derivados del ácido clavulánico y se conjugaron a las proteínas albumina de suero humano, histona y a la proteína inmunogénica hemocianina de lapa californiana. Los resultados obtenidos revelaron la importancia de la estructura química ya que dos de estos haptenos generaron anticuerpos tipo IgG con una elevada selectividad al ácido clavulánico. Concretamente, la selectividad fue tres veces mayor (la reactividad cruzada pasó del 30 al 10 %) que para otras familias de antibióticos  $\beta$ -lactámicos. Finalmente, solo el antígeno conjugado a albumina de suero humano preparado tras la esterificación del clavulanato potásico permitió la detección de IgE específica de pacientes alérgicos. Así pues, la sensibilidad diagnóstica obtenida en el ensayo fue del 50 % y la especificidad diagnóstica del 100 %.

A pesar de las mejoras obtenidas con las estrategias llevadas a cabo, se estudiaron otras vías no clásicas para la síntesis de nuevos haptenos con mayor diversidad química. Este enfoque se basa en la generación de antígenos en librerías químicas de compuestos con diversidad estructural para encontrar nuevos haptenos biológicamente activos. Dichas estrategias, hasta el momento, no han sido empleadas para la generación de antígenos y el análisis de muestras de suero de pacientes alérgicos. Con el fin de incorporar diversidad estructural, se sintetizaron, mediante la técnica combinatoria *diversity-oriented synthesis*, 22 compuestos de los precursores de las penicilinas y cefalosporinas, ácido 6-aminopenicilánico y ácido 7-amino-desacetoxicefalosporánico, respectivamente, y de los antibióticos amoxicilina y ampicilina. Su evaluación con el inmunoensayo *in vitro* basado en disco compacto ha demostrado que la incorporación de diversidad permite el reconocimiento de epítomos causantes de episodios alérgicos. Concretamente, se observó que estos antígenos eran capaces de detectar anticuerpos tipo IgG e IgE específicos procedentes de suero de conejos inmunizados y de suero humano de pacientes alérgicos, siendo especialmente selectivos los determinantes de amoxicilina y ampicilina. Concretamente, se obtuvo una sensibilidad diagnóstica del 79 % y una especificidad diagnóstica del 100 %.



## RESUM

Al voltant del 10% de les reaccions adverses a medicaments són degudes a al·lèrgies, sent els antibiòtics  $\beta$ -lactàmics aquells que més episodis al·lèrgics ocasionen. Encara que la incidència real continua sent desconeguda, els individus sospitosos de presentar al·lèrgia a algun medicament acaben sent prescrits amb altres medicaments, menys efectius, més cars o perjudicials. Així doncs, un correcte diagnòstic resulta clau per a disminuir els costos econòmics derivats i procedir a un adequat 'desetiquetatge' de la població. En l'actualitat, les proves de diagnòstic d'al·lèrgies a antibiòtics  $\beta$ -lactàmics es basen en assaigs *in vivo* i *in vitro*. Aquests assaigs mostren baixes prestacions, ja que són invasius i perillosos i proporcionen falsos positius i/o negatius. A més a més, la sensibilitat diagnòstica està lluny de ser l'esperada, possiblement perquè encara no s'ha aconseguit reconèixer tots els epítopes causants dels episodis al·lèrgics. En aquest sentit, la preparació d'antígens s'ha basat fins al moment, en major mesura, en la unió directa dels antibiòtics a les molècules portadores mitjançant la formació d'un enllaç amida entre els grups amino de les lisines de la molècula portadora i el grup carboxilat de l'antibiòtic. Així i tot, les IgE específiques són vagament detectades amb aquests antígens.

En aquesta tesi s'ha abordat la síntesi d'haptens i la generació de determinants antigènics a antibiòtics  $\beta$ -lactàmics amb els quals poder realitzar un diagnòstic *in vitro* més fiable d'al·lèrgies a aquests fàrmacs. La seua avaluació s'ha dut a terme mitjançant assaigs *in vitro* multiplex basats en tecnologia de disc compacte.

Aquesta investigació comença centrant-se en la síntesi i preparació d'antígens de penicil·lina. Per a això, en una primera fase s'ha estudiat l'efecte de la incorporació de braços espaciadors alifàtics en la generació d'antígens, considerant la possibilitat que s'obtinga un millor reconeixement molecular en allunyar l'haptè de la proteïna portadora. Es van sintetitzar tretze haptens derivats de bencilpenicil·lina i amoxicil·lina amb els quals es van preparar antígens amb la proteïna albúmina de sèrum humà. L'avaluació dels antígens va revelar que malgrat ser prou immunogènics i ser reconeguts per anticossos IgG de conill, aquests no van ser reconeguts per IgE específiques de mostres de pacients al·lèrgics. Així bé, d'altra banda, l'estratègia de cationització de les proteïnes albúmina de sèrum humà i histona va ser abordada tenint en compte que la modificació dels grups

carboxilats de la proteïna a grups amino augmenta la relació molar hapten/proteïna. Aquesta estratègia va permetre la generació de 5 antígens, 4 dels quals (els antígens d'histona), aquesta vegada sí, van incrementar l'especificitat de la resposta immunològica obtinguda, reconeixent IgE específiques. Concretament, s'han determinat IgE específiques en sèrum de pacients al·lèrgics a baixes concentracions (LOD = 0.07 IU/mL) amb una especificitat diagnòstica del 100 % i una sensibilitat del 60 i 31 % per a bencilpenicil·lina i amoxicil·lina, respectivament, millorant la sensibilitat un 60 % en comparació amb l'assaig *in vitro* de referència.

A continuació, es va abordar la idea de preparar antígens menors basats en metabòlits de penicil·lina. Així doncs, els àcids penicil·loic, penil·loic, penicíl·lic i 6-aminopenicil·lànica, juntament amb la penicil·lamina van ser conjugats a les proteïnes albúmina de sèrum humà i histona. La seua avaluació mitjançant l'assaig *in vitro* va mostrar que tots, excepte l'àcid penil·loic, van ser reconeguts específicament i selectivament en analitzar un conjunt de mostres de sèrum de pacients positius. L'especificitat diagnòstica obtinguda va ser del 100 %, 94 % en el cas de l'àcid penicíl·lic, i la sensibilitat es va situar entre el 67 i 100 %.

Avaluada la preparació d'antígens de penicil·lina, la tesi es va centrar en la producció d'antígens per a altres famílies d'antibiòtics  $\beta$ -lactàmics. La generació d'antígens per a les cefalosporines, carbapenems, monobactàmics o els inhibidors  $\beta$ -lactàmics resulta imprescindible, ja que no es troben actualment assaigs *in vitro* comercials per a la detecció d'al·lèrgies a aquests antibiòtics. Així bé, en primer lloc, es van avaluar els resultats obtinguts després de la preparació d'antígens majoritaris i minoritaris per als antibiòtics cefuroxima, cefotaxima, ceftriaxona, meropenem i aztreonam. Mentre els antígens majors no van ser reconeguts en analitzar mostres de sèrum de pacients, els menors sí. La utilització d'aquests antígens menors, preparats després de l'acidificació de les seues corresponents sals, va millorar les prestacions analítiques del microimmunoassaig desenvolupat. Així, es van obtenir alts nivells de sensibilitat analítica (LOD < 0.01 IU/ml) i especificitat diagnòstica (100 %). Finalment, es van obtenir valors de sensibilitat diagnòstica del 35, 49 i 51 % per als antibiòtics cefuroxima, cefotaxima i meropenem.

A continuació, el treball es va orientar a la preparació d'antígens de l'àcid clavulànic, ja que la combinació del mateix amb amoxicil·lina, comunament conegut com Augmentin, és hui dia el medicament més prescrit. Fins ara, no existeix un assaig *in vitro*

per a la determinació de IgE específica d'àcid clavulànic, per la qual cosa resulta molt interessant abordar la síntesi d'haptens. En aquest cas, es van sintetitzar tres haptens derivats de l'àcid clavulànic i es van conjuguar a les proteïnes albúmina de sèrum humà, histona i a la proteïna immunogènica hemocianina de llepessa californiana. Els resultats obtinguts van revelar la importància de l'estructura química, ja que dos d'aquests haptens van generar anticossos tipus IgG amb una elevada selectivitat a l'àcid clavulànic. Concretament, la selectivitat va ser tres vegades major (la reactivitat creuada va passar del 30 al 10 %) que per a altres famílies d'antibiòtics  $\beta$ -lactàmics. Finalment, només l'antigen conjugat a albúmina de sèrum humà preparat després de l'esterificació del clavulanat potàssic va permetre la detecció de IgE específica de pacients al·lèrgics. Així doncs, la sensibilitat diagnòstica obtinguda en l'assaig va ser del 50 % i l'especificitat diagnòstica del 100 %.

Malgrat les millores obtingudes amb les estratègies dutes a terme, es van estudiar altres vies no clàssiques per a la síntesi de nous haptens amb major diversitat química. Aquest enfocament es basa en la generació d'antígens en llibreries químiques de compostos amb diversitat estructural per a trobar nous haptens biològicament actius. Aquestes estratègies, fins al moment, no han sigut emprades per a la generació d'antígens i l'anàlisi de mostres de sèrum de pacients al·lèrgics. Amb la finalitat d'incorporar diversitat estructural, es van sintetitzar, mitjançant la tècnica combinatòria *diversity-oriented synthesis*, 22 compostos dels precursors de les penicil·lines i cefalosporines, àcid 6-aminopenicil·lànic i àcid 7-amino-desacetoxicefalosporànic, respectivament, i dels antibiòtics amoxicil·lina i ampil·lina. La seua avaluació amb l'immunoassaig *in vitro* basat en disc compacte ha demostrat que la incorporació de diversitat permet el reconeixement d'epítops causants d'episodis al·lèrgics. Concretament, es va observar que aquests antígens eren capaços de detectar anticossos tipus IgG i IgE específics procedents de sèrum de conills immunitzats i de sèrum humà de pacients al·lèrgics, sent especialment selectius els determinants d'amoxicil·lina i ampil·lina. Concretament, es va obtenir una sensibilitat diagnòstica del 79 % i una especificitat diagnòstica del 100 %.

## DISSEMINATION OF RESULTS

The results derived from this thesis have led to the follow scientific contributions.

*Published articles in indexed scientific journals:*

1. Edurne Peña-Mendizabal, Sergi Morais, Ángel Maquieira. “Boosting the sensitivity of *in vitro*  $\beta$ -lactam allergy diagnostic tests”.  
*Chem. Commun.* 2020, **56**, 11973-11976.  
Impact factor (5-year): 6.086
2. Edurne Peña-Mendizabal, Sergi Morais, Ángel Maquieira. “Neo-antigens for the serological diagnosis of IgE-mediated drug allergic reactions to antibiotics cephalosporin, carbapenem and monobactam”.  
*Sci. Rep.* 2020, **10** (16037), 1-12  
Impact factor (5-year): 4.576
3. Edurne Peña-Mendizabal, Bruce K. Hua, Ethel Ibañez-Echevarria, Dolores Hernández-Fernández de Rojas, Sergi Morais, Ángel Maquieira and Stuart L. Schreiber. “Synthesis of skeletally diverse  $\beta$ -lactam haptens for the *in vitro* diagnosis of IgE-mediated drug allergy”  
*Under review*

*Communications in national and international conferences:*

1. Sergi Morais, María José Juárez-Rodríguez, Salvador Mas-García, Pedro Quintero-Campos, Edurne Peña-Mendizabal, María Estrella Fernández-Sánchez, Luis Antonio Tortajada-Genaro, Ángel Maquieira “*Moving towards advanced in vitro diagnostics for drug allergy*” 13th Rapid Methods Europe Conference, Amsterdam (The Netherlands), **2021**.
2. Edurne Peña-Mendizabal, Sergi Morais, Ángel Maquieira. “*Effect of aliphatic diamine length as chemical strategies for designing structural determinants for  $\beta$ -lactam allergy*”, XIII International Workshop on Sensors and Molecular Recognition, Valencia (Spain), **2019**.
3. Sergi Morais, María José Juárez-Rodríguez, Edurne Peña-Mendizabal, Salvador Mas-García, Luis Antonio Tortajada-Genaro, Ángel Maquieira “*Covalent anchoring of beta-lactam determinants on digital surfaces for developing in vitro tests for the diagnosis of allergy*” 8th Drug Hypersensitivity Meeting, Amsterdam (The Netherlands), **2018**.
4. Edurne Peña-Mendizabal, Sergi Morais, Ángel Maquieira. “*Selección de determinantes antigénicos con potencial para el diagnóstico de alergias a antibióticos  $\beta$ -lactámicos*”, XI International Workshop on Sensors and Molecular Recognition, Valencia (Spain), **2017**.
5. Edurne Peña-Mendizabal, Sergi Morais, Ángel Maquieira. “*Selección de determinantes antigénicos para la detección de alergias a antibióticos  $\beta$ -lactámicos*”, XXI Reunión de la Sociedad Española de Química Analítica, Valencia (Spain), **2017**.

6. Sergi Morais, María José Juárez-Rodríguez, Edurne Peña-Mendizabal, Luis Antonio Tortajada-Genaro, Salvador Mas-García, Estrella Fernández-Sánchez, Ángel Maquieira. “*Chemical strategies for designing structural determinants for  $\beta$ -lactam allergy*” XXXVI Reunión Bienal de la Real Sociedad Española de Química, Sitges (Spain), **2017**.
  
7. Edurne Peña-Mendizabal, Sergi Morais, Ángel Maquieira. “*Diagnóstico de alergias a antibióticos  $\beta$ -lactámicos*”, IV Encuentro de Estudiantes de Doctorado en la UPV, Valencia (Spain), **2017**.

## ACRONYMS AND ABBREVIATIONS

6-APA	6-aminopenicillanic acid
7-ACA	7-aminocephalosporanic acid
ADE	Adverse drug event
ADR	Adverse drug reaction
AHS	Abacavir hypersensitivity syndrome
AMP	Ampicillin
AMX	Amoxicillin
APC	Antigen-presenting cell
ATC	Anatomical therapeutic chemical classification system
AZT	Aztreonam
BAT	Basophil activation test
BLC	$\beta$ -lactam antibiotic
BPL	Penicilloyl-polylysine
BPA	Benzylpenicillanyl
BPO	Benzylpenicilloyl
BSA	Bovine serum albumin
CAF	Child-appropriate formulation
CDC	Center for Disease Control and Prevention
CFO	Cefotaxime
CFR	Cefuroxime
CFT	Ceftriaxone
CNBr	Cyanogen bromide
CTL	Cytotoxic T lymphocyte
CVL	Clavulanic acid
DCC	N,N'-dicyclohexylcarbodiimide
DDD	Defined daily dose
DEL	DNA-encoded library
DHR	Drug hypersensitivity reaction
DNA	Deoxyribonucleic acid

DOS	Diversity-oriented synthesis
DPT	Drug provocation test
DVD	Digital Versatile Disc
EAACI	European Academy of Allergy and Clinical Immunology
EC <sub>50</sub>	Half maximal effective concentration
ECDC	European Centre for Disease Prevention and Control
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ENDA	European Network on Drug Allergy
EU	European Union
FDA	U.S. Food and Drug Administration
GAM-HRP	Goat anti-mouse antibody labelled with horseradish peroxidase
GAR-HRP	Goat anti-rabbit antibody labelled with horseradish peroxidase
GT	Glycosyltransferase
H1	Hystone from calf thymus
HR	Hypersensitivity reaction
HRMS	High-resolution mass spectrometry
HSA	Human serum albumin
HTS	High-throughput screening
IDHR	Immediate drug hypersensitivity reaction
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHR	Immediate hypersensitivity reaction
IL	Cytokine interleukin
IU	International units
KLH	Keyhole limpet hemocyanin
LC/MS <sup>n</sup>	Data-dependent multiple-stage tandem mass spectrometry
LOD	Limit of detection
LRMS	Low-resolution mass spectra
mAb	Monoclonal antibody
MCR	Multicomponent reaction



MDM	Minor determinant mixture
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex
MRP	Meropenem
MRSA	Methicillin-resistant <i>S. aureus</i>
MS-MALDI-TOF	Matrix assisted laser desorption/ionization mass spectrometry
NHS	N-hydroxy succinimide
NMR	Nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
(P-I) model	Pharmacological interaction model
PAMAM	Polyamidoamine
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PG	Benzylpenicillin (or Penicillin G)
PLL	Poly-L-lysine
PPL	Penicilloyl polylysine
PPR	Piperacillin
PV	Phenoxymethylpenicillin (or Penicillin V)
RAST	Radioallergosorbent test
sIgE	Specific IgE
SIT	Skin intradermal test
SPT	Skin prick test
SSRIs	Selective serotonin reuptake inhibitor
ST	Skin testing
TCR	T-cell receptor
TLC	Thin layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
TOS	Target-oriented synthesis
tIgE	Total IgE
US	United States
WAO	World Allergy Organization
WHO	World Health Organization

## **GLOSSARY OF TERMS**

- Hapten

An organic compound of low molecular weight (<1,000 Da) chemically reactive that can be covalently conjugated to high molecular weight molecules in order to elicit an immune response.

- Antigen

Any foreign substance that generates a specific immune response when introduced in the organism, well by producing specific antibodies, by sensitizing T-cells or both.

- Antigenic determinant

The immunodominant part of the antigen that is recognized by the immune system and consequently stimulates an immune response. It is also referred to as epitope.

- Diagnostic sensitivity

The ability of a test, expressed in percentage, to correctly identify populations of individuals with the disease.

- Diagnostic specificity

The ability of a test, expressed in percentage, to correctly identify populations of individuals without the disease.

- Analytical sensitivity

The lowest concentration of an analyte determined or detected by an assay in a sample.

- Analytical selectivity

The ability of a method to determine particular analytes in mixtures or matrices exhibiting a degree of preference for the substance of interest.

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# **1. INTRODUCTION**



# 1. INTRODUCTION

## 1.1. Adverse drug reactions

A drug is pharmacologically a chemical substance, obtained naturally or synthetically, capable of producing a biological effect in an individual when consumed.<sup>1</sup> However, drugs not only produce a therapeutic benefit, but they can also cause adverse reactions. In this respect, the World Health Organization (WHO), in 1972, coined the term “**adverse drug reaction**” (ADR) to underline the noxious and unintended response to a drug occurring at doses normally used in individuals for the prevention, diagnosis or therapy of a disease or for the modification of physiological functions.<sup>2</sup> Although any drug can lead to an ADR, the most common drug groups causing ADRs are listed in Table 1.<sup>3</sup>

**Table 1.** Therapeutic groups suspected of causing most ADRs

<b>Therapeutic group</b>	<b>Drugs</b>
Antithrombotic agents	Aspirin, clopidogrel, heparin, warfarin
Antibacterials for systemic use	Amoxicillin, azithromycin, cephalexin, ciprofloxacin
Psycholeptics	Clozapine, haloperidol, olanzapine, quetiapine
Psychoanaleptics	Amitriptyline, citalopram, sertraline, venlafaxine
Antineoplastic agents	Protein kinase inhibitors (imatinib, nilotinib, ribociclib)
Antiinflammatory and antirheumatics	Celecoxib, diclofenac, ibuprofen, ketoprofen
Analgesics	Hydrocodone, metamizole, morphine, paracetamol
Antiepileptics	Carbamazepine, diazepam, lamotrigine, levetiracetam
Agents acting on the renin-angiotensin system	Benazepril, captopril, losartan, valsartan
Sex hormones and modulators of the genital system	Norethindrone and ethinylestradiol, norgestimate and ethinylestradiol

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ADR should not be confused with the term “adverse drug event” (ADE), which refers to those injuries resulting from the use of a drug. In this sense, these events include the harm caused by both the drug, such as ADRs and overdoses, and medication errors, from the interaction between drugs or drug and food.<sup>4</sup>

Presently, both immune and non-immune ADRs are major causes of morbidity and mortality worldwide.<sup>5</sup> It was recently reported that 3 to 6 % of all hospital admissions of a study including 4,802 patients was due to ADRs.<sup>6</sup> Furthermore, they occurred in 10 to 15 % of hospitalized patients, as observed in several studies<sup>7-9</sup> accounting for up to 3,695 patients.

ADRs were commonly distinguished between dose and non-dose-dependent, classified as type A and type B reactions.<sup>10</sup> More recently, four new categories have been proposed,<sup>11,12</sup> namely type C, D, E and F reactions. The classification of ADR are listed in Table 2. It is worth noting, however, that not all individuals have to be affected in the same way nor do it has to suffer from the same type of reaction.<sup>13</sup> Indeed, ADR may not even develop for a specific individual.<sup>14</sup>

**Table 2.** Classification of adverse drug reactions

Type of ADR	Features	Reactions
A	Intrinsic and predictable Related to the pharmacological action of a drug Dose-dependent Common (80 % of ADRs)	Digoxin toxicity Respiratory depression with opioids
B	Unpredictable Non-dose-dependent Idiosyncratic drug reactions	Drug hypersensitivity reactions Enzyme defects
C	Dose and time-dependent Correlates to the cumulative dose	Hypothalamic pituitary-adrenal axis suppression by corticosteroids
D	Time- and usually dose-dependent Delayed-reactions	Carcinogenesis Teratogenesis
E	Occurs soon after use of the drug	Myocardial ischaemia
F	Unexpected failure of therapy Dose-dependent Commonly caused by drug interaction	Administration of an inappropriate dose of an oral contraceptive in combination with other drugs

## 1.2. Hypersensitivity reactions

Clemens von Pirquet, in 1906, defined the term “allergy” as the unusual biological response shown by some individuals to develop symptoms and signs of sensitivity when exposed to certain substances.<sup>15</sup> These **hypersensitivity reactions** (HRs) are exaggerated or inappropriate responses that cause the immune system to respond abnormally.<sup>16</sup> HRs include allergies and various autoimmune disorders, produced by exogenous or endogenous substances, called antigens or autoantigens, respectively.<sup>17</sup>

The hypersensitivity reaction is developed at early and later stages, namely as sensitization and effector phases. Firstly, individuals are sensitized by means of an asymptomatic primary immune response to allergens,<sup>18</sup> in which this predisposition is certainly influenced by genetics<sup>19,20</sup> and age.<sup>21</sup> In this respect, however, genetic factors alone cannot explain the distinctiveness of allergic sensitizations, and sensitivity differences among individuals to the noxious effects of allergens is another factor to consider.<sup>22</sup> Secondly, a symptomatic harmful secondary immune response, referred to also as the effector phase, occurs. It is in this phase when the acute inflammatory response is commonly developed. However, only those individuals previously sensitized can produce acute inflammatory response.<sup>18</sup>

In early stages of the development of the hypersensitivity, the antigen-presenting cells (APCs) cause a response in T<sub>H</sub>2 lymphocytes that produces the cytokines interleukin (IL)-4 and specific immunoglobulin E (sIgE) in response to the allergen by interaction with B-cells. The sIgE binds to high-affinity receptors located on mast cells, basophils and activated eosinophils, and triggers the secondary immune response that finally ends in an acute inflammatory response.<sup>23</sup> This acute inflammatory response differs in terms of the the effector mediating the HR and the adaptive immune response. Thus, according to the deleterious consequences to the host, Coombs and Gell<sup>24</sup> classified HRs into four forms as type I, II, III and IV (Figure 1).<sup>25</sup>

**Type I** reactions are caused by sIgE cross-linked by contact with free soluble antigens that are bound to mast cells and basophils and cause their degranulation. These reactions release histamine and other inflammatory mediators.

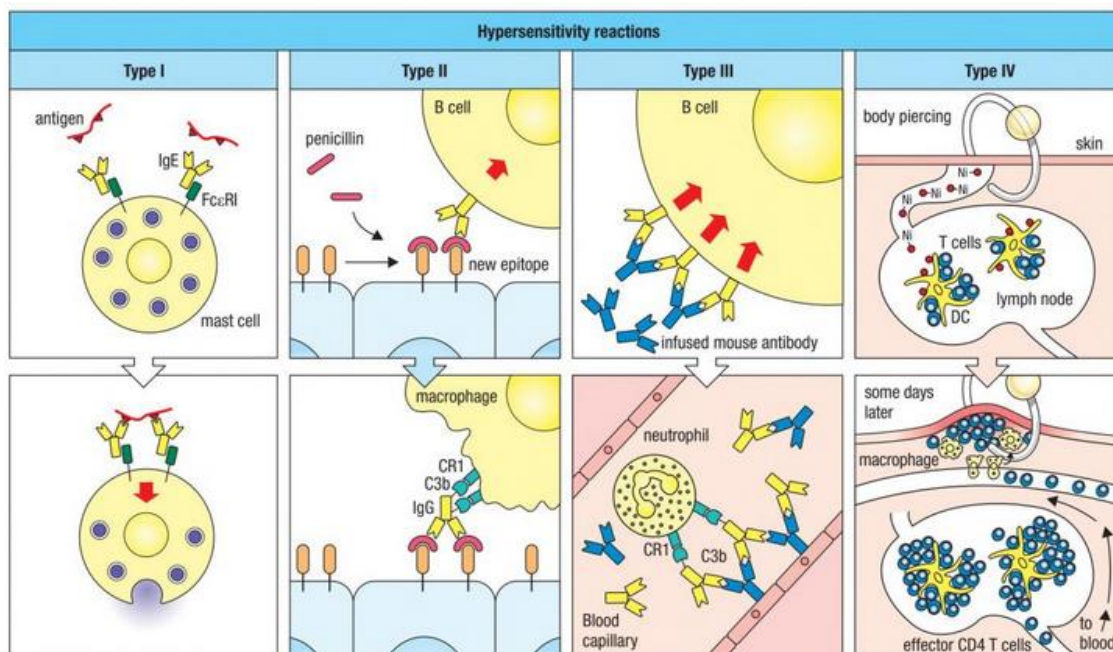
**Type II** reactions are cytotoxic and caused by IgG and IgM antibodies, which are selective to particular tissues. These antibodies bind inappropriately to new epitopes

## Introduction

on the surface of the cells by chemical modifications and activate the complement cascade.

**Type III** reactions are immune complex-mediated and involve IgG antibodies raised against a soluble foreign protein. The deposition of this antigen-IgG antibody complex initiates the complement cascade, which activates neutrophils and results in tissue damage.

**Type IV** reactions are caused by T-cells that respond either to peptides or to the epitopes of foreign proteins, and are the only HRs that involve sensitized T-lymphocytes rather than antibodies.<sup>26</sup> Type IV HRs were further subdivided in IVa-IVd reactions, according to the immune reactant and the effector involved.<sup>27</sup>



**Figure 1.** Scheme of the different mechanisms involved in HRs<sup>25</sup>

Another common classification for HRs is based on the delay of the onset of the reaction. Therefore, they can be classified as (I) immediate hypersensitivity reactions (IHRs) because they occur within 1 hour,<sup>28</sup> and (II) non-immediate (delayed) hypersensitivity reactions, occurring between 1 hour and up to several days. Type I, II and III reactions are considered as IHRs<sup>29</sup> and type IV reactions are classified as non-immediate, or delayed, HRs.



### 1.3. Drug hypersensitivity reactions and drug allergy

The World Allergy Organization (WAO) defined **drug hypersensitivity reactions** (DHRs) as those reactions with reproducible responses or symptoms observed after exposure to a drug at doses normally tolerated by non-hypersensitive individuals.<sup>30</sup> These responses, comprising both allergic and non-allergic reactions, are common in clinical practice and are type B, accounting for up to 15 % of all ADRs.<sup>31</sup> Furthermore, DHRs can be mediated by both specific and non-specific immunological mechanisms.

In this respect, in 2003, the WAO defined **drug allergy** as only those immunologically mediated DHRs. In total, drug allergy is estimated to account for about 10 % of all ADRs.<sup>32,33</sup> However, one controversial issue is that patients with a suspected drug allergy are commonly instantaneously categorized as “allergic” without further clinical evaluation. As a result, morbidity, mortality and economical costs may arise and that is why delabeling initiatives are now part of drug stewardship programs.<sup>34,35</sup>

Based on a study by Zhou et al., in 2016, the most reported drug classes causing an allergic reaction, or immunologically mediated DHR, are –in descending order of prevalence–: penicillins (12.8 %), sulfonamides (7.4 %), opiates (6.8 %), non-steroidal anti-inflammatory drugs (3.5 %), macrolides (2.6 %), angiotensin-converting enzyme inhibitors (2.0 %), cephalosporins (1.7 %),  $\beta$ -hydroxy  $\beta$ -methylglutaryl-CoA reductase inhibitors (1.5 %), fluoroquinolones (1.3 %), tetracyclines (1.2 %), selective serotonin reuptake inhibitors (0.6 %) and phenothizines (0.5 %).<sup>36</sup> In terms of specific drugs, the most reported were codeine (3.1 %), amoxicillin (2.1 %), and aspirin (1.6 %).

Drug allergy can be antibody-mediated or cell-mediated and is clinically classified according to the deleterious consequences to the host, proposed by Coombs and Gell (see section 1.2).<sup>24</sup>

**IgE-mediated** reactions (Type I, Figure 2) mainly cause urticaria, angioedema, maculopapular exanthema and anaphylaxis.<sup>30</sup> Urticaria and angioedema are common clinical problems whose lifetime prevalence is around 20 %.<sup>37</sup>

Urticaria manifests as raised, erythematous, and usually very itchy wheals, and can occur at any time. The mechanism involves the activation and degranulation of dermal mast cells, releasing histamine, serotonin, proteases, tumor necrosis factor  $\alpha$  and cytokines, such as IL-1.<sup>38</sup> This results in an increase of the permeability and distension of

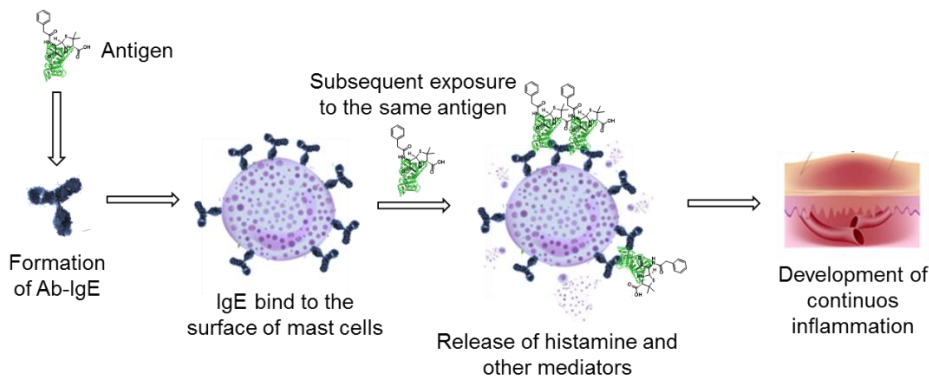
## Introduction

blood capillaries which trigger the development of a continuous inflammation and, eventually, itchy skin rash that regresses within 24 hours.

Angioedema is initiated through a similar mechanism as for acute urticaria, but may last for several days.<sup>39</sup> It occurs in the deep dermis and subcutaneous tissue and is commonly present with urticaria, as a non-pitting swelling of the submucosal or subcutaneous tissue.<sup>40</sup>

Maculopapular exanthema manifests as a diffuse cutaneous erythema in which some skin areas present maculopapules of different sizes and can develop to vesicles or papules.<sup>41</sup> It is considered as a non-immediate reaction.

Anaphylaxis is an immediate, severe, life-threatening, systemic reaction that occurs when a previously sensitized individual is re-exposed to an allergen.<sup>42</sup> It involves respiratory and/or cardiovascular problems, caused by a massive release of mediators.<sup>43</sup> Anaphylaxis incidences in western countries account to approximately 4-50 per 100,000 individuals per year<sup>44</sup> and its frequency is approximately 50 to 2,000 events per 100,000 individuals.<sup>45</sup>



**Figure 2.** Pathogenic mechanism of drug allergy

**Non-IgE-mediated** drug allergies can be cytotoxic (type II), drug immune-complex (type III) or T-cell mediated (type IV) reactions. Immune hemolytic anemia or thrombocytopenia, among others, are considered type II reactions, while serum sickness and drug-induced lupus are drug immune-complex reactions. Some type IV reaction examples include severe cutaneous adverse reaction, Stevens-Johnson syndrome and toxic epidermal necrolysis, and acute generalized exanthematous pustulosis.<sup>46</sup> These latter reactions are the most largely represented<sup>47</sup> and account for the most prevalent ones.<sup>48</sup>

#### 1.4. Mechanisms of action of allergic reactions to drugs

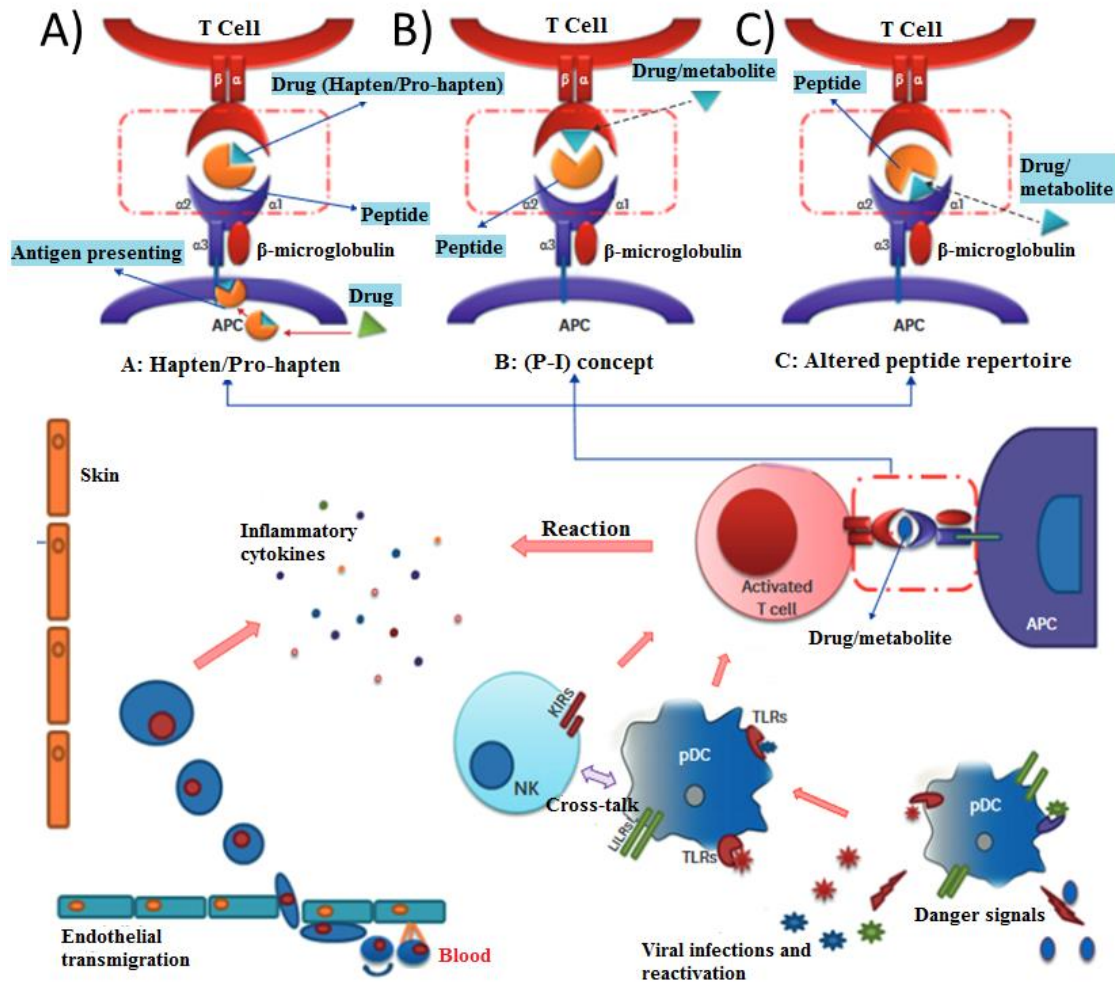
DHRs are explained, according to their mode of action (Figure 3),<sup>49,50</sup> with the hapten/pro-hapten model, the pharmacological interaction with immune receptors model or the altered peptide repertoire model.

In the **hapten/pro-hapten model**, the concept of “hapten” is introduced. Drugs (< 1000 Da) are considered haptens if they are chemically reactive and can be covalently bind to molecules, such as carrier proteins, dendrimers or polypeptides. This drug complex is processed intracellularly and directly bound to the immunogenic major histocompatibility complex (MHC), to the enclosed peptide, or to the MHC/peptide complex on APCs; leading to the formation of distinct epitopes that can generate a warning signal. This signal results simultaneously in both humoral and cellular immune responses. T-cells, in particular, recognize carrier proteins and help B-cells to identify the hapten. On the other hand, the “pro-hapten” concept includes those drugs that are not chemically reactive and therefore cannot form covalent bonds with soluble or cell-bound molecules. In this case, these drugs require metabolic activation to become chemically reactive and act as haptens following the hapten model. The hapten/pro-hapten model can elicit any type of specific immune responses.<sup>51</sup> Sensitive reactions to  $\beta$ -lactam antibiotics (BLCs) are one example of the hapten/pro-hapten model.

In the **pharmacological interaction with immune receptors (P-I) model**, drugs fit into specific proteins or enzymes blocking their function,<sup>52</sup> and are not necessarily covalently bound to MHC molecules. Since chemically inert drugs cannot covalently bind to soluble or cell-bound molecules, they link directly to specific T-cell receptors (TCRs) or MHC molecules by non-covalent bonds without the intracellular processing of the drug.<sup>10</sup> Under certain conditions, the reversible drug-receptor interaction may lead to an immune response of T-cells specific for peptide antigens. This enables to expand and cause different types of inflammatory reactions without the need of occurring a primary immune response to the drug to occur. Allergic reactions due to the drug sulfamethoxazole are a good example of this model as they are reported to bind in a non-covalent way to the TCRs.<sup>53</sup>

The **altered peptide repertoire model** consists of the drug binding inside the MHC (peptide binding groove) during the human leukocyte antigen assembly in the endoplasmic reticulum. This results in an alteration of the presented peptide self-

repertoire, leading to the releasing of an erroneous T-cell response as the TCR does not recognize this altered peptide self-repertoire as self anymore.<sup>54</sup> Furthermore, the pathomechanism of the altered peptide repertoire model may involve co-stimulators accompanied by cross-talk between dendritic cells and natural killer cells. All this leads to the formation of different profiles of severe cutaneous ADRs, depending on the key cells and cytokines involved. The drug abacavir and the abacavir hypersensitivity syndrome (AHS) was the first example of this model.<sup>55</sup>



**Figure 3.** Mechanisms of action of allergic reactions to drugs.<sup>50</sup> A) Hapten and Pro-hapten hypothesis, B) pharmacological interaction (P-I) model and C) altered peptide repertoire model. APC, antigen-presenting cell; KIRs, killer immunoglobulin-like receptor; NK, natural killer cell; pDC, plasmacytoid dendritic cell; TLRs, Toll-like receptors.

### 1.5. Antibiotics

The term **antibiotic**, first coined in 1942,<sup>56</sup> is defined, according to the WHO, as any medicine used for the prevention and treatment of bacterial infections by killing or inhibiting the growth of bacteria.<sup>57,58</sup> Antibiotics were first considered as those

compounds that were produced by one specific microorganism, which selectively inhibited the growth of another. Today, most of the antibiotics are semisynthetic.

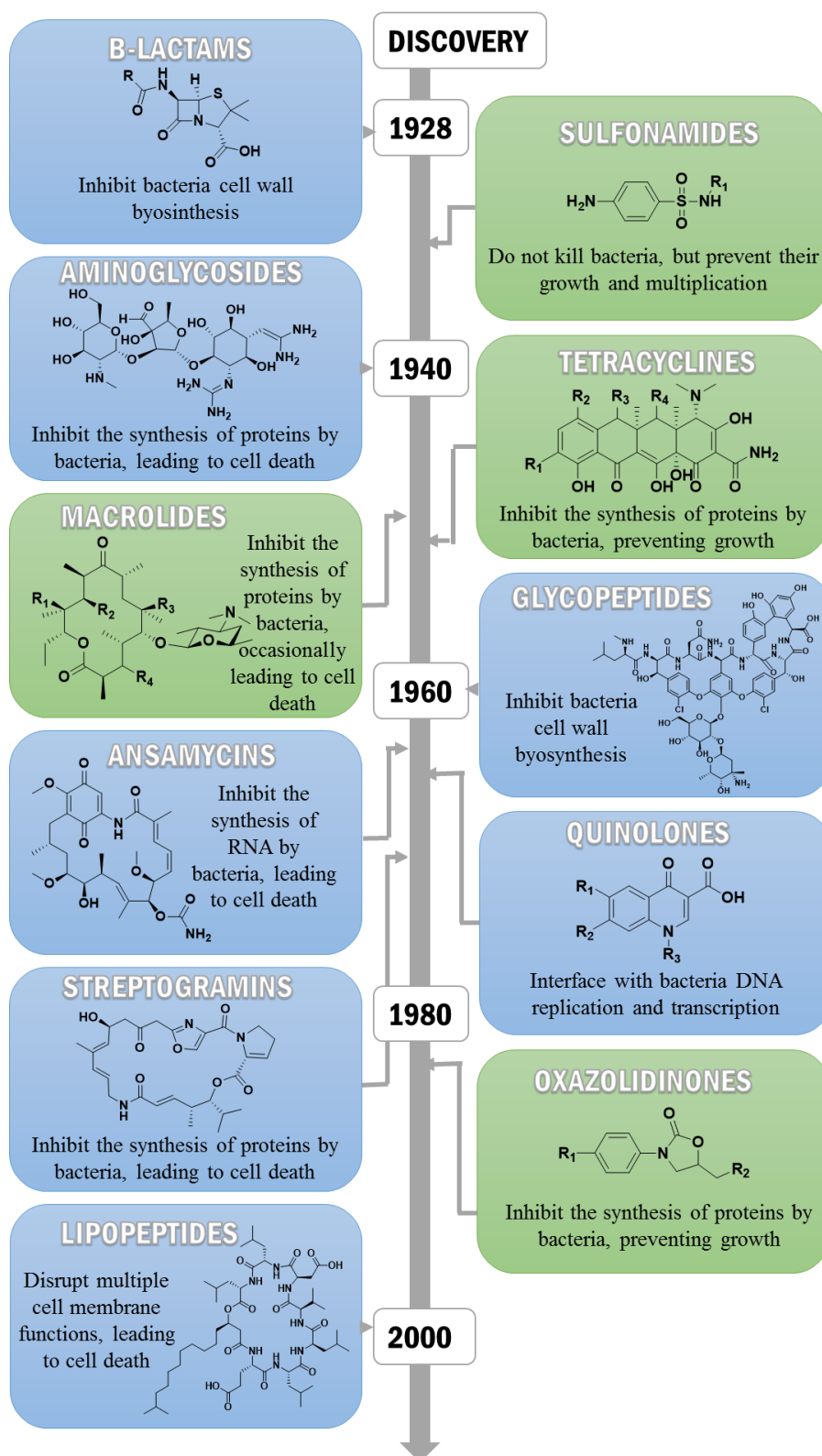
Antibiotics can be classified according to their chemical structure, spectrum<sup>59</sup> or mode of action (Table 3 and Figure 4).<sup>60,61</sup>

**Table 3.** Classification of antibiotics

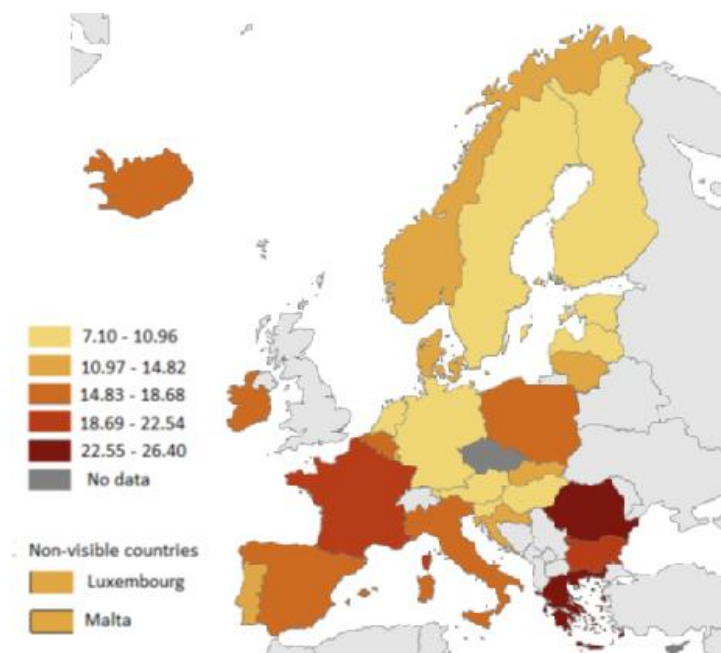
	<b>Antibiotics</b>
Chemical structure	$\beta$ -lactam antibiotics, sulfonamides, aminoglycosides, tetracyclines, macrolides, glycopeptides, ansamycins, fluoroquinolones, streptogramins, oxazolidinones and lipopeptides
Spectrum	Broad- and narrow- spectrum antibiotics (depending on whether the specific type of the microorganism is known or not)
Mode of action	Bactericidal: kill bacteria by inhibiting cell wall synthesis Bacteriostatic: reduce bacteria's growth by inhibiting the synthesis of bacterial proteins, the replication of DNA, and other aspects related to the bacterial cellular metabolism.

### 1.5.1. Consumption pattern of antibiotics

According to the last report from the European Centre for Disease Prevention and Control (ECDC), the total consumption of antibiotics in the community in the European Union (EU) ranged from 7.1 to 26.4 DDDs in 2020 with an average of 15.0 defined daily doses (DDDs) (Figure 5).<sup>62</sup> The DDD is a metric used to calculate the assumed mean maintenance dose of an antibiotic. This metric considers only the number of adults who are consuming antibiotics per 1,000 inhabitants per day. In relation to the gross worldwide consumption pattern of antibiotics, the surveillance report of antibiotic consumption from the WHO,<sup>63</sup> ranged from 4.4 to 64.4 DDDs per 1,000 inhabitants per day during 2015. In the last years, antibiotic consumption increased 65 %, from 21.1 to 34.8 billion DDDs, and its rate increased 39 %, from 11.3 to 15.7 DDDs. The rise of the domestic product per capita in low- and middle-income countries between 2000 and 2015, benefited this increase.<sup>64</sup> This also correlates with the evolution of allergies to antibiotics, which allergy pattern changed over the years. In this respect, a study by Doña et al.<sup>65</sup> evaluated the variation of DHRs, from 2005 to 2010, in a population of 4,460 subjects which developed an allergy reaction. While the general prevalence of allergies to antibiotics remained similar (from 44 to 43 %), there was an increase in DHRs to, particularly, quinolones (from 1.8 to 4.6 %) and macrolides (from 2.7 to 6.2 %), which can be attributed to the increase in their consumption rate.



**Figure 4.** Classes of antibiotics and their mode of action in chronological order of development. Green charts: Antibiotics that commonly act as bacteriostatic agents; Blue charts: Antibiotics commonly considered as bactericidal agents. General structure of each class of antibiotic is shown, unless expressed. Examples of some antibiotic classes are: penicillins (for  $\beta$ -lactams), streptomycin (for aminoglycosides), vancomycin (for glycopeptides), geldanamycin (for ansamycins), pristinamycin IIA (for streptogramins), and surfactin (for lipopeptides).



**Figure 5.** Map of consumption of antibiotics for systemic use in the EU, expressed in DDDs (adapted from <sup>62</sup>)

Antibiotics are also widely used in veterinary medicine and agriculture.<sup>66</sup> However, their use is often non-therapeutic, it is meant to lower production costs, promote faster growth, reduce commercialization time, maintain animal's health and prevent losses.<sup>67</sup> Human consumption of products derived from food-producing animals treated with antibiotics may still contain residues or metabolites, as they can remain in the tissues or cells of the animals.<sup>68</sup> Therefore, the total consumption of antibiotics in the population goes beyond that due to their therapeutic use, and increases after the intake of meat and dairy products. In conclusion, the use of antibiotics by the livestock industry is an importance concern since it can increase the number of allergic and/or toxic reactions as well as aggravate the counteraction of bacteria to the bactericidal effect of antibiotics, known as antibiotic resistance.<sup>69</sup> To mention some figures, about 75 % of all antibiotics annually consumed in the United States (US) are used by the livestock industry.<sup>56</sup> Concretely, a report from the US Food and Drug Administration (FDA) on antibiotics distributed for use in food-producing animals reported domestic annual sales of more than 11 million kg of active antibiotics in 2018.<sup>70</sup> The largest amounts accounted for tetracyclines (66 %), BLCs (13 %), macrolides (8 %), sulfonamides (5 %), aminoglycosides (5 %) and quinolones (< 1 %). The European Medicines Agency (EMA) on antibiotic sales for use in food-producing animals reported domestic annual sales of

ca. 6.6 million kg of active antibiotics in 2017 in the EU. The overall sales of antimicrobials accounted for tetracyclines (30 %), penicillins (27 %) and sulfonamides (9 %). In addition, annual sales of antibiotics for animal use in the EU between 2011 and 2017 dropped by more than 32 %, <sup>71</sup> coinciding with the European prohibition in 2006 on the use of antibiotics as feed additives for growth promotion. <sup>69</sup>

Further to this, antibiotic resistance, aroused as an emerging problem. <sup>72</sup> In this respect, the U.S. Center for Disease Control and Prevention (CDC) calculated \$20 billion of costs for antibiotic resistance related to overspending time in hospitals. <sup>56</sup> Moreover, the Organisation for Economic Co-operation and Development (OECD) predicted that 2.4 million people in Europe, North America and Australia will die from infections related to antibiotic resistance in 30 years. <sup>73</sup> This means \$3.5 billion costs per year.

## **1.6. $\beta$ -lactam antibiotics**

Considered as one of the most prescribed drugs,  $\beta$ -lactam antibiotics (BLCs) are currently also the most widely-used group of antibiotics. <sup>74</sup> These drugs are chemically characterized for having a common feature, the  $\beta$ -lactam ring, that is highly reactive. The antibacterial activity associated to these antibiotics is assigned to it, whereas the lateral chain determines the antibacterial spectrum of action and the pharmacological properties of each specific BLC. <sup>75</sup> These antibiotics are used to treat numerous clinical infections, such as upper respiratory tract infections, pharyngitis, skin/soft tissue infections or intra-abdominal infections. <sup>76</sup>

The first natural BLC, benzylpenicillin (PG), was discovered in 1928 by Fleming who showed that the fungus *P. notatum* prevented the growth of *Staphylococci* bacterium. Since the clinically approval of PG as a therapeutic agent in the 1940s, <sup>72</sup> several natural and semi-synthetic BLCs, categorized in families according to their chemical structure (Figure 6), <sup>74,76</sup> have been developed.

**Penicillins** are characterized for having the nucleus of the 6-aminopenicillanic acid (6-APA) that presents both a  $\beta$ -lactam and a thiazolidine ring, and a lateral chain. This group includes natural penicillins, such as PG and penicillin V (PV), penicillase-resistant penicillins, such as oxacillin and cloxacillin, aminopenicillins, such as (AMX) and ampicillin (AMP), carboxypenicillins, such as carbenicillin and ticarcillin, and



ureidopenicillins, such as piperacillin (PPR) and azlocillin.<sup>77</sup> Another classification of penicillins is based on the Anatomical Therapeutic Chemical (ATC) code J01, which classifies antibacterials with an alphanumeric code for systemic use, and is a therapeutic subgroup of the ATC classification system. Within this system, penicillins, encoded as J01C, are classified as penicillins with extended spectrum, such as AMX, AMP, PPR, carbenicillin;  $\beta$ -lactamase-sensitive penicillins, such as PG and PV; and  $\beta$ -lactamase-resistant penicillins, like oxacillin and cloxacillin.

**Cephalosporins** are semisynthetic BLCs that contain a 7-aminocephalosporanic acid (7-ACA) nucleus and a side-chain containing a 3,6-dihydro-2 H-1,3-thiazane ring. According to the antibacterial spectrum of action, they are classified into five generations. This antibacterial spectrum has, however, to be redefined as cephalosporin resistance increases due to extended-spectrum  $\beta$ -lactamases and carbapenemases, especially for Gram-negative bacteria. Some examples include: cefazolin and cefadroxil, from the 1<sup>st</sup> generation; cefaclor and cefuroxime (CFR), from the 2<sup>nd</sup>; ceftriaxone (CFT) and cefotaxime (CFO), from the 3<sup>rd</sup>; cefepime and ceftiprome, from the 4<sup>th</sup>; and ceftaroline and ceftobiprole, from the 5<sup>th</sup> generation.

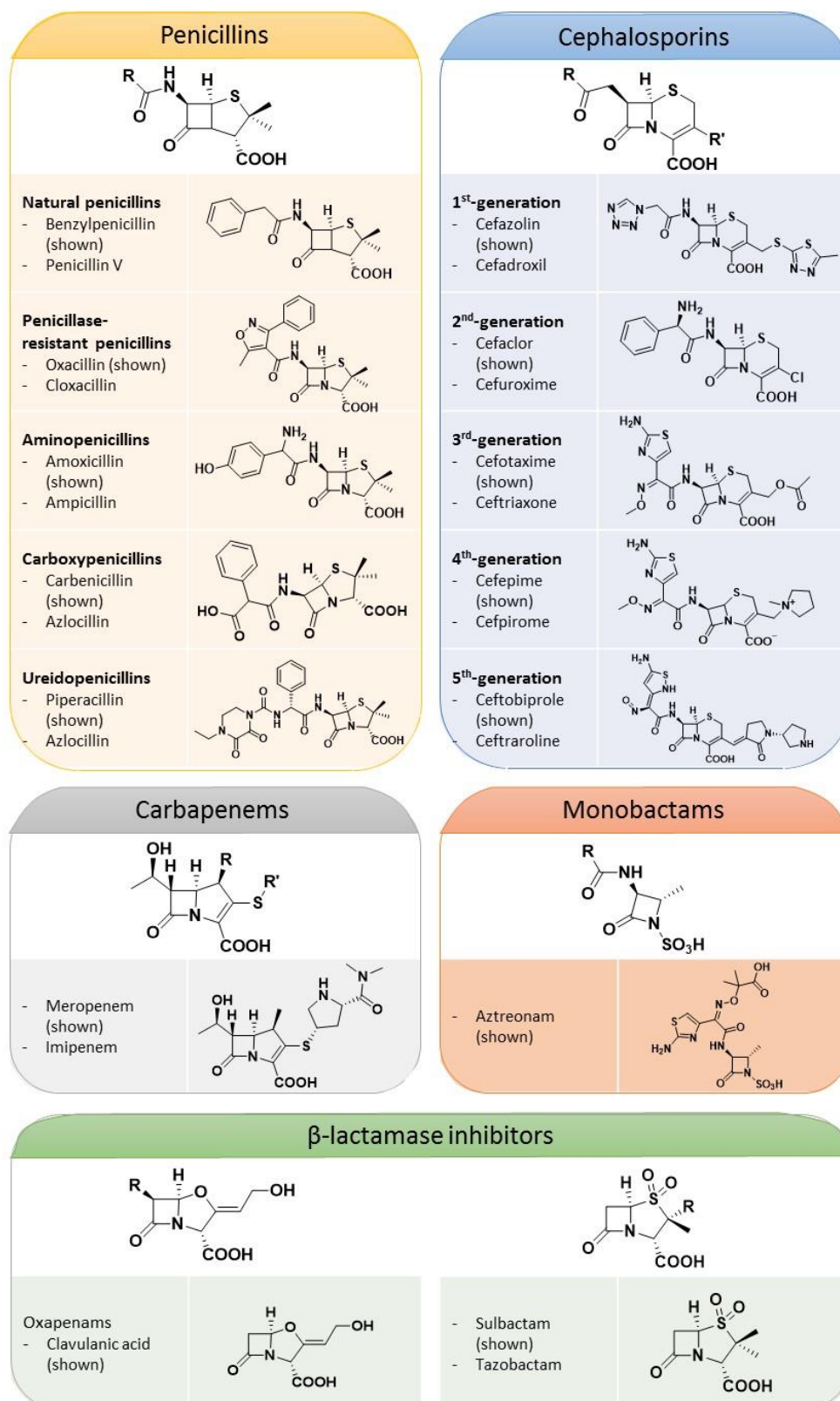
The defining structure of **carbapenems** is similar to that of the penicillins, but a carbon atom has been placed where the sulphur atom of the thiazolidine ring of penicillin was, together with the increase of the degree of unsaturation. They exhibit a broader spectrum of activity compared with penicillins and cephalosporins. However, it is the lateral chain placed next to the  $\beta$ -lactam ring, which gives protection against most  $\beta$ -lactamases. Nevertheless, Gram-negative organisms to counteract their effectiveness produce carbapenemases. This group includes antibiotics such as meropenem (MRP) and imipenem.<sup>78</sup>

**Monobactams** contain a  $\beta$ -lactam ring that is not fused to any other ring and a sulfonate moiety. They are active against aerobic Gram-negative organisms. Aztreonam (AZT) is the only monobactam currently approved by the FDA and in the EU.<sup>79</sup>

**$\beta$ -lactamase inhibitors** inactivate serine  $\beta$ -lactamases and are normally prescribed in conjunction with a BLC, such as AMX or piperacillin. 1<sup>st</sup> generation  $\beta$ -lactamase inhibitors include clavulanic acid (CVL), sulbactam and tazobactam; while newer generations, such as avibactam and vaborbactam, are active against carbapenemases. When used in conjunction with other BLC, this combination is active

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against aerobic Gram-negative, Gram-positive and anaerobic organisms that produce  $\beta$ -lactamases; increasing their antibacterial spectrum of action.



**Figure 6.** Chemical structures of BLCs

### 1.6.1. Consumption pattern of $\beta$ -lactam antibiotics

The consumption pattern of BLCs in the human population generates over \$15 billion in annual sales, making up close to 65 % of the total antibiotics sales.<sup>80</sup>

Penicillins, which is the most prescribed group of BLCs, had a median consumption rate of 195 DDDs, according to the last WHO report on antibiotic consumption.<sup>63</sup> In particular, the global antibiotic consumption rate of penicillins was up to 114 DDDs for penicillins with extended spectrum, 17 DDDs for  $\beta$ -lactamase-sensitive penicillins, 23 DDDs for  $\beta$ -lactamase-resistant penicillins and 41 DDDs for those combinations of penicillins with  $\beta$ -lactamase inhibitors. In the EU, they represent the 43 % of total antibiotic consumption in the community with a median population-weighted consumption of 6.5 DDDs in 2020.<sup>62</sup> The highest values of consumption of penicillins in the EU were observed in Romania (10.5 DDDs), France (10.1 DDDs), Spain (9.9 DDDs), and Greece (8.7 DDDs). By contrast, lowest values were observed in Germany (3.1 DDDs), Finland (3.1 DDDs), and the Netherlands (2.4 DDDs). In particular, AMX, a penicillin considered the most administrated BLC worldwide and the main culprit drug involved in drug allergy reactions,<sup>81,82</sup> is the most consumed BLC in southern European countries. However, PV was the most administrated penicillin in Scandinavia.<sup>83,84</sup>

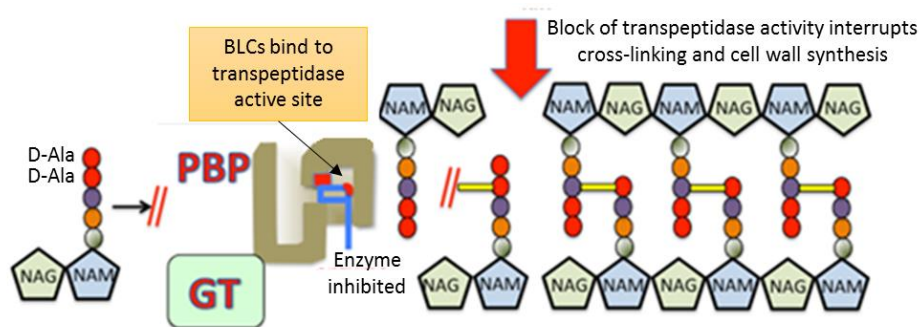
On the other hand, the global antibiotic consumption rate for other families of BLCs, encoded as J01D, is increasing over the years. Therefore, the global antibiotic consumption rate for cephalosporins, carbapenems, monobactams and  $\beta$ -lactamase inhibitors were 135.45, 11.26, 6.23 and 1.00 DDDs, respectively, between 2016 and 2018.<sup>63</sup> In fact, the population-weighted consumption in the community of all these other BLCs in the EU in 2020, was 1.7 DDDs, accounting for around 11 % of total European intake.<sup>85</sup> The highest values of consumption of J01D antibiotics in the EU were observed in Greece (5.8 DDDs), Bulgaria (4.0 DDDs) and Romania (3.9 DDDs). By contrast, Denmark and the Netherlands reported values below 0.1 DDDs.

Changes in the consumption pattern of BLCs have influenced the evolution of DHRs to them. Therefore, on the one hand, allergy prevalence to the first discovered penicillin PG is decreasing, as well as its prescription. On the other hand, AMX and its use in conjunction with CVL has been observed to increase over the years.<sup>65,86</sup>

### 1.6.2. Mechanisms of action of $\beta$ -lactam antibiotics

DD-transpeptidases, also known as penicillin binding proteins (PBPs), are enzymes located beneath the bacterial cell wall and the cell surface membrane. In the absence of a BLC, they catalyze crosslinks between adjacent peptidoglycan chains, removing a terminal D-alanine residue from one of the former peptidoglycans. Then, covalent bonds are created by glycosyltransferases (GT) between adjacent NAM and NAG sugar molecules. These covalent bonds help creating a rigid cell wall that protects the bacterial cell from osmosis and cell rupture.

However, BLCs work by inhibiting bacterial cell-wall synthesis (Figure 7) and when a BLC is present, it binds to these PBPs. This disrupts the structure of the cell wall and interferes with the transpeptidation process, through an activation of bacterial cell wall hydrolases and autolysins which leads to an osmotic lysis.<sup>87</sup> They therefore induce a futile cycle of peptidoglycan synthesis and degradation that may end in the inhibition of bacterial cell division, induction of morphology changes and cell death.<sup>88,89</sup>



**Figure 7.** Mechanisms of action of BLCs (adapted from<sup>90</sup>). NAG: N-acetylglucosamine; NAM: N-acetylmuramic acid.

### 1.6.3. Immediate allergic reactions to $\beta$ -lactam antibiotics

Allergic reactions to BLCs are important since they can cause all four types of DHRs. Furthermore, anaphylaxis, maculopapular exanthema and urticaria are the most common clinical manifestations of BLC allergy,<sup>91</sup> with allergic reactions caused by anaphylaxis being of great concern due to the risk that they entail and the sunset onset of the reactions. For instance, a study showed that the incidence of allergic reactions due to anaphylaxis was between 1 and 5 in 10,000 patients after PG was administrated,<sup>92</sup> corresponding to relatively high morbidity. Another study revealed that PG caused an estimated 75 % of all anaphylactic deaths in the USA, approximately 500 to 1000 per

year.<sup>93</sup> Consequently, special attention is given to immunologically IgE-mediated reactions (Type I). Discovered in 1960, IgE is one of the five immunoglobulin antibodies produced by the immune system,<sup>94</sup> and is specifically mainly found in the lungs, skin and mucous membranes of mammals. IgE antibodies are associated for their role in allergic type I reactions since they bind ( $K_a \sim 1 \cdot 10^{-10} \text{ M}^{-1}$ ) to receptors, FcεRI and FcεRII-CD23, on the surface of mast cells and basophils. They upregulate the expression of both types of Fcε receptors and their participating role in allergic reactions, and their interaction with the complementary allergens.<sup>95</sup>

Generally, total IgE (tIgE) levels increase when the allergic episode occurs, with common levels in allergic patients, in international units (IU), ranging from 100 to up to 2,500 IU/mL (1 IU/mL = 1 kU/L = 2.4 ng IgE/mL). In this respect, total IgE levels diminish over time in tune with specific IgE (sIgE) levels to BLCs.<sup>96,97</sup> Even though sIgE levels decrease, it does not mean that the patient would tolerate the β-lactam antibiotic. A study by Hjortlund et al.<sup>98</sup> showed a boosting of tIgE levels after a controlled BLC readministration in ‘formed’ allergic patients, which might lead to new allergic episodes. Regularly, total IgE levels are not considered since they do not seem to be useful to diagnose allergies to BLCs.<sup>99</sup> However, the determination of the ratio of sIgE to the corresponding β-lactam to tIgE was found to be useful for the diagnosis of BLC allergies in those allergic patients with tIgE levels up to 200 IU/mL.<sup>99</sup>

The presence of sIgE is at most from 20 to 50 % of the tIgE.<sup>100</sup> Despite almost the entire population has detectable IgE, only from 20 to 30 % of this IgE is specific IgE. It has been stated that the remainder IgE could have similar characteristics with other immunoglobulin classes or isotype IgE could randomly switch in B cell clones.<sup>100</sup> Still, these low figures are likely related to the lack of the appropriate diagnostic tools and knowledge.

In order to determine the level of specific IgE, the radioallergosorbent test (RAST) classification regarding the prevalence of allergy is used. It ranges from absent or undetectable sIgE to extremely high level of sIgE to an allergen (Table 4).<sup>101</sup>

**Table 4.** RAST classification of drug allergy

<b>IgE level (IU/mL)</b>	<b>Level of sIgE to an allergen</b>
< 0.35	Absent or undetectable
0.35 - 0.69	Low
0.70 - 3.49	Moderate
3.50 - 17.49	High
17.50 - 49.99	Very high
50.00 - 100.00	Ultra high
> 100.00	Extremely high

#### 1.6.4. $\beta$ -lactam metabolites and structural antigens

In 1935, Landsteiner and Jacobs described the hapten model, reporting the inability of small molecules (haptens) to induce an immune response unless they were chemically coupled to soluble molecules as carrier proteins.<sup>102</sup> In this sense, haptens would act as epitopes (antigenic determinants) being recognized by antibodies due to the immunogenicity provided by the carrier proteins, which successfully trigger the cascade of events and stimulate an immunological response in the form of anti-hapten antibodies.<sup>103</sup> The carrier proteins are most commonly proteins of different origin, like hemocyanins, albumins, globulins, and other structures such as virus-like particles or toxoids.<sup>104</sup> This model provides a good explanation for the allergic reactions caused by  $\beta$ -lactam antibiotics,<sup>49</sup> since they present a highly reactive  $\beta$ -lactam ring and other functional groups which have the possibility to covalently bind to carrier molecules and form antigens.<sup>105</sup>

In this respect, IgE antibodies will be able to recognize small chemical differences between the formed antigens.<sup>106</sup> The side-chain structures of the BLCs have been found to induce allergic episodes without the participation of the  $\beta$ -lactam ring and the fused ring attached to it.<sup>107-109</sup> It is worth mentioning that, in addition to side-chain recognition, other studies revealed the  $\beta$ -lactam ring and/or fused ring moieties are responsible for IgE production in allergic patients.<sup>110-112</sup> Therefore, it is proposed that all BLCs may induce DHRs due to the different metabolites, chemical moieties and degradation products formed *in vivo* when the desired antibiotic is administered and bound to a carrier protein under physiological conditions.

##### a) Penicillins

The penicillin-induced allergic reactions were the first IDHRs whose mechanism was studied in detail. Levine discovered that benzylpenicilloyl (BPO) is a PG metabolite that linked to polylysine forms, what he called, the major PG antigenic determinant A2,<sup>113</sup> shown in Figure 8. To do this, Levine and Ovary<sup>114</sup> conjugated D-benzylpenicillenic acid with various soluble molecules, forming antigens by binding both with amino groups from lysine residues and with disulfide bonds from cysteines. The characterization of the prepared antigens by quantitative hapten inhibition by precipitation, PCA inhibition, rabbit immunization and evaluation of skin-test responses in individuals demonstrated that the penicilloyl determinant was extremely important as a responsible determinant of many of the IDHRs to PG. This determinant was therefore considered the most predominant antigen,<sup>115</sup> since about 95 % of penicillin molecules bound to a carrier protein under physiological conditions were thought to form penicilloyl groups that play a role in 75 % of IgE-mediated allergic reactions.<sup>116</sup>

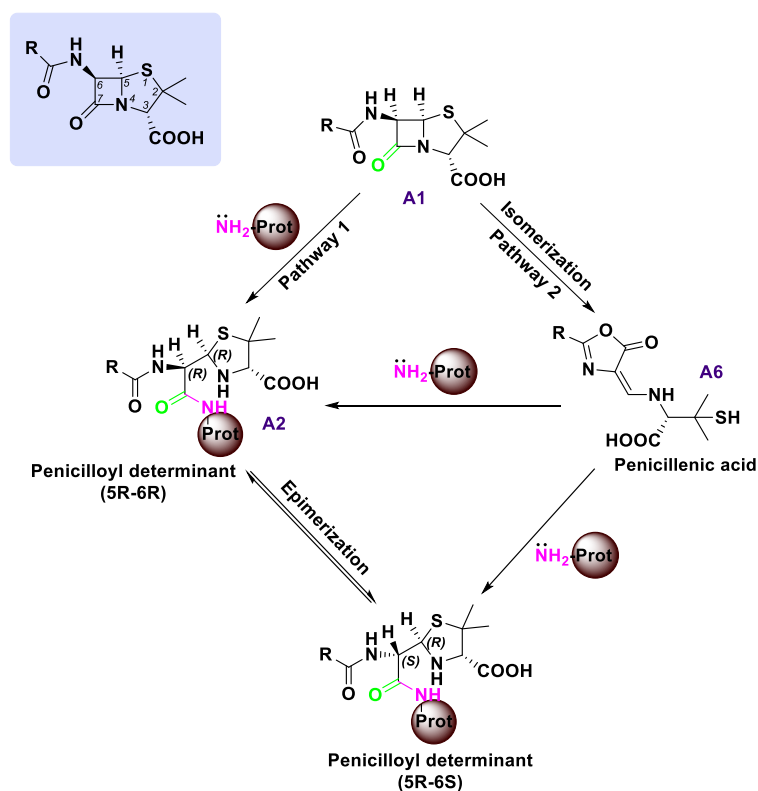
**Major determinants** form -oyl groups, by predomination of the 5R,6R diastereoisomer configuration Amidation between the carboxylic acid of the  $\beta$ -lactam ring and the  $\epsilon$ -amino groups of lysine residues, such as Lys<sub>199</sub>, opens the  $\beta$ -lactam ring (Figure 8, pathway 1).<sup>117,118</sup> Levine first demonstrated the formation of this determinant for PG at a high pH.<sup>119</sup> However, later studies showed that this reaction could likewise proceed at physiological conditions.<sup>120,121</sup> This penicilloyl determinant can also be formed after rearrangement to its isomer, penicillenic acid A6, under neutral conditions by nucleophilic attack, which has been observed to bind selectively to e.g., Lys<sub>199</sub> and Lys<sub>525</sub> of human serum albumin (HSA), and form BPO-lysine adducts (Figure 8, pathway 2).<sup>122</sup> The preferred configuration in this case is the 5R,6S diastereoisomer as was characterized after continuous incubation of penicillenic acid A6 with HSA.<sup>118</sup> Furthermore, BPO linked to HSA between Met<sub>123</sub> and Met<sub>297</sub> residues, and between Met<sub>297</sub> and the C-terminal residue have been observed.<sup>123</sup>

Insulin and lysozyme have also been used as carrier proteins to form antigens. With regard to insulin, PG antigens are formed via amidation well with the  $\alpha$ -amino group of the N-terminus of the A chain, or with the  $\epsilon$ -amino groups of lysine residues. For lysozyme, the  $\epsilon$ -amino group of Lys<sub>116</sub> was observed to react with PG.<sup>124</sup>

The other 5 % of ADRs to penicillins are caused by **minor determinants**. These molecules are related to induce anaphylaxis in 95 % of the cases,<sup>115,125</sup> although their implication in the mechanism of formation of IDHRs is still unknown.<sup>107</sup> In

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particular, penicillins are supposed to remain in equilibrium with pseudopenicillin A3 under neutral conditions. However, they are characterized by susceptible ring-opening under different conditions, such as the presence of acids, bases, nucleophiles, oxidizing agents, heat, UV light, or polar solvents, as observed in Figure 9.

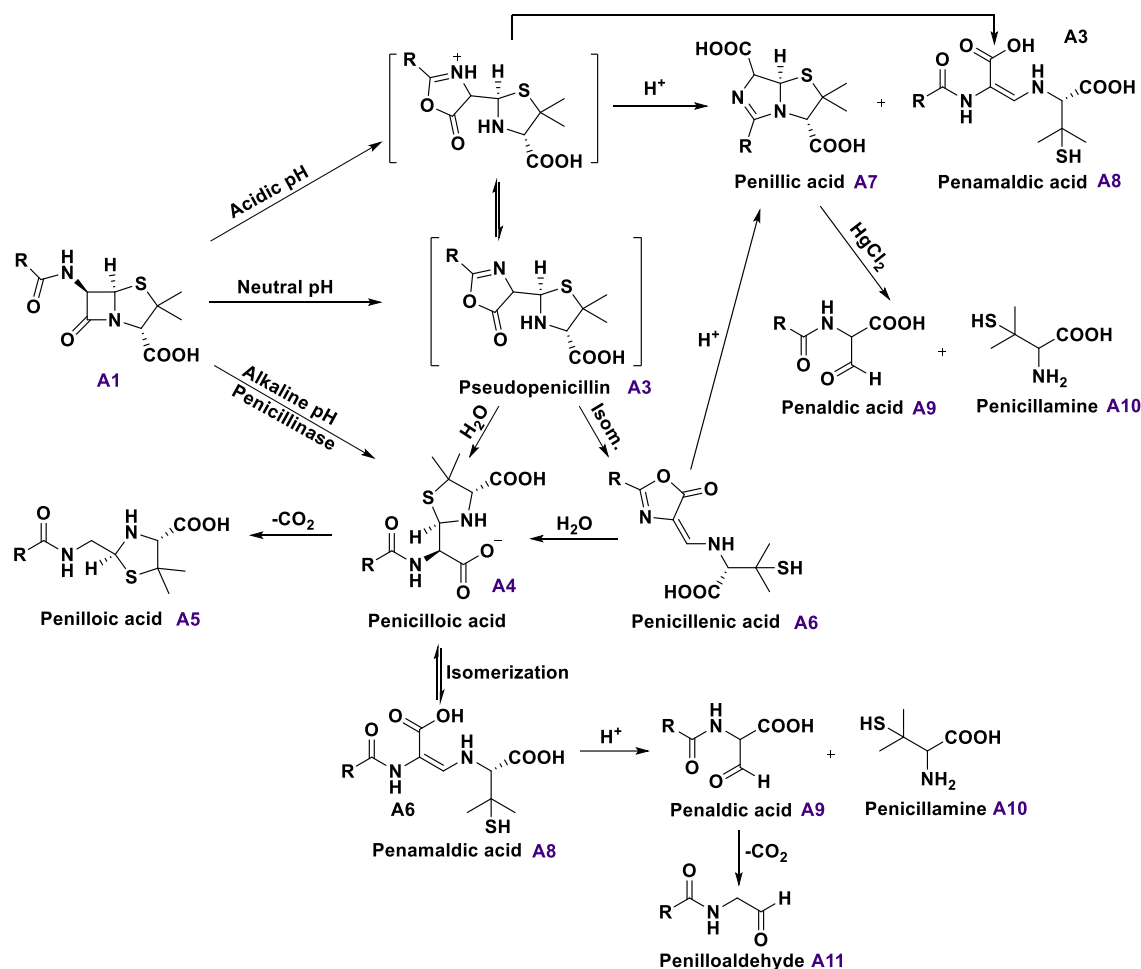


**Figure 8.** Scheme of the penicilloyl formation

Penicillin metabolites, or degradation products, that can form minor determinants include penicillin A1, penicilloic acid A4 (penicilloate in its salt form), penilloic acid A5 (or penilloate), penicillenic acid A6 (or penicillenate), penillic acid A7 (or penillate), penamaldic acid A8 (or penamaldate), penaldic acid A9, penicillamine A10, penilloaldehyde A11, 6-APA A12 and penicoyl A13 (see Figure 11).<sup>126–128</sup>

Several studies using penicilloic acid A4 and its decarboxylated penilloic acid A5 revealed positive skin results in a range between 21 and 55 % of the selected patients.<sup>129–132</sup> However, the specificity of penicilloic acid-directed reactions still remains unclear and it is the hapten and not its determinant the one that has been used in skin testing.<sup>129</sup> Moreover, *in vivo* penicilloic acid A4 is believed to react with cysteine disulfide via its penamaldic acid A8 to form penamaldic acid cysteine mixed disulfide and finally, penicillamine and penilloaldehyde A11, as shown in Figure 9.<sup>122</sup>



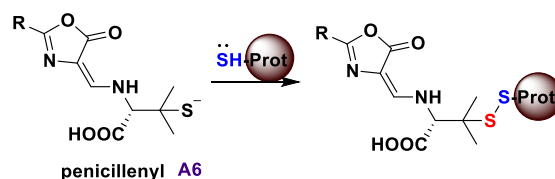


**Figure 9.** Pathways of degradation of penicillins in acidic and alkaline conditions

Penicillenate A6, penamaldate A8 and penicillamine A10 can be bound via disulfide bonds with cystine residues to form antigens (Figure 10).<sup>133</sup> In particular, patients allergic to PG, even when showed higher responses to BPO, have been observed to have IgE antibodies directed against the penicillenate A6 and penicillamine A10 determinants and therefore exhibit heterogeneity in those IgE responses.<sup>134</sup> Furthermore, penicillamine A10 determinants linked to poly-L-lysine (PLL) and HSA tested positive by skin testing in 35 to 41 % of the studied patients, respectively.<sup>122</sup> In the case of the penamaldate A8 and penaldate A9 determinants, a study using guinea pigs showed that they could act as antigens, but no specific antibodies were detected in pigs immunized with BPO.<sup>129</sup> Moreover, disulfide metabolite-metabolite interactions can occur as well as penicillin polymerized metabolites. Nevertheless, no recent studies are found in the literature related to the formation of antigens from penicillin metabolites, and their

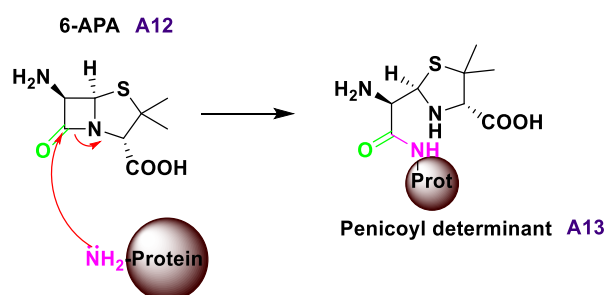
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allergenic significance therefore still lacks substantial evidence. More research is needed to discover new antigens with epitopes responsible to generate sIgE.



**Figure 10.** Formation of the penicillanyl antigen

6-APA (compound A12) can react with lysine  $\epsilon$ -amino groups of the protein under physiological conditions to give the penicoyl determinant A13, shown in Figure 11.<sup>120</sup> What is more, polymeric 6-APA has been observed in stored solutions of potassium PG.<sup>126,135</sup> Still, the clinical significance of 6-APA and related antigens needs to be fully evaluated since it has been stated that their immunogenicity is related to contamination with penicilloyl moieties.<sup>117,136</sup>

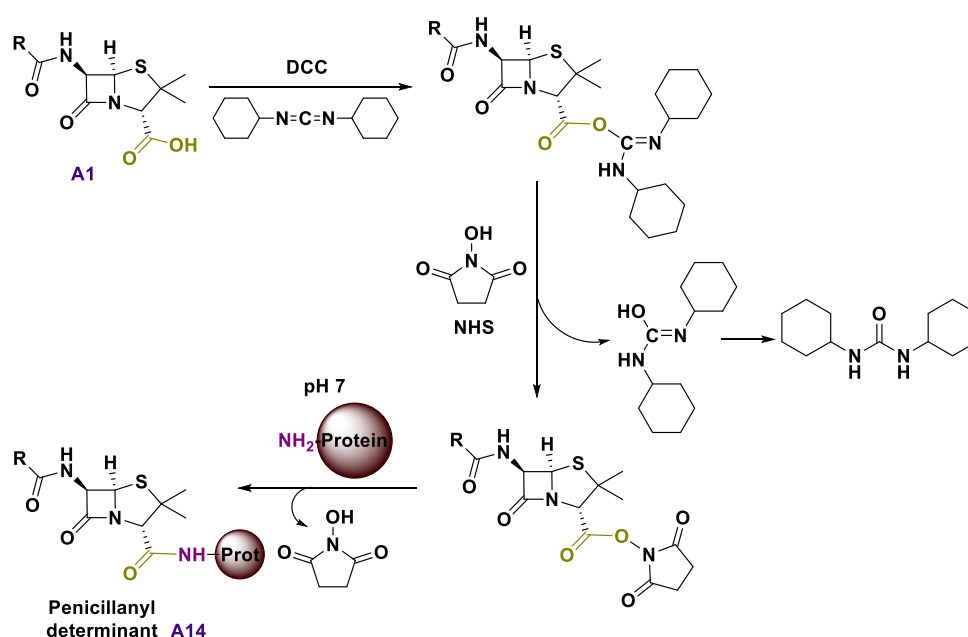


**Figure 11.** Scheme of the formation of the penicoyl determinant

Other minor determinants have been proposed for penicillins. For instance, amidation through the carboxyl group presented in the C-3 of the thiazolidine ring of the penicillin with amino groups of lysine residues of carrier proteins leads to penicillanyl (BPA) antigen A14 (Figure 12), proposed by Feinberg in 1970. Although formation of this determinant under physiological conditions seemed improbable, clinical studies revealed that sIgE from allergic patients commonly recognize this determinant, inducing the formation of specific antibodies for the whole penicillin molecule, that differ from those to the penicilloyl.<sup>111,134</sup> For instance, in a study by Zhao et al.<sup>138</sup> they compared the *in vitro* reactivities of BPO and BPA determinants coupled to cyanogen bromide (CNBr) activated papers and nitrocellulose (NC) discs with serum from 47 allergic patients. The determinants were linked to different carrier molecules, HSA and PLL. The results revealed that IgE from the allergic patients detected BPA in more sera than BPO. On CNBr-activated papers, 55.3 vs. 83.0 % (BPO vs. BPA) and 41.5 vs. 58.1 % with PLL-

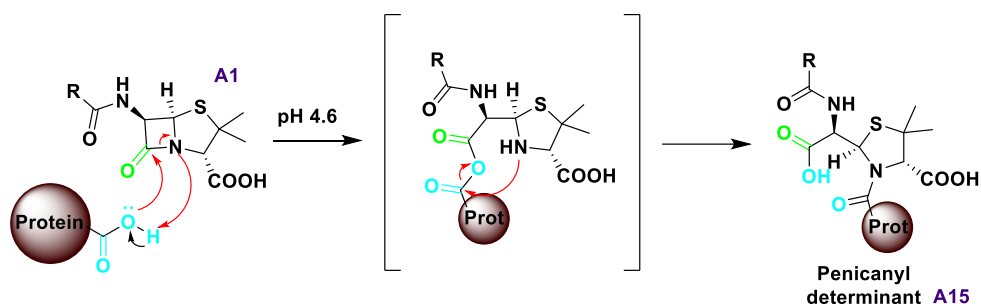
and HSA-based determinants, respectively, were positive, whereas on NC discs, 38.6 vs. 50.0% (BPO vs. BPA) and 75.6 vs. 70.7 % (PLL- vs. HSA-based determinants) tested positive.

Penicillanyl antigens are prepared via the mixed anhydride or the carbodiimide methods. Nevertheless, the carbodiimide method is commonly preferred over the mixed anhydride since the formation of a N-hydroxy succinimide (NHS) ester avoids the presence of cross-linking problems with carrier proteins.<sup>134</sup> Theoretically, minor penicillanyl antigens of penicilloate A4, penilloate A5 and penillate A7 can be therefore obtained.



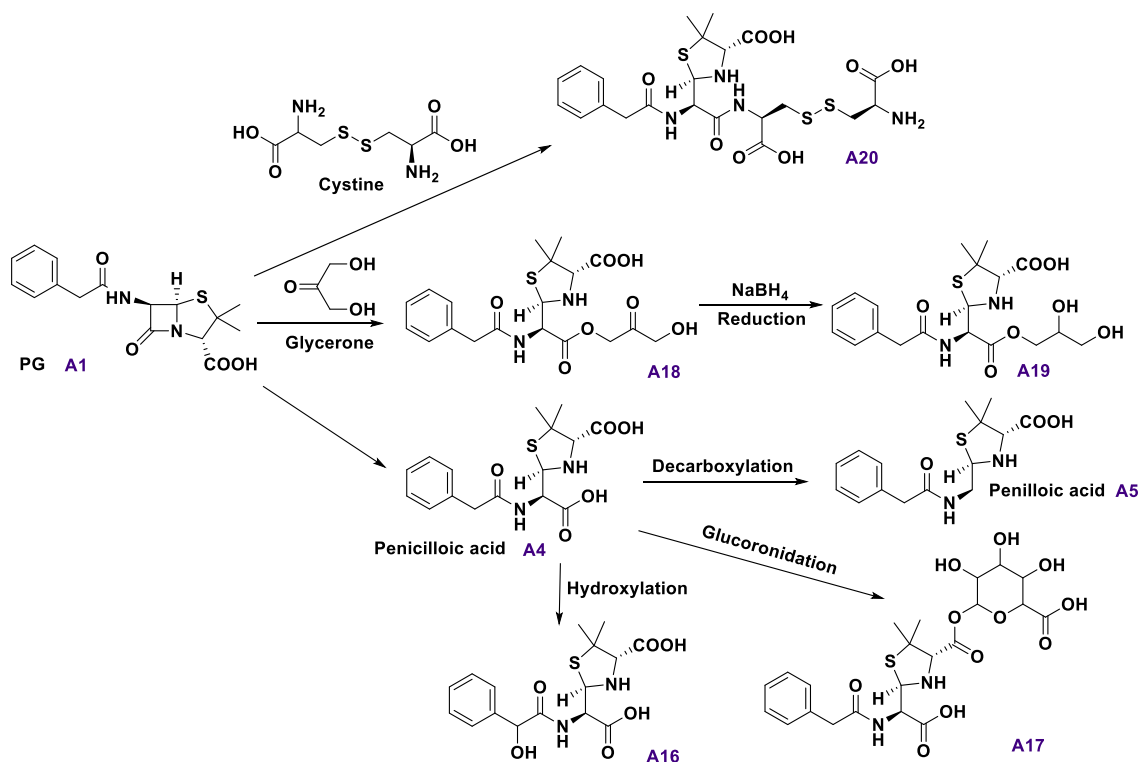
**Figure 12.** Pathway of penicillanyl formation via the carbodiimide method

Matas et al.<sup>137</sup> proposed penicanyl (A15) as a new metabolite for penicillins, after the covalent attachment of the carrier protein through the formation of a mixed anhydride intermediate, following the pathway shown in Figure 13. For that, they prepared AMX antigens linked to modified peptides, which have attached a biotin group. They were evaluated with sera from 15 allergic patients to penicillin through a magneto-enzyme-linked immunosorbent assay (ELISA). Results revealed that the new proposed epitope “penicanyl” was recognized by around 80 % of the samples, whereas only 40 % were recognized by the amoxicilloyl configuration. However, it is worth mentioning that these antigens were tested only with one serum from a pool of control patients and the diagnostic specificity remains unknown.



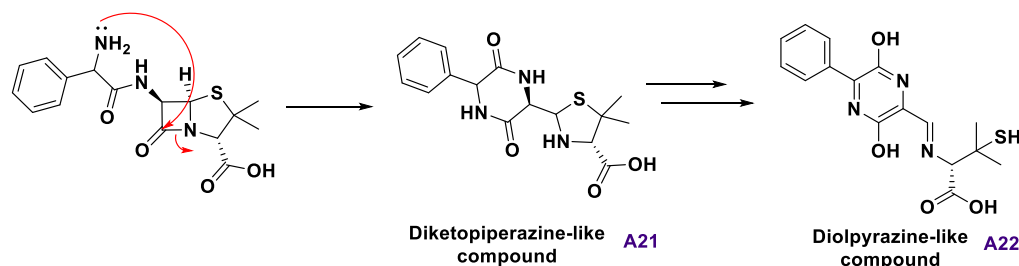
**Figure 13.** Proposed structure for the formation of the metabolite penicanyl

Ho et al. identified 5 minor metabolites of PG in real human serum samples.<sup>138</sup> Figure 14 shows the structures of the 5 new minor metabolites of PG and the proposed pathway for their formation. In brief,  $\beta$ -lactam ring opening affords penicilloate A4 (labelled as its corresponding acid, penicilloic acid) and its decarboxylation gives penilloate A5 (or penilloic acid). From here, the formation of A16 and A17 might be based on hydroxylation and glucuronide conjugation of penicilloic acid, respectively. The reactive metabolite A18 was proposed to be generated *in vivo* by reaction with glycerone, followed by a reduction to afford A19. Finally, reaction of PG with cysteine could form the compound A20. However, these metabolites A16-A20 have not been evaluated in sera from allergic patients.



**Figure 14.** Proposed pathway for the formation of minor metabolites of PG

Aminopenicillins can also experience intramolecular acylation by the amino group in the C-6- $\beta$ -acyl substituent of the R side chain of aminopenicillins to form diketopiperazines-like compounds.<sup>139</sup> These compounds can slowly undergo oxidation of the diketopiperazine ring in basic or neutral conditions followed by thiazolidine ring opening to give diolpyrazine-like compounds. Figure 15 shows the formation of compounds A21 and A22 with AMP.<sup>140</sup> However, several studies have shown that skin testing and *in vitro* sIgE quantification using this determinant does not improve the clinical diagnosis. For instance, Torres et al.<sup>139</sup> revealed that in a study of 61 patients allergic to AMX, only 3 (diagnostic selectivity of 4.9 %) were positive to diketopiperazine by skin testing, and very little inhibition was observed. Positive patients were furthermore recognized with AMX itself, highlighting that the diagnosis of allergies to aminopenicillins was not enhanced after these diketopiperazine-like determinants were used. Therefore, the implementation of such determinants does not seem to be helpful for the *in vitro* drug allergy testing of aminopenicillins.



**Figure 15.** Pathway of the formation of the diketopiperazine and diolpyrazine-like compounds with ampicillin

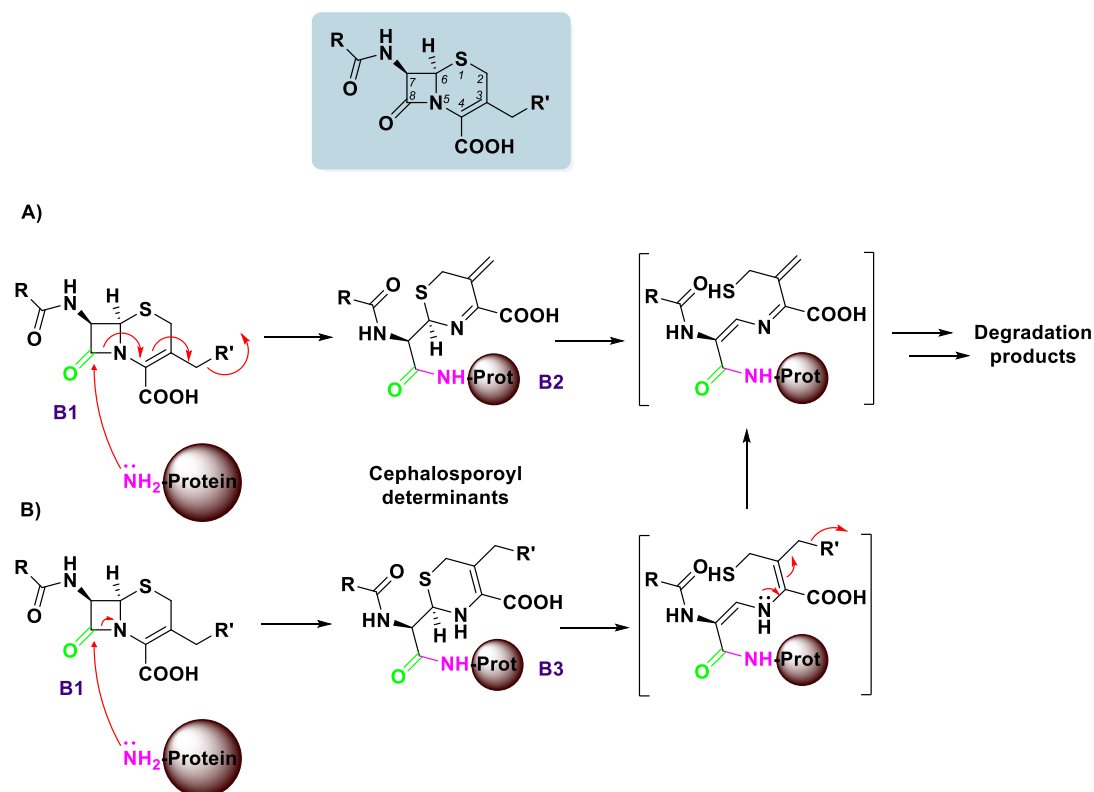
## b) Cephalosporins

The dihydrothiazine ring attached to the  $\beta$ -lactam ring confers more stability to cephalosporins in comparison to penicillins. However, they are prone to undergo a variety of chemical and enzymatic reactions whose degradation products, or metabolites, depend on both the R and R' side chains at C-3 and C-7, respectively.<sup>126</sup>

Furthermore, the allergenic structures of cephalosporins are not characterized as well as penicillins are. Preparation of **major determinants** of cephalosporins under alkaline conditions through aminolysis of cephalosporins with a carrier protein, by nucleophilic  $\beta$ -lactam ring opening, afforded products not well characterized. However, they are used in the same way as those for penicillins, even when uncertainty existed.<sup>106,141,142</sup> Later on, studies demonstrated the influence of the leaving group in the

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C-3 position of the dihydrothiazine ring for its own stability and reactivity of the  $\beta$ -lactam ring (Figure 16).<sup>136</sup>

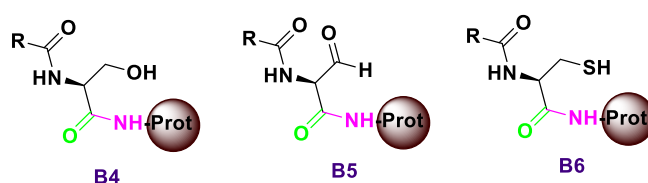


**Figure 16.** Proposed formation of the cephalosporoyl antigens. A) Good R' leaving group.  
B) Poor R' leaving group

Thus, nucleophilic attack and amidation through amino groups of lysines of carrier proteins and the  $\beta$ -lactam ring result in  $\beta$ -lactam ring opening and elimination of the R' leaving group, yielding labile compounds and allowing for the detection of sIgE to cephalosporin in sera from allergic patients.<sup>143</sup> This procedure is different according to the properties of the R' leaving group at C-3. First, aminolysis in presence of a good leaving group at C-3, as Cl in cefaclor or acetoxy in cephalothin, forms cephalosporoyl determinant B2, which presents a double bond at  $\Delta$ -4 and a methylene group at C-3, and eliminates the R' leaving group (Figure 16a). Second, aminolysis in presence of a poor leaving group, as the methyl group in cephalixin and cefadroxil, forms the cephalosporoyl structure B3, via its major metabolite anhydrodeacetyl cephalosporoic acid, without the immediate breakdown of the dihydrothiazine ring or cleavage of the leaving group, but only temporarily (Figure 16b). Finally, unlike penicillins, both procedures end with the decomposition of these unstable cephalosporoyl intermediates to compounds presenting well the R side chain, the attached amide or parts of the  $\beta$ -lactam

ring.<sup>112</sup> They form penaldate and penamaldate-like structures, whose amino group was observed to be part of the lysine residue and not part of the dihydrothiazine ring of the cephalosporin.<sup>136</sup>

In this respect, Montañez et al.<sup>144</sup> proposed the structures B4-B6 of these degradation products (Figure 17). For that, they designed and synthesized structures that mimicked a fragment of the cephalosporin determinants proposed, consisting of molecules with low molecular weight but with different functional groups at C-3 and R side chains. Specific IgE from five allergic patients recognized some of these prepared skeletons in those epitopes related to the R lateral acyl side chain of the cephalosporin. However, the number of samples from allergic patients used in this study was very low and only two concentrations (10 and 100 mM) were tested. Therefore, more studies related to their recognition by IgE antibodies need to be performed.

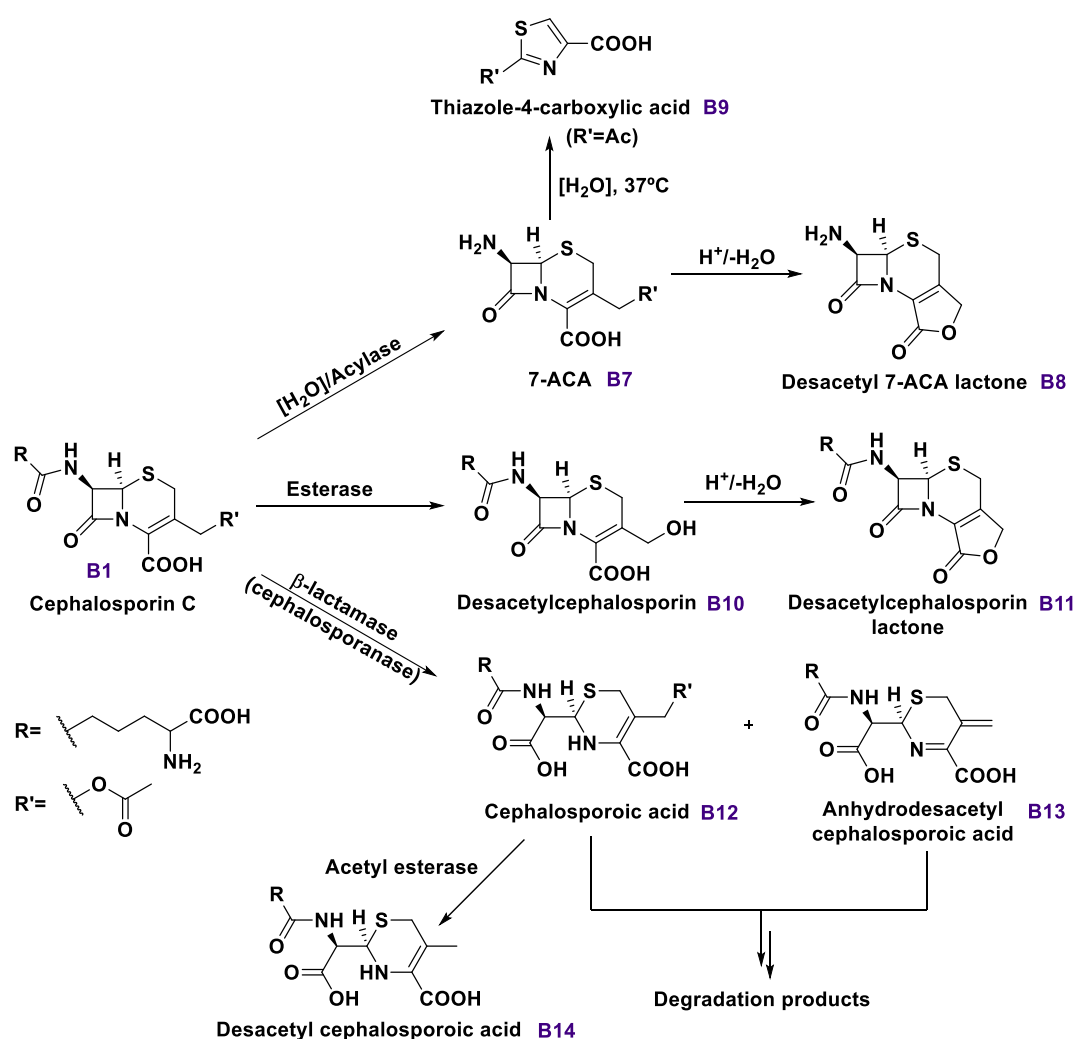


**Figure 17.** Hypothetical degradation structures for cephalosporin antigens B4-B6

Regarding the formation of **minor determinants** of cephalosporins, enzymatic degradation of cephalosporin C in particular gives different metabolites (Figure 18), which lead to many fragmented and rearranged products.<sup>126</sup> First, 7-ACA (compound B7) is obtained after enzymatic degradation with acylase, which undergoes lactonization to give the desacetyl-7-ACA lactone B8. Second, cephalosporin C gives desacetyl cephalosporin B10 followed by desacetyl cephalosporin lactone B11 in presence of the enzyme esterase. Finally, treatment of cephalosporin C with  $\beta$ -lactamases (as cephalosporanases) gives cephalosporoic acid B12 and anhydrodesacetyl cephalosporoic acid B13, which leads to the degradation products shown in Figure 17. Moreover, the action of acetyl esterase to B12 affords desacetyl cephalosporoic acid B14.

Venemalm<sup>145</sup> showed that a different pattern (Figure 19) involving  $\alpha$ -amino cephalosporins, such as cefaclor, cephalexin or cefadroxil, can also undergo aminolysis through the amino group in the 7- $\beta$ -acyl substituent of the R side chain. Thus, the formation of pyrazinone degradation determinant B16 after nucleophilic attack of amino groups of lysine residues of carrier proteins and the  $\beta$ -lactam ring permitted the detection

of pyrazinone-specific IgE antibodies, as observed in a study by Martin-Serrano et al.<sup>146</sup> They showed that five out of the eight allergic patients included in the study recognized this determinant (diagnostic sensitivity of 63 %). Even so, aminolysis of these compounds can also lead to other determinants (as the cephalosporoyl B3), apart from the pyrazinone-like determinant, involving the R side chain, because it is not unusual to find patients allergic to  $\alpha$ -amino cephalosporins who are not allergic to other BLCs with the same or similar R side chain.<sup>147</sup> Again, as stated for penicillins, both studies on the subject and the quantity of sera available from allergic patients are limited, so the results are not very conclusive.

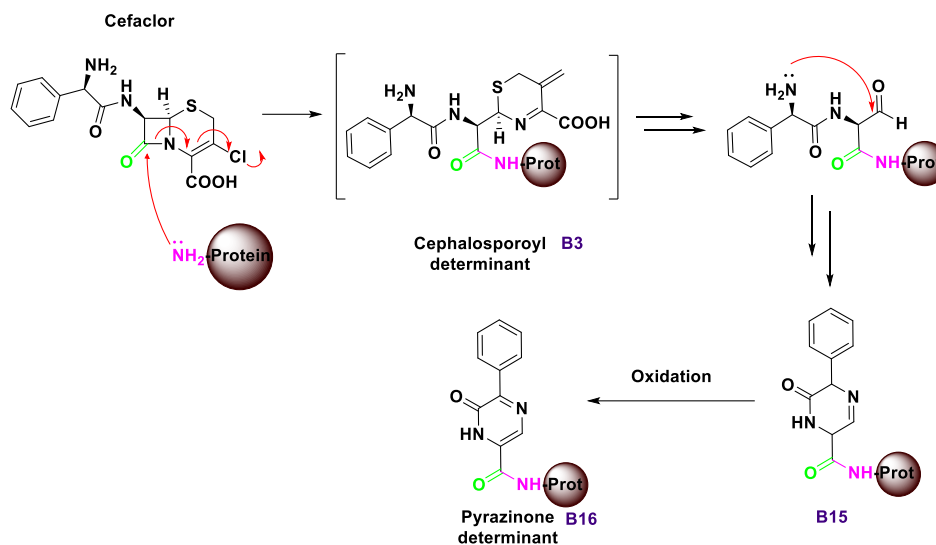


**Figure 18.** Hypothetical pathway of degradation of cephalosporin C

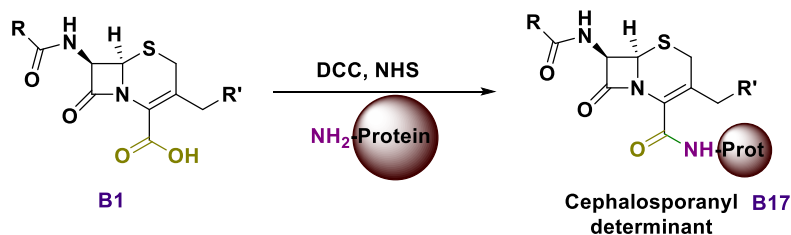
As with penicillins, formation of the cephalosporanyl determinant B17 via carbodiimide coupling has been studied,<sup>106,117</sup> treating to maintain the structural integrity



of the cephalosporins (Figure 20). These studies revealed the presence of immunogenic activity.



**Figure 19.** Pathway of the formation of the pyrazinone-like antigen with cefaclor



**Figure 20.** Formation of the cephalosporanyl determinant

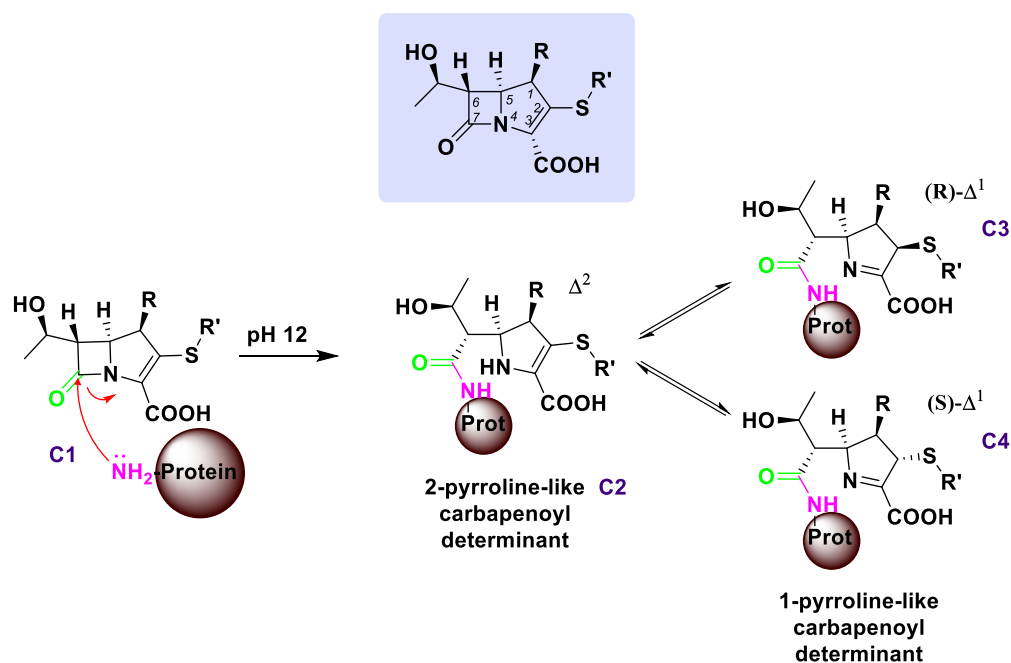
For instance, in a study by Pham and Baldo,<sup>142</sup> cephalosporanyl antigens were prepared from different cephalosporins. The prepared antigens showed activity when hapten inhibition studies were performed using 12 sera from allergic patients to cefaclor, enlightening differences in the recognition pattern of the IgE antibodies regarding to the R and R' lateral chains of the cephalosporins. Another study by Zhao et al.,<sup>117</sup> used the cephalothinanyl antigen, linked to PLL, as a solid phase and studied its inhibition by some penicillins, related molecules, and cephalothin, as a model of the cephalosporins. The diagnostic selectivity ranged from 20 to 68 %. Nevertheless, this study evaluated the recognition of the cephalothinanyl antigen by IgE testing only one serum sample from an allergic patient to AMX, so this result is not conclusive.

### c) Carbapenems

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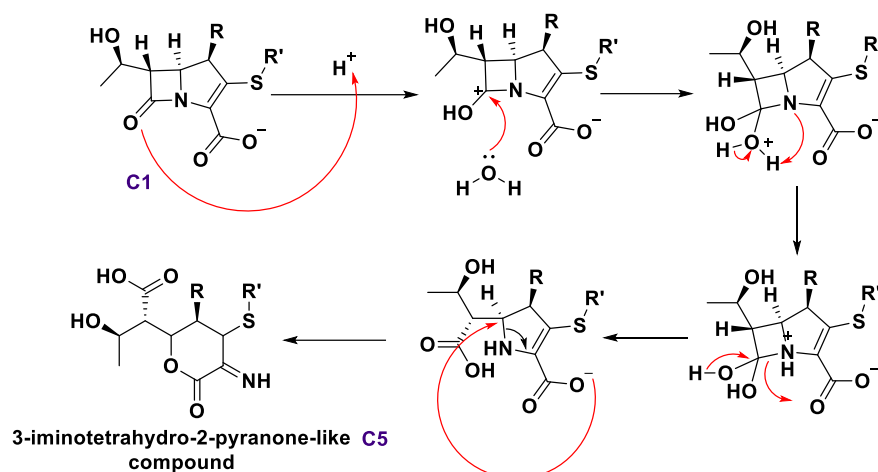
Carbapenems have significant structural similarity with penicillins and the **major determinant** is a stable carbapenoyl compound, similar to that of penicillins and capable of binding to amino acid residues (Figure 21).<sup>148</sup>

Lohans et al.<sup>149</sup> examined the stereoselectivity of the alkaline hydrolysis of both MRP and imipenem by sodium hydroxide and observed rapid formation of the  $\Delta^2$  (enamine) hydrolysis, or 2-pyrroline-like, product C2 after the corresponding  $\beta$ -lactam ring opening. Its rapid tautomerization formed the (R)- $\Delta^1$  (imine) hydrolysis product epimer C3, which slowly epimerized to afford the (S)- $\Delta^1$  hydrolysis product C4. In any case, tautomerization of the  $\Delta^1$ , or 1-pyrroline-like, epimers C3-C4 back to the hydrolytically-susceptible  $\Delta^2$  form C2 is potentially possible.<sup>149</sup>



**Figure 21.** Scheme of the formation of the carbapenoyl determinant

However, presently, the immunochemistry of carbapenems is not fully elucidated and the possible **minor determinants** therefore remain unknown. A study by Sun et al.<sup>150</sup> about the characterization of carbapenem compounds revealed the loss of one  $\text{CO}_2$  molecule for the acid hydrolysis of both MRP and imipenem. This finding contradicted what was previously reported about the formation of 1-pyrroline or 2-pyrroline structures with two carbonyl acid units. Thence, they proposed the formation of the six-membered cyclic, 3-iminotetrahydro-2-pyranone compound C5 after acid hydrolysis of one of the carbonyl acids (Figure 22).

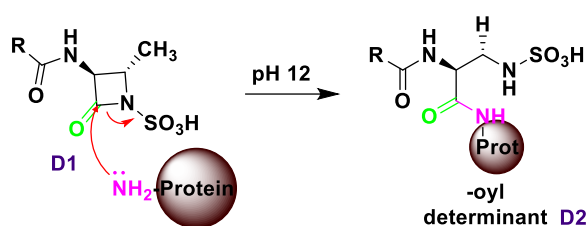


**Figure 22.** Proposed pathway of the formation of the 3-iminotetrahydro-2-pyranone-like structure

On the other hand, cross-reactivity of carbapenems with other BLC families has been reported to be very low and rare (< 4.4 %), with the observation that IgE antibodies against the  $\beta$ -lactam ring were not formed.<sup>136</sup> It is therefore thought that IgE antibodies against carbapenems are directed towards the R' lateral chain at the sulfur atom at C-3 and at C-6.

#### d) Monobactams

The **major determinant** of monobactams D2 is considered to be formed similarly to that of penicillins (Figure 23).<sup>136</sup>

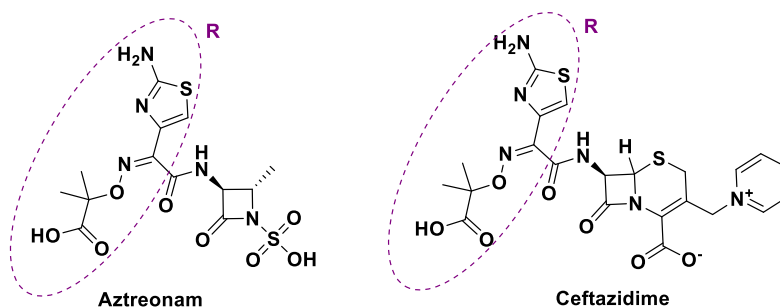


**Figure 23.** Scheme of the -oyl formation for monobactams

Nevertheless, the unique chemical structure of the monobactams and their metabolites, formed only by the  $\beta$ -lactam ring, is responsible for the structural difference to other BLCs. Hence, no cross-reactivity has been observed for AZT with neither other monobactams with a different R side chain nor different BLCs.<sup>136,151</sup> However, when AZT antibodies were tested against the cephalosporin ceftazidime, which has the same R

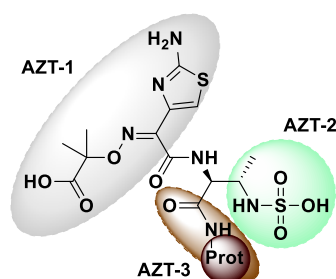
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side chain (Figure 24), cross-reactivity existed.<sup>152,153</sup> This result therefore reveals that the R side chain might be the major epitope of monobactam allergy.<sup>148</sup>



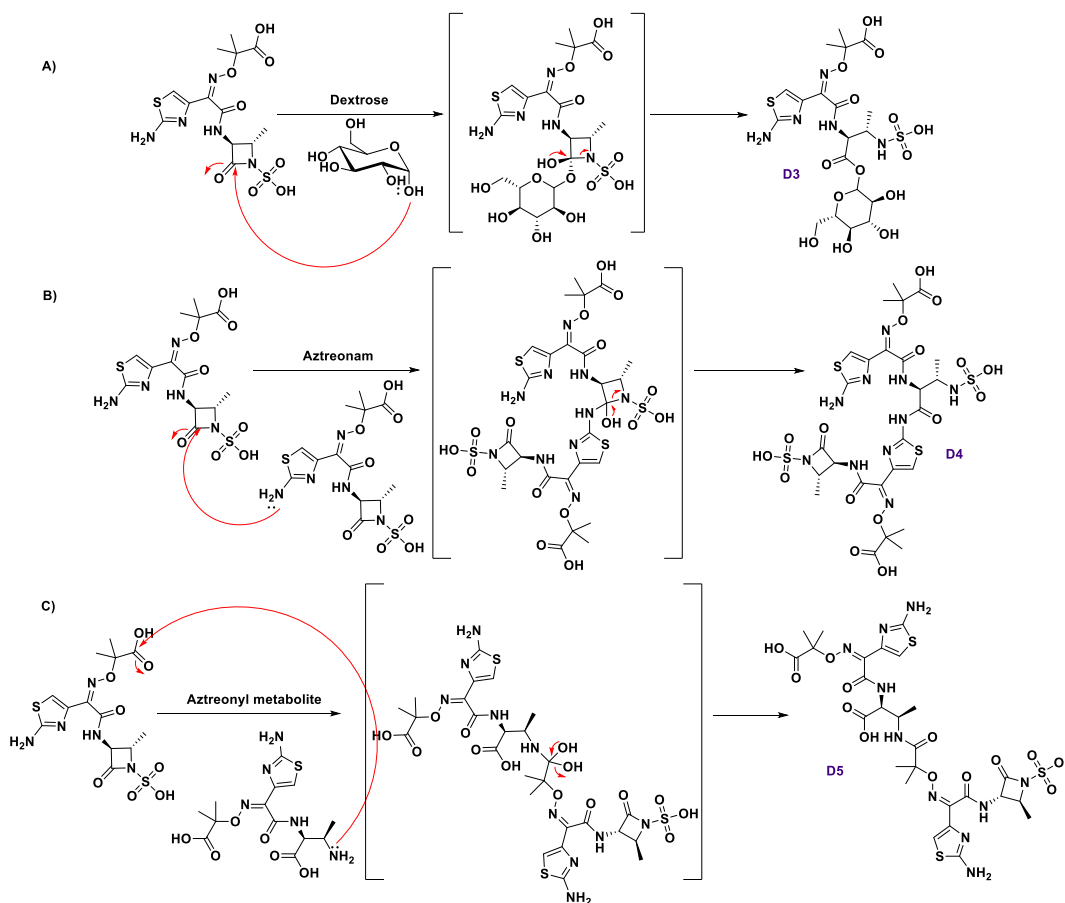
**Figure 24.** Chemical structures of aztreonam and ceftazidime with the same R lateral chain

Furthermore, Shimizu et al.<sup>154</sup> described the production of three monoclonal antibodies (mAbs) against different epitopes on the aztreonyl antigen (Figure 25). They observed that the clone AZT-3 of mAb-producing hybridoma exhibited cross-reactivity with many other BLCs.



**Figure 25.** Possible epitopes (AZT-1, AZT-2 and AZT-3) of the aztreonyl antigen D2

Related to other metabolites of monobactams, Ye et al.<sup>155</sup> identified and characterized three new metabolites of AZT. From these metabolites (Figure 26), the first metabolite D3 is proposed to be formed via reaction of AZT with dextrose and metabolite D4 is an AZT pseudo-dimer. D5 was characterized as that pseudo-dimer linked by an amide bond between AZT and the aztreonyl metabolite. These structures could be linked to carrier molecules to generate antigens with which to evaluate IgE from sera from allergic patients and controls.



**Figure 26.** Proposed pathways for the formation of AZT metabolites A) D3, B) D4 and C) D5

#### e) $\beta$ -lactamase inhibitors

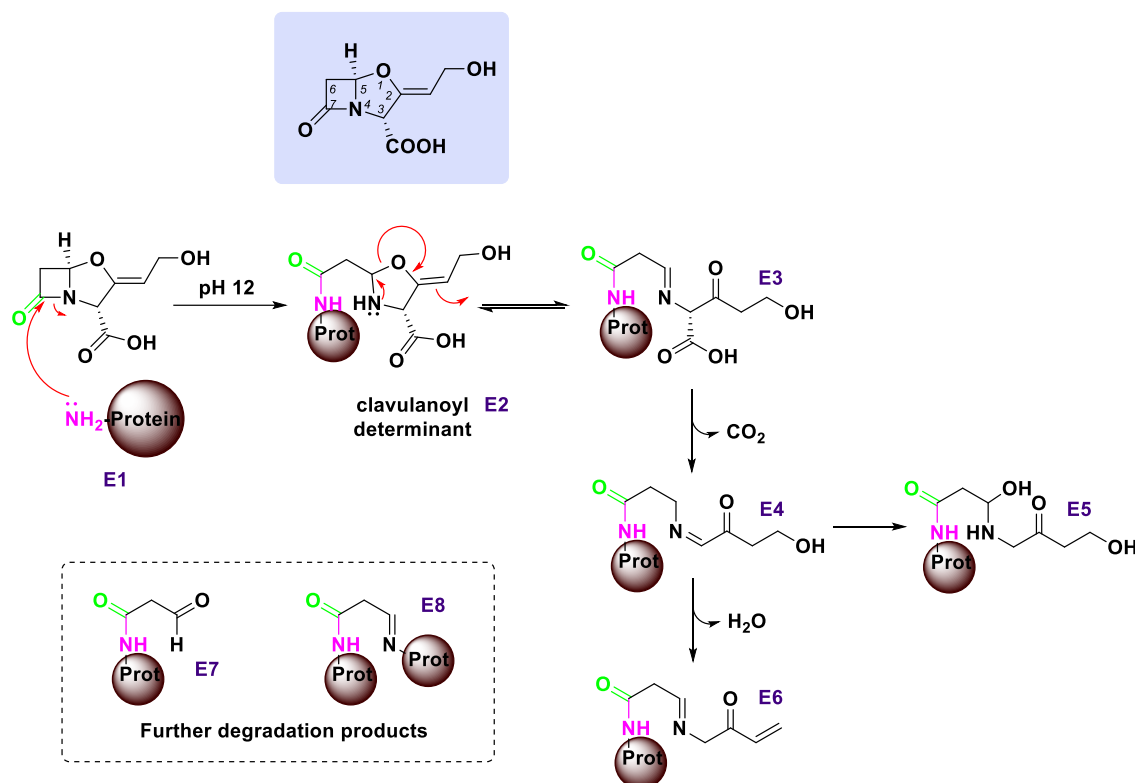
The most common prescribed  $\beta$ -lactamase inhibitors include: i) the oxapenam CVL and ii) the bactams sulbactam and tazobactam. Although there is a large focus on DHRs to BLCs, until now, little information has been reported related to the preparation of  $\beta$ -lactamase inhibitor antigens within the aim of allergic diagnosis.

CVL forms covalent adducts with *Mycobacterium tuberculosis*  $\beta$ -lactamase, which is the reason why CVL is considered a  $\beta$ -lactamase inhibitor.<sup>156</sup> CVL has a  $\beta$ -hydroxyethylidene at C-2 and no substituent at C-6. At the beginning, the formation of antigens of CVL was believed to be non-immunogenic and not the cause of DHRs to Augmentin.<sup>157</sup> However, the number of IgE-mediated allergic reactions to CVL has increased and allergic patients showed the presence of IgE antibodies to CVL.<sup>83</sup>

Amidation between amino groups of lysine residues of carrier proteins and the carbonyl carbon of CVL at C-7 results in  $\beta$ -lactam ring opening forming the clavulanoyl

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antigen E2 (Figure 27). Nevertheless, this clavulanoyl determinant forms multiple metabolites following further decomposition.<sup>83,158,159</sup>



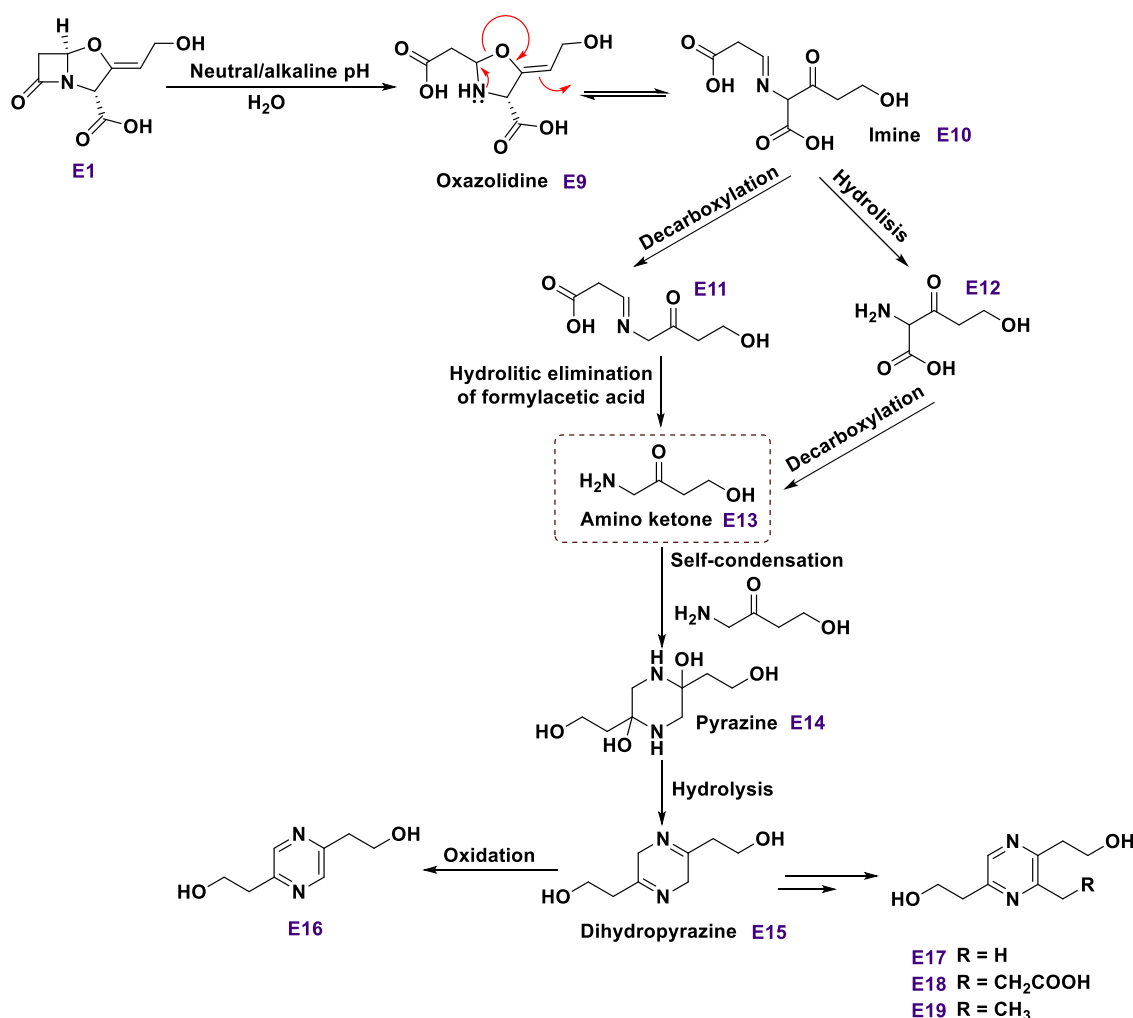
**Figure 27.** Proposed pathway of the formation of CVL antigens

The clavulanoyl determinant E2 is an oxazolidine-like intermediate which is not stable and quickly tautomerizes to the imine form E3,<sup>160</sup> and results in the formation of antigens that do not have the five-membered oxazolidine ring. Determinant E3 decarboxylates to generate E4 that can be hydrolyzed or dehydrated to generate E5 and E6, respectively. Further degradation of E3-E6 affords E7 and the cross-linking E8 determinants after stabilization by NaCNBH<sub>3</sub> reduction.<sup>159</sup>

Moreover, CVL can also undergo spontaneous hydrolysis under neutral or alkaline pH (Figure 28) to generate a variety of metabolites that can further suffer from decomposition and self-condensation.<sup>160,161</sup>

Neutral or alkaline hydrolysis of the  $\beta$ -lactam ring of the CVL results in  $\beta$ -lactam ring opening and formation of the oxazolidine-like compound E9 which, as well as the clavulanoyl determinant, rapidly tautomerizes to the corresponding imine E10. Thereupon, the amino ketone E13 can be formed after decarboxylation of E10 followed by a hydrolytic elimination of formylacetic acid from E11. At the same time, hydrolysis

of the imine E10 affords the  $\alpha$ -amino- $\beta$ -keto acid E12, which decarboxylates to give also E13.<sup>160</sup> This amino ketone E13 is stable under acidic conditions. Nevertheless, its self-condensation is undergone in neutral or alkaline conditions leading, first, the pyrazine-like metabolite E14 and later, the dihydropyrazine-like compound E15 after hydrolysis of E14. The spontaneous oxidation of E15 leads to the formation of E16 and the reaction of formaldehyde, formylacetic acid and acetaldehyde can form metabolites E17-19.<sup>161</sup> A study by Meng et al.<sup>159</sup> characterized the antigens formed by these structures as well as the epitopes involved by employing spectrometric methods. However, the origin and involvement of these aldehydes in the pathway still remains unclear, with multiple adducts identified *in vitro* at high concentrations in four patients exposed to the drug. Further to this, these antigens have not been tested for diagnostic purposes.

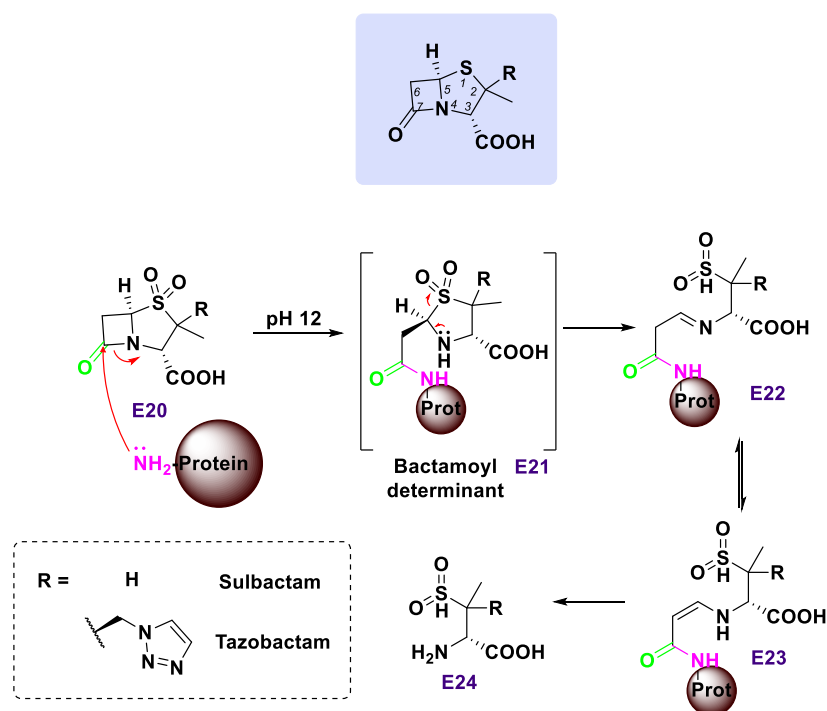


**Figure 28.** Hypothetical pathway of degradation of CVL

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Regarding **bactams**, only a few reports documented DHRs to sulbactam and tazobactam, but no reports about allergic reactions to other bactams, such as avibactam or vaborbactam, were found.<sup>162</sup>

The proposed pathway of degradation of sulbactam and tazobactam is shown in Figure 29.<sup>163</sup> First, just the same as with the other BLC families, nucleophilic attack on the carbonyl group of the  $\beta$ -lactam ring by lysine amino groups results in  $\beta$ -lactam ring opening and amidation to form the bactamoyl determinant E21. Second, elimination gives the imine E22 that quickly tautomerizes to give the determinant E23 that can undergo deacylation to afford the aminosulfenic acid E24. This could react with amino acid residues of carrier molecules through the carboxyl group presented in the C-3 of the thiazolidine ring of the bactam.<sup>164</sup> Considering that it has been observed that the aminosulfenic acid E24 of tazobactam is stable and was detected in human serum,<sup>163</sup> the prepared antigen of E24 could be tested with human sera. However, its evaluation with sera from allergic patients and controls to diagnose allergies to this BLC has not yet been carried out.



**Figure 29.** Proposed degradation pathway of the  $\beta$ -lactam inhibitors sulbactam and tazobactam



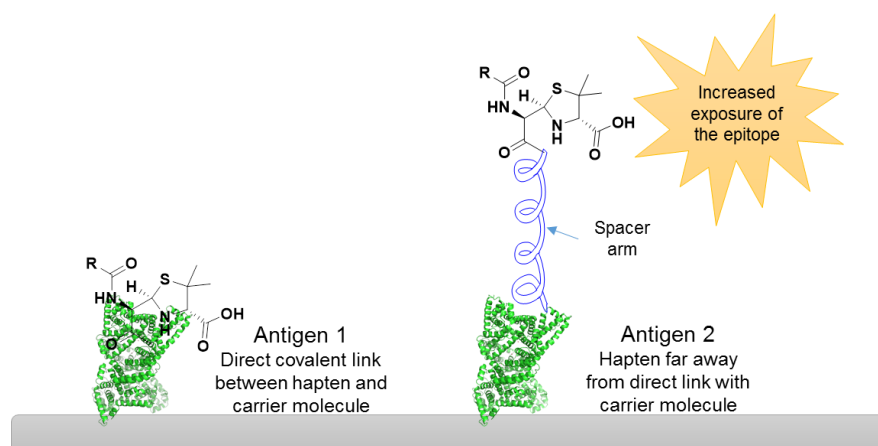
### 1.6.5. Other approaches to form antigens

There is a great effort focused on the recognition of different major and minor BLC antigens. On the one hand, IgE antibodies from allergic patients still sometimes did not recognize the antigens prepared classically. On the other hand, it has been stated that some sensitive individuals can have more than one type of BLC-IgE antibodies, as observed in their recognition pattern.<sup>106</sup> All this highlights the need to continue exploring the relationship between IgE antibodies and antigens that will help to improve the diagnosis of  $\beta$ -lactam allergy. Such exploration involves the search of other strategies referring to both the synthesis of haptens with different chemical structures that can widen the available epitopes.

Traditionally, the formation of antigens implies amide bonds where carboxylate groups of the hapten are linked to amine groups of carrier proteins, commonly through lysine residues. As observed, BLC antigens, in particular, are generally prepared by direct aminolysis with a carrier by nucleophilic  $\beta$ -lactam ring opening. However, the direct attachment of carrier molecules to chemically reactive moieties of specific compounds can leave these moieties less accessible, which may lead to the loss of generating high-affinity antibodies.<sup>104</sup>

Different synthetic strategies to prepare antigens include the study of the R lateral chain,<sup>108,165</sup> the use of spacer arms,<sup>166,167</sup> cationized proteins,<sup>168</sup> polymeric structures<sup>169–171</sup> and dendrimers<sup>172</sup>, among others.

For instance, the use of **spacer arms** to haptenize small molecules has been reported in the literature. The introduction of spacer arms cannot only move the hapten away from the direct interaction with carrier molecules, but also to avoid all the possible steric effects may permit an effective covalent link between the hapten and carrier molecules (Figure 30).<sup>166</sup> Henceforth, these features award spacer arms with the possibility of improving molecular recognition, since the generated antigens would have their conformational epitopes accessible what might increase the degree of IgE recognition.<sup>167,173,174</sup> Still, spacer arms encounter difficulties which rang from the nonspecific adsorption and the effect of the length of the spacer arm involved<sup>175</sup> to the own stability of the final complex formed.<sup>176</sup>

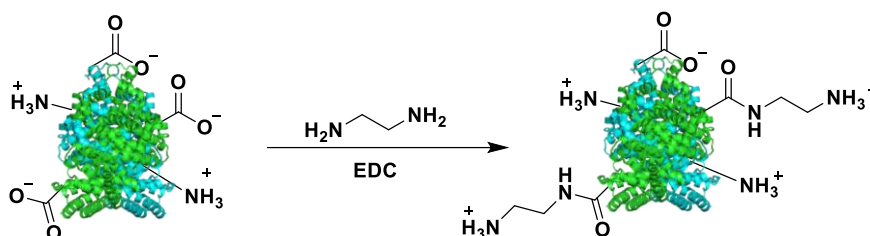


**Figure 30.** Schematic representation of antigens without (antigen 1) and with (antigen 2) spacer arms

Spacer arms have been widely studied in the literature. Some examples include the preparation of haptens for antibody production and the detection of organophosphorus pesticides, such as fenthion, as reported in studies by Kim et al.<sup>166</sup> and Brun et al.<sup>174</sup> However, limited experimental studies on drug-antigens are available in the bibliography. In this regard, most of them have been oriented to the preparation of antibiotic-based haptens. In particular, the studies by Peng et al.<sup>167</sup>, Peng et al.<sup>173</sup> and Pastor-Navarro et al.<sup>177</sup> are based on the synthesis of quinoxalines, macrolides and tetracyclines-based haptens, respectively.

The use of spacer arms for the synthesis of BLC haptens remains quite unexploited. Some achievements include the preparation of BLC-anyl antigens (explained in Section 1.6.4), which involves the use of carboxylate activating agents, such as zero-length crosslinkers based on carbodiimides, in conjunction with NHS to form NHS-esters. These esters would therefore react with amines from carrier molecules to form the BLC-anyl antigens. To achieve this, several carbodiimides are used depending on the desired conditions.<sup>168</sup> On the one hand, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) is water-soluble and its use together with sulfo-NHS allow for the preparation of reactive NHS esters in those buffers commonly used for biomolecular conjugations. The by-product formed can be removed by dialysis. On the other hand, the formation of reactive esters using dicyclohexyl carbodiimide (DCC) and NHS is performed in organic media and therefore hydrolysis problems that can arise with EDC/sulfo-NHS are avoided. Furthermore, the by-product formed is an insoluble salt that can be easily removed by filtration.

Apart from the preparation of BLC-anyl antigens, only one study was found in the literature regarding the synthesis of BLC-based haptens with spacer arms. In this study,<sup>144</sup> they prepared cephalosporin-based structures in order to help understand the relationship of these structures in the development of allergies. The evaluation of these structures in *in vitro* studies proved that molecular recognition existed, but few samples from allergic patients were used. Furthermore, the use of diamines and dicarboxylates of different alkyl lengths might be a promising practice not only to ensure that the desired functional groups are placed at a suitable distance from the epitope, but also to modify the carrier molecules via **protein cationization**. In this respect, cationization of the carrier protein bovine serum albumin (BSA) has been reported in the literature.<sup>168</sup> From the 59 lysine  $\epsilon$ -amine groups that BSA possesses, only 30-35 of them are thought to be available for bioconjugation.<sup>168</sup> In this regard, protein cationization allow for the conversion of the native carboxylates to positively charged amines. To achieve this, the model protein, BSA, is treated with EDC and the original carboxylates converted to active esters, which then react with the selected diamine to create amide bonds (Figure 31). This will help obtain electrically charged proteins with more available amino groups to link to the desired hapten, reducing crosslinking and steric hindrance.<sup>168</sup>



**Figure 31.** Scheme of the cationization of protein molecules using ethylene diamine and EDC

Cationized proteins are known to improve the cellular uptake and intracellular delivery compared with the unmodified carrier proteins, which is the reason why they are frequently used in drug delivery.<sup>178-180</sup> Within the capabilities of protein haptenization, it could be expected that cationized proteins were also frequently used to generate antigens. However, they had been rarely used in the preparation of antigens and therefore few data are found in the literature related to drug antigen preparation using cationized proteins. One example is the study by Zhang et al.<sup>181</sup> In this study, they prepared tetracycline-based immunogens using BSA and cationized BSA to develop antibodies for the detection of tetracycline residues in milk. The sera raised with the immunogens using cationized BSA

rendered more sensitive and selective immunoassays, highlighting the utility of this strategy for the preparation of antigens. Nevertheless, any study was found in the literature regarding the preparation of antigens for their application to allergic diagnosis.

**Dendrimers**, well-defined globular, highly-branched macromolecules,<sup>182</sup> are another alternative to the conventional preparation of antigens since their surface is segregated. Therefore, all haptens linked to the dendrimer remain in the peripheral area and become accessible for binding.<sup>172</sup> Several studies used polyamidoamine (PAMAM) dendrimers to diagnose allergy to AMX and PG.<sup>172,183</sup> However, the number of human samples studied is far from becoming a representative population and the results obtained has to be taken with caution.

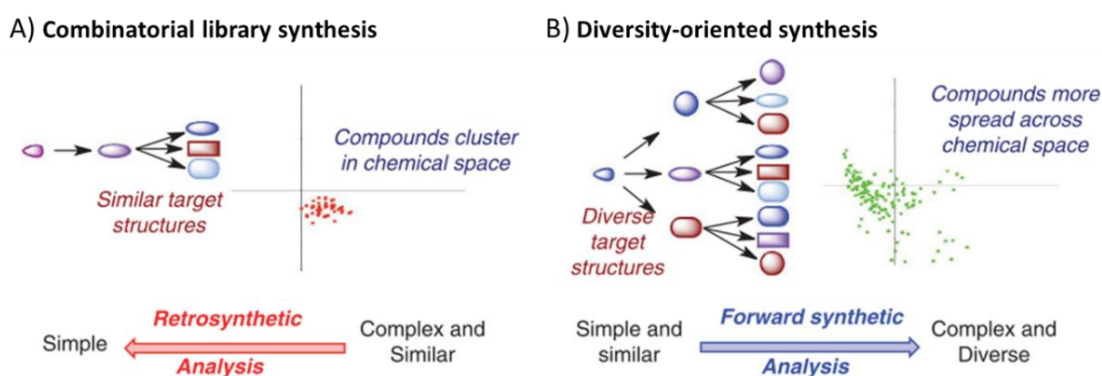
However, the preparation of antigens, either through traditional methods or through the synthetic strategies just presented herein, as well as the selection of the most promising ones has, to a wide extent, been based on previous knowledge of the chemical structure of the natural or synthetic desired compound (hapten). Modern methodologies have also claimed the importance of assessing the ability of these haptens to bind to preselected carrier proteins, but, amazingly, have suggested that greater possibilities of finding biologically active molecules exist when samples with higher number of compounds are screened and when functionally diverse compound libraries are evaluated.<sup>184,185</sup> Therefore, it makes sense to consider also these techniques in the preparation of antigens that can be recognized by IgE antibodies provoking allergic episodes.

In regards to the study of diverse compound libraries, **diverse structural techniques** range from combinatorial libraries, high-throughput screening (HTS) to target-oriented synthesis (TOS) and diversity-oriented synthesis (DOS). On the one hand, HTS allows automated testing of a biochemical or cellular event against chemical structures.<sup>186</sup> These HTS libraries are focused on specific chemistry areas and therefore do not cover a wide chemical spectrum, which may lead to the lack of important biological targets of interest.<sup>187</sup> TOS of haptens, individually or as collections (focused libraries), for its part, are directed to the identification of preselected targets rather than the reactants, which commonly are small molecules. In this sense, TOS libraries are planned retrosynthetically (Figure 32a). This means that starting from a chemically complex target, the repetitive application of the process would increase the knowledge of

these preselected targets and would therefore allow to find those small molecules able to initiate the synthetic pathway proposed.<sup>188</sup>

On the other hand, DOS, first coined by Stuart Schreiber in 2000, underlines the necessity to generate small molecules that would permit the identification and regulation of biological targets.<sup>189</sup> DOS libraries are aimed at the direction of any chemical reaction, forward synthetic analysis, from reactants to products, which in this case is from small molecules to biological targets (Figure 32b). Furthermore, the probability of finding out small molecules able to bind to any biological target in a novel manner boosts as the structural diversity between these small molecules also increases.<sup>190</sup>

DOS is an attractive route to synthesize compounds that have both structural complexity and diversity containing familiar chemical scaffolds of natural products, such as  $sp^3$ -hybridized basic nitrogen atoms, stereogenic elements, and novel skeletons.<sup>191</sup> The need for a divergent synthesis, where small molecules are transformed into many distinct structures, implies the use of complexity-generating reactions to develop novel chemical scaffolds, which allow for the use of the product of the former reaction to be the substrate for the next. These complexity-generating reactions commonly consist of multi-component reactions (MCRs), which use three or more starting materials and are one-step reactions.<sup>192</sup> Moreover, the use of combinatorial techniques involves chemical building blocks to be systematically mixed and fitted to finally generate libraries of small molecules by different methods, such as parallel synthesis or split-pool synthesis.<sup>187</sup>



**Figure 32.** Synthetic strategies. A) traditional combinatorial library synthesis. B) diversity-oriented synthesis<sup>184</sup>

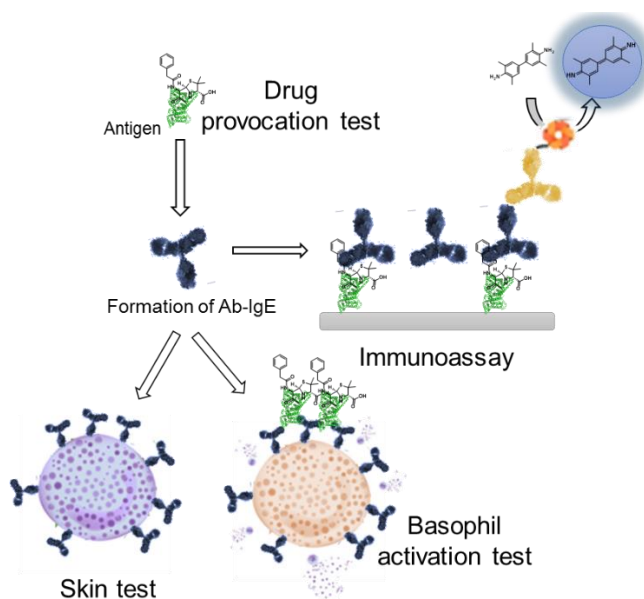
Despite the high potential of combinatorial techniques, up to now, it has been more directed to create or select new drug candidates. For instance, Sarabia and Sánchez-Ruiz<sup>193</sup> synthesized bengamide analogues by using a diversity-oriented approach to

modify specific moieties where the interaction with enzymes proved to be important. Hergenrother et al.<sup>194</sup> prepared small molecule microarrays by their covalent attachment on alcohol-containing glass slides by using DOS technique. They were then screened with proteins labeled with Cy5 and streptavidin and demonstrated the process of split-pool synthesis on glass slides and the detection of the interaction between these small molecules and carrier proteins. Moreover, Gordon et al.<sup>195</sup> described the synthesis of a library including  $\beta$ -lactam and pyrrolidine heterocycles, and Kumagai et al.<sup>196</sup> used a MCR and intramolecular cyclization reactions to yield derived 6-APA compounds, among others. More recently, deoxyribonucleic acid (DNA)-encoded libraries (DELs) arose, which identify small molecules from DOS libraries that bind to unique DNA-encoded sequences. In this respect, Gerry et al.<sup>197</sup> synthesized and validated a DEL of saturated N-heterocycles and showed the success of some compounds as high carbonic anhydrase inhibitors.

However, the use of combinatorial techniques in the synthesis of haptens that allow the preparation of antigens to diagnose allergies remains unexplored. Given that the diagnostic sensitivity and specificity with the antigens commonly used for the diagnosis of allergies remains low, it is challenging and scientifically interesting to apply these techniques as a very promising way to generate and discover new haptens. Specifically, the ability of these techniques to increase both the spatial and the structural diversity could allow for the detection and coverage of a wider range of epitopes responsible for allergic episodes.

### **1.7. Diagnostic approaches to evaluate and detect immediate allergic reactions to $\beta$ -lactam antibiotics**

According to the general European Network for Drug Allergy (ENDA)/European Academy of Allergy and Clinical Immunology (EAACI) guidelines, diagnostic approaches to IHRs to BLCs comprise clinical histories, physical examinations, and *in vivo* (skin and oral provocation tests) and *in vitro* (basophil activation test and specific IgE quantification) assays (Figure 33). However, a common diagnosis frequently only includes a detailed clinical history with physical examination, skin testing and sometimes IgE quantification. Lack of sensitivity of these assays often requires the use of drug provocation tests too.



**Figure 33.** Diagnostic approaches to IHRs to BLCs

The first approach to diagnose BLC allergy is reporting the clinical history, which implies knowing all prescription drugs taken, clinical symptoms and their duration, together with a list of possible previous ADRs.<sup>32</sup> Furthermore, in order to reduce differences in evaluation, it is recommended to complete the ENDA questionnaire that includes 43 possible symptoms related to DHRs in addition to the time lapse between the last drug administration and the reaction.<sup>91</sup>

### 1.7.1. *In vivo* tests

The term *in vivo* testing refers to those medical tests performed directly in patients. The most used *in vivo* assays include skin tests and oral provocation tests. Nevertheless, prior to performing any *in vivo* test, it has to be first evaluated the likely risk of adverse acute reactions or resistance to the medical treatment, together with a real evaluation of the utility of performing it.<sup>198</sup> These tests should be avoided in patients with any previous severe allergic reaction to the drug or with chronic medical conditions, such as angina and pregnancy.<sup>199</sup> Furthermore, *in vivo* tests can sensitize people to the antibiotics used when screening the response of them in drug studies.

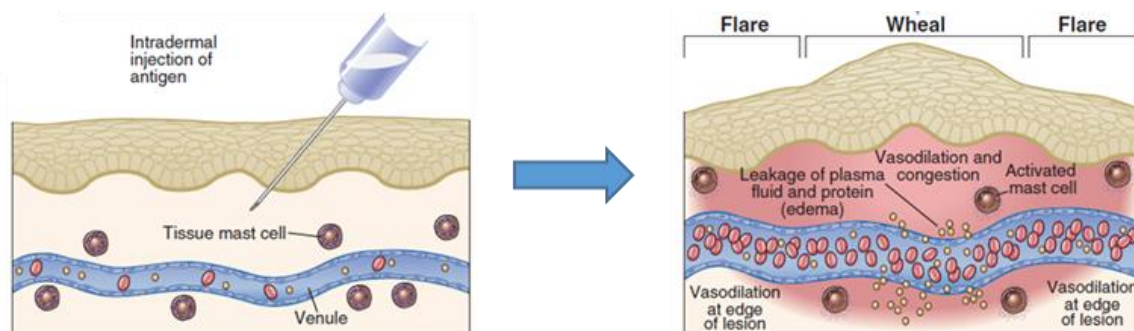
#### a) Skin testing

The skin testing (ST) approach is a well known assay to diagnose BLC allergy that comprises prick, intradermal and patch tests.<sup>200</sup> Skin prick tests (SPTs) consist on pricking the skin with the antigen of interest in the volar forearm, and are commonly the

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most used as they not only are rapid and simple but also cheap. A positive SPT consists on a wheal larger than 2 mm together with erythema. Only when the result is negative, skin intradermal tests (SITs) are performed. For that, a small bleb is marked followed by an injection of 0.02-0.05 mL of the desired antigen, and the result is positive if there is an increase in diameter greater than 3 mm in the wheal area (Figure 34). Finally, patch testing involves applying patches with the allergen solution on a person's back, although these tests are rather used for delayed HRs to BLCs.<sup>201</sup>

In the case of PG, available antigen commercial solutions to perform STs ranged from the minor determinant mixture (MDM) to BPO linked to different carriers: BPO-polylysine (BPL), BPO-HSA and BPO-poly-L-lysine (PPL). This selection raises as HSA is a suitable carrier protein to *in vitro* haptenize molecules because it presents a high ligand-binding capacity<sup>202</sup> and is considered as the carrier protein responsible for the generation of IgE antibodies *in vivo*, as observed in about 90 % of serum-bound penicilloyl groups.<sup>203</sup> Poly-L-lysine, PLL, for its side, is the most used carrier to prepare drug antigens because it is easily available in different polymeric sizes, presents low toxicity, high reactivity, and yields determinants with higher hapten-carrier density ratios.<sup>204</sup> On the other side, MDM is formed by the combination of PG, penicilloate, and penilloate. They can be administered together because they do not inhibit or degrade the reactivity of each other. However, reactions to PPL can be inhibited, and therefore PPL must be given separately.<sup>125</sup> In respect to other BLCs, it can be any BLC solution freshly prepared from the intravenous form under sterile conditions. However, these antigen commercial solutions have to be soluble in saline media at a non-irritant concentration, ranging from 2 to 20 mg/mL for most BLC antigens.<sup>205</sup>



**Figure 34.** Immunopathological basis of allergy skin testing<sup>206</sup>

The sensitivity of these tests is not so high and varies according to the studies. In one study of 290 patients, sensitivities of 22, 21, 43 and 33 % were obtained for PPL,



MDM, AMX and AMP, respectively, with specificity values of 98-99 % in all cases.<sup>207</sup> However, ST alone is not a good technique since its sensitivity decreases with time and non-exposure to the desired BLC.

b) Drug oral provocation tests

Drug oral provocation tests (DPTs) are widely considered to be the method of reference, a good standard for a definitive BLC allergy diagnosis, because they reproduce both allergic symptoms and other adverse clinical manifestations that are non-mechanism related.<sup>31</sup> In this respect, DPTs can discriminate patients with a BLC allergy from those without it. For instance, Bousquet et al have demonstrated that 17.4 % of allergic patients with negative skin tests to a BLC were positive when using the DPT technique.<sup>208</sup>

Nevertheless, DPTs are time-consuming and should be carried out only when other diagnostic tests do not allow relevant conclusions since DPT is of high risk. For this reason, DPT must be performed under medical surveillance with controlled administration.

### 1.7.2. *In vitro* tests

*In vitro* tests refer to those assays performed in the laboratory using samples of serum containing the targeted IgE. The main goal of these tests is the determination of sIgE to the desired antigen, in the form of the drug itself or its metabolites.<sup>209</sup> For that, basophil activation tests and immunoassays related to quantification of sIgE are the most currently used *in vitro* methods. However, sIgE levels in blood are very low, compromising the clinical sensitivity of these tests and their incorporation in regular clinical protocols. Furthermore, to induce an immune response, BLCs have to be able to covalently bind to carrier proteins in the form of haptens. Thus, the search for the best conjugation proteins and antigens is key to achieve an appropriate IgE recognition together with an improvement in the clinical performances of these *in vitro* methods.

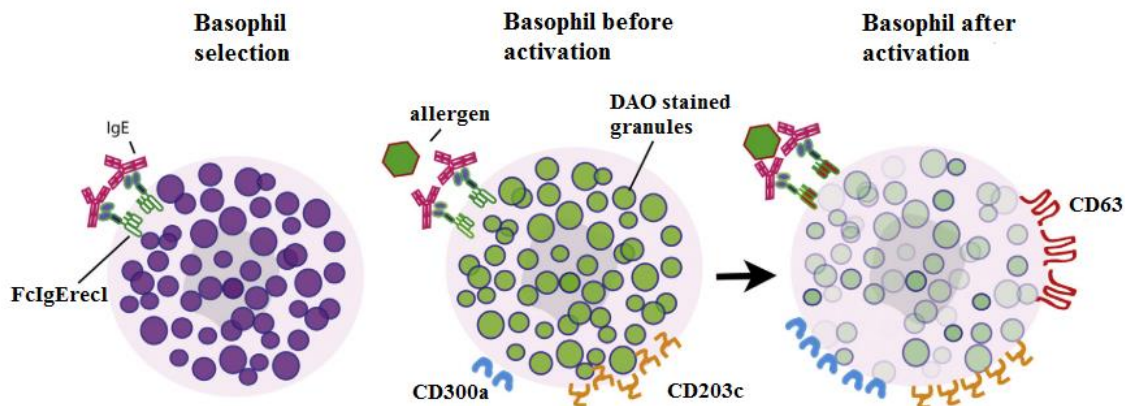
a) Basophil activation test

The basophil activation test (BAT), also referred to as *ex vivo* testing in the bibliography, consists of the determination of basophil degranulation using flow cytometry.<sup>210,211</sup> Basophils express the high-affinity IgE receptor, FcεRI, resulting from antigen or anti-IgE stimulation and carrying sIgE antibodies on their surface.<sup>212</sup> For that, the antigens normally used are BPO-PLL, MDM, PLL, and the desired BLC

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antibiotic.<sup>213,214</sup> Activation markers, such as CD63 CD203c, and the expression of the inhibitory receptor CD300a are then expressed and degranulated following the cross-linking of these sIgE/ FcεRI complexes (Figure 35), and quantified by flow cytometry techniques.<sup>215</sup>

Different protocols are available, but the most common is based on the analysis of whole blood. In order to maximize viability and functionality of basophils, this test is performed within a 4-hour time-frame after blood recollection. Yet, BAT clinical sensitivity and specificity values from BLCs range from 50 to 77.7 % and from 89 % to 97 %, respectively, according to previous studies,<sup>213,214,216</sup> which is far from what would be desired for an *in vitro* diagnosis test.

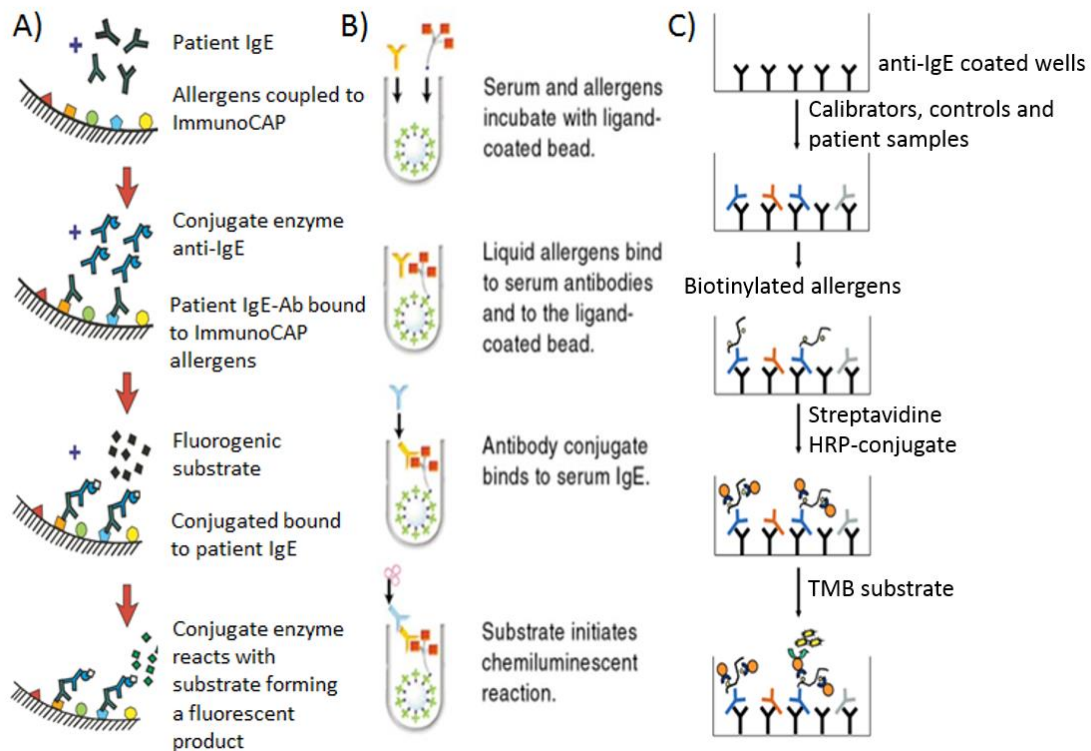


**Figure 35.** Principle of the BAT.<sup>201</sup> DAO: Intracellular fluorochrome-labeled diamine oxidase

### b) Specific IgE quantification

In order to determine sIgE to the desired drug allergen, the currently used commercial immunoassays are mainly ImmunoCAP (PHADIA, Thermo Fisher Scientific), the 3gAllergy™ Universal Kit (IMMULITE, Siemens) and ALLERG-O-LIQ (Dr. Fooke-Achterrath Laboratorien). These tests are based on fluorescence, enzyme-enhanced chemiluminescence and reversed enzyme-linked immunosorbance using biotinylated reagents, respectively (Figure 36).<sup>217</sup>

Their clinical sensitivities depend on the BLC involved, but they are rather low, insufficient, and variable (0 - 50 %), with high specificity values (83.3 - 100 %).<sup>81</sup> These tests have a limit of detection (LOD), related to the WHO IgE standard, of 0.35 IU/mL, which is also far from being optimal. Furthermore, all are devoted to detect only sIgE to 4 penicillins (PG, PV, AMX and AMP) and 1 cephalosporin (Cefaclor).



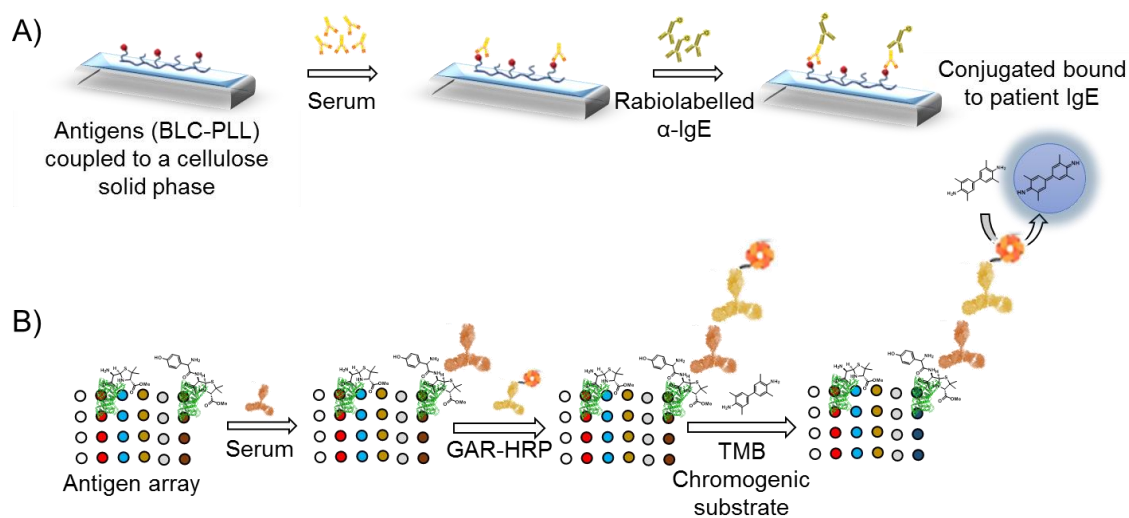
**Figure 36.** Principles of A) ImmunoCAP (adapted from <sup>218</sup>), B) 3gAllergy<sup>219</sup> and C) ALLERG-O-LIQ (adapted from <sup>220</sup>)

Other immunoassays developed to detect sIgE comprise singleplex methods, such as NOVEOS (HYCOR Biomedical) and Euroimmun (PerkinElmer), which are based on chemiluminescence and dot-blot technique, respectively. On the other hand, multiplex methods are referred to MeDALL-chip, developed within the MeDALL European project; ALEX (Allergen explorer, Macro- ArrayDX); and EUROLINE (PerkinElmer). These multiplex tests are based on fluorescence, nano-particles and western blot, respectively.<sup>201</sup> The goal of the multiplex techniques is to allow simultaneous detection and quantification of IgE specific to a large number of allergen molecules with the use of small serum sample volumes.<sup>221</sup> However, these aforementioned immunoassays are only available for the detection of food, inhalation and/or insect venom allergies. Therefore, they do not allow for the detection of drug allergies and, more specifically,  $\beta$ -lactam allergy testing. This is most likely because they were not able to identify the appropriate determinant causing the allergic episode, compromising the sensitivity.

The limited availability of these tests to only a few BLCs (if any) has led to the use of other in-house immunoassays (Figure 37), such as the radioallergosorbent test

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(RAST) based on gamma spectroscopy, developed by Garcia et al.,<sup>222</sup> and the colorimetric<sup>223,224</sup> and chemiluminescence<sup>225</sup> assays prepared in our lab. The polycarbonate surface used in our immunoassay platform is a commercial digital versatile disc (DVD), allowing for its chemical functionalization, mass production, and multiplex screening capacity in a short time.<sup>226</sup> Furthermore, it allows for the evaluation and the selection of antigens, feature not possible with the reference *in vitro* test, ImmunoCAP. This, therefore, would permit the selection of the most appropriate antigens that would help us to set-up and develop good *in vitro* sIgE quantification tests.



**Figure 37.** Principles of in-house A) RAST and B) colorimetric assay. BLC-PLL,  $\beta$ -lactam antibiotic-poly-L-lysine antigen;  $\alpha$ -IgE, anti-human IgE; GAR-HRP, goat anti-rabbit labelled with horseradish peroxidase (HRP); TMB, 3,3',5,5'-tetramethylbenzidine.

## 1.8. Recapitulation

The evaluation and management of  $\beta$ -lactam allergy is still an ongoing challenge. The commercially available *in vivo* assays used in the clinical practice are, in brief, singleplex, invasive, can give false positives and are not recommended in patients with a positive allergy history. In this respect, the aforementioned limitations have been partially overcome by the application of *in vitro* tests that quantify sIgE. However, current *in vitro* tests show poor sensitivity for  $\beta$ -lactam allergy diagnosis and the number of BLCs that can be tested is low. These limited clinical performances are a consequence of multiple factors, which the antigens used (both major and minor) are a crucial point. Moreover, the number of serum samples available is still very low for those less prescribed antibiotics and the concentration determined by the current *in vitro* tests is very low as well.

So far, the preparation of antigens has been commonly based on the conjugation of the desired  $\beta$ -lactam directly to a carrier protein through the functional groups available in the antibiotic. All this in order to keep the chemical structure of the antibiotic minimally modified to mimic the *in vivo* behavior. In consequence, the effects that the proximity of the carrier protein may have when generating the antigens or the effect of incorporating other functional groups in the haptens may allow for a better recognition of the antigens by IgE antibodies. In this regard, there is still much to improve on the development of suitable  $\beta$ -lactam antigens for the *in vitro* diagnosis of allergy to these drugs. For these reasons, there is an important need to synthesize novel haptens for the preparation of antigens, which may support the development of *in vitro* methods with better sensitivity and specificity.



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## **2. OBJECTIVES**



## 2. OBJECTIVES

The main objective of the present dissertation is to establish the chemical basis to improve the clinical performances of the current *in vitro* tests for  $\beta$ -lactam allergy diagnosis. To that end, haptens will be synthesized with which to prepare antigens that permit the detection of specific IgE causing the allergic episodes to  $\beta$ -lactam antibiotics. This will allow the development of immunoanalytical approaches to determine specific IgE antibodies to the most common  $\beta$ -lactam antibiotic *in vitro* tests. This main objective is pursued throughout the following specific objectives:

1. To study the effect of the incorporation of spacer arms in the development of antigens accessible and free of hindrance effects for improving the diagnosis of allergies to  $\beta$ -lactam antibiotics. To this end, aliphatic spacer arms of different length will be used in the synthesis of diamine-based haptens to benzylpenicillin and amoxicillin.
2. To evaluate the use of cationized proteins as a novel strategy for the production of selective antigens, pursuing the generation of additional amino residues in the carrier proteins to increase the hapten/protein molar ratio.
3. To evaluate the antigens based on benzylpenicillin metabolites 6-aminopenicillanic acid, penicillamine, penicillic acid, benzylpenicilloic acid, and benzylpenilloic for  $\beta$ -lactam *in vitro* testing, using samples from allergic patients and controls. The results obtained will be compared with those using both *in vivo* and *in vitro* assays.

## *Objectives*

4. To explore the applicability of major and minor determinants to  $\beta$ -lactam antibiotics cefuroxime, cefotaxime, ceftriaxone, meropenem and aztreonam to quantify specific anti- $\beta$ -lactam IgE antibodies with high sensitivity and specificity.
5. To synthesize novel clavulanic haptens to rise specific immunoreagents and quantify specific IgE antibodies.
6. To determine the feasibility of the incorporation of structural diversity into  $\beta$ -lactams in the development of unique antigens. A cohort of skeletally diverse compounds will be synthesized using diversity-oriented synthesis for the penicillin and cephalosporin precursors, 6-aminopenicillanic acid and 7-amino-deacetoxycephalosporanic acid, and the  $\beta$ -lactam antibiotics amoxicillin and ampicillin. The haptens will be conjugated to carrier proteins in order to be evaluated by specific IgG and IgE from rabbits and human samples.



### **3. EXPERIMENTAL RESULTS**



*Chapter 1*

**3.1. Penicillin determinants for the diagnosis of  
IgE-mediated allergic reactions**



Penicillins are by far responsible for most of the immunologically mediated drug hypersensitivity reactions. Even though penicillin allergy has been extensively studied, diagnostic tests are often either not performed, leading to the direct classification of patients as allergic when in fact they are not, or lack the necessary diagnostic specificity and sensitivity for a good diagnosis test. Therefore, the goal is to synthesize new haptens with which several antigens will be prepared within the aim to improve the diagnostic sensitivity of the *in vitro* immunoassays.

Concretely, the first part of this chapter (Section 3.1.1.) describes the utilization of aliphatic diamines of different alkyl chain lengths by two different approaches. First, the use of diamines of different alkyl chain lengths as spacer arms to haptinize benzylpenicillin and amoxicillin antibiotics is attempted. With these haptens, the corresponding antigens are prepared after conjugation to two carrier proteins (human serum albumin and histone) through two different conjugation routes, generating major and minor determinants. On the other hand, the second approach is based on the use of the aliphatic diamines of different alkyl chain lengths as modifier compounds to generate cationized proteins. In this respect, the incorporation of additional amino groups in carrier proteins can result in an increase of the hapten-carrier protein molar ratio.

On the other hand, although the immunological properties of penicillin metabolites were studied in detail in the past, the results obtained were not conclusive. Furthermore, recent studies are more focused in discovering new structures with the immunological properties needed than in the evaluation of the well known *in vivo*-formed metabolites, as stated in the Introduction (Section 1.6.4). Therefore, the second part of this chapter (Section 3.1.2.) deals with the evaluation of the metabolites 6-aminopenicillanic acid, penicillamine, penicillic acid, benzylpenicilloic acid and benzylpenilloic acid. For that, benzylpenicilloic acid and benzylpenilloic acid are synthesized from sodium benzylpenicillin and the other metabolites used as commercially supplied. Then, all these metabolites are conjugated to the aforementioned carrier proteins by three different routes, depending on the corresponding metabolite, to generate the metabolite-based antigens.

Finally, all the produced antigens are evaluated in a multiplex *in vitro* colorimetric microimmunoassay to establish the molecular recognition pattern against sera from immunized rabbits and from a cohort of allergic patients and controls.



### **3.1.1. Boosting the sensitivity of *in vitro* $\beta$ -lactam allergy diagnostic tests**








## Boosting the sensitivity of *in vitro* $\beta$ -lactam allergy diagnostic tests†

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### ABSTRACT

The synthesis of structurally new haptens and the development of suitable antigens are essential for boosting the sensitivity of drug allergy diagnostic testing. Unprecedented structural antigens for benzylpenicillin and amoxicillin are characterised and evaluated in a cohort of 70 subjects with a turnkey solution based on consumer electronics.

#### 1. Introduction

$\beta$ -lactams (BLCs) are the most extensively used antibiotics for the treatment of bacterial infections,<sup>1</sup> but also the most frequently involved in allergic reactions.<sup>2,3</sup> Penicillins - BLC antibiotics - represent 37 % of the total antibiotic consumption in the European Union. Between 2016 and 2018, the median intake was 7.1 defined daily doses per 1000 inhabitants per day.<sup>4</sup>

According to the hapten hypothesis on the mechanism of antigen presentation, a drug (e.g., a hapten) covalently binds to specific lysine residues of endogenous proteins (carrier molecule) to be processed and presented by cells, eliciting an immune response.<sup>5</sup> This mechanism involves the production of major (-oyl) and minor (-anyl) antigens.<sup>6,7</sup>

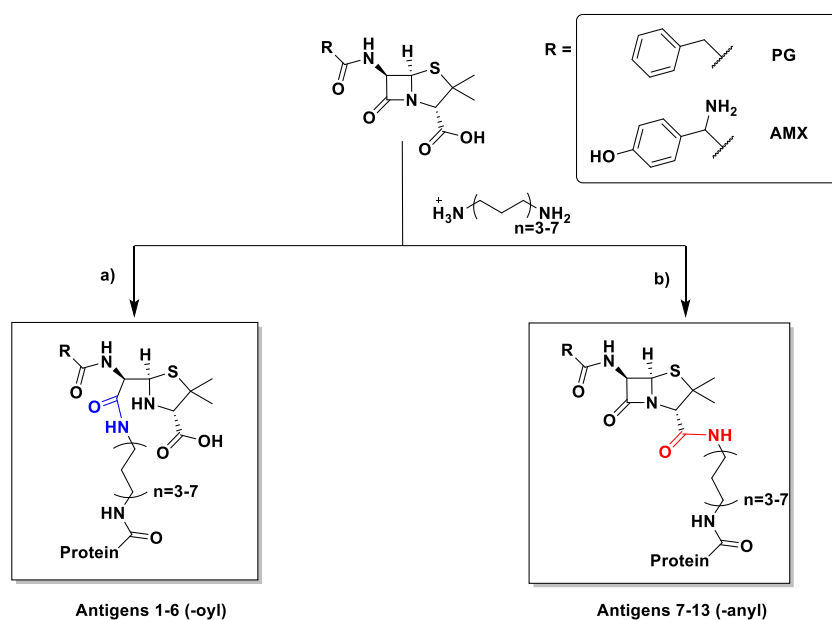
Approximately 95 % of penicillin molecules are in the penicilloyl configuration, involved in 75 % of IgE-mediated allergic reactions.<sup>8,9</sup>

Quantitative inhibition methods have allowed the establishment of a correlation between fine structural features of haptens and allergic sensitivities in patients.<sup>10,11</sup> For effective development of sensitive and selective *in vitro* tests, the chemical structure of the hapten, among other critical variables, is essential to define the specific epitope that binds to the carrier molecule. Spacer arms have been widely used in the haptenization of small molecules,<sup>12-14</sup> to increase the exposure of the epitope, and also to covalently link the hapten to a carrier molecule, to reduce steric hindrances.<sup>15</sup> Experimental data on drug-derived antigens are limited. Montañez *et al.* synthesized different cephalosporin skeletons conjugated to carrier molecules to establish the structure-IgE molecular recognition relationships,<sup>16</sup> while Pastor-Navarro *et al.* synthesized various haptens for tetracyclines using spacer arms to induce heterology in their chemical structures.<sup>17</sup> Some results suggest that the conformational epitope should be accessible to generate antigens that are better recognized by specific IgEs.<sup>18,19</sup> According to certain researchers the ideal spacer arm should have:<sup>14</sup> (I) a proper length (at least three atoms), (II) no active centre that could lead to non-specific adsorption, and (III) bifunctional groups to react with the hapten and the carrier molecule. Another strategy to enhance drug-IgE molecular recognition is to prepare effective antigens with a higher hapten:carrier ratio to increase the strength and specificity of the immune response and subsequent antibody generation.<sup>20</sup> This may be achieved by forming additional amines on the carrier protein by using cross-linkers to convert negatively charged carboxylates to positively charged amines.<sup>21</sup> Cationized proteins are known to increase the immune response compared to their native forms making them possible promising molecules to boost the molecular recognition events.<sup>22</sup>

## **2. Results and discussion**

In this work, a collection of 13 diamine-derived haptens for benzylpenicillin (PG) and amoxicillin (AMX) was synthesized with aliphatic diamines of different alkyl chain lengths. To this end, the spacer arm was placed (1) at the carbonyl carbon after  $\beta$ -lactam ring opening (-oyl) and (2) at the carboxylic acid of the thiazolidine ring (-anyl), maintaining the  $\beta$ -lactam ring common to all BLCs, maximizing exposure to the lateral

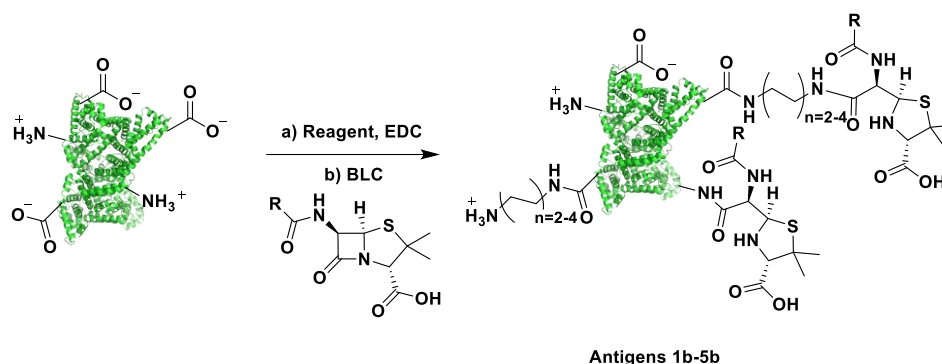
chain. Of the 13 haptens (see nuclear magnetic resonance spectra, ESI<sup>†</sup>), six were -oyl (**1-6**, Scheme 6.1.S1) and seven were -anyl (**7-13**) haptens, using PG (haptens **1-3** and **7-9**) and AMX (haptens **4-6** and **10-13**) as the starting materials. These haptens were used to develop antigens (Scheme 1.1.1) conjugated to human serum albumin (HSA) prepared following the carbodiimide chemistry. HSA was employed due to its content of glutamic and aspartic residues, 60 and 30, respectively. Next, five antigens, one for HSA and four for histone H1, were prepared using cationized carrier proteins (Scheme 1.1.2). The effect of diamine chain length on molecular recognition of the diamine-based antigens was assessed using a multiplex microimmunoassay developed on a DVD with colorimetric detection (Figures 6.1.S1 and 6.1.S2, ESI<sup>†</sup>), using a hacked disc drive as the detector.<sup>23</sup>



**Scheme 1.1.1.** Reaction pathway for antigen preparation, a) protein, Na<sub>2</sub>CO<sub>3</sub>, pH=11, and rt; b) 0.1 M DCM/DMF, then protein, PBS 1X, pH=7,4, and rt. PG, benzylpenicillin; AMX, amoxicillin.

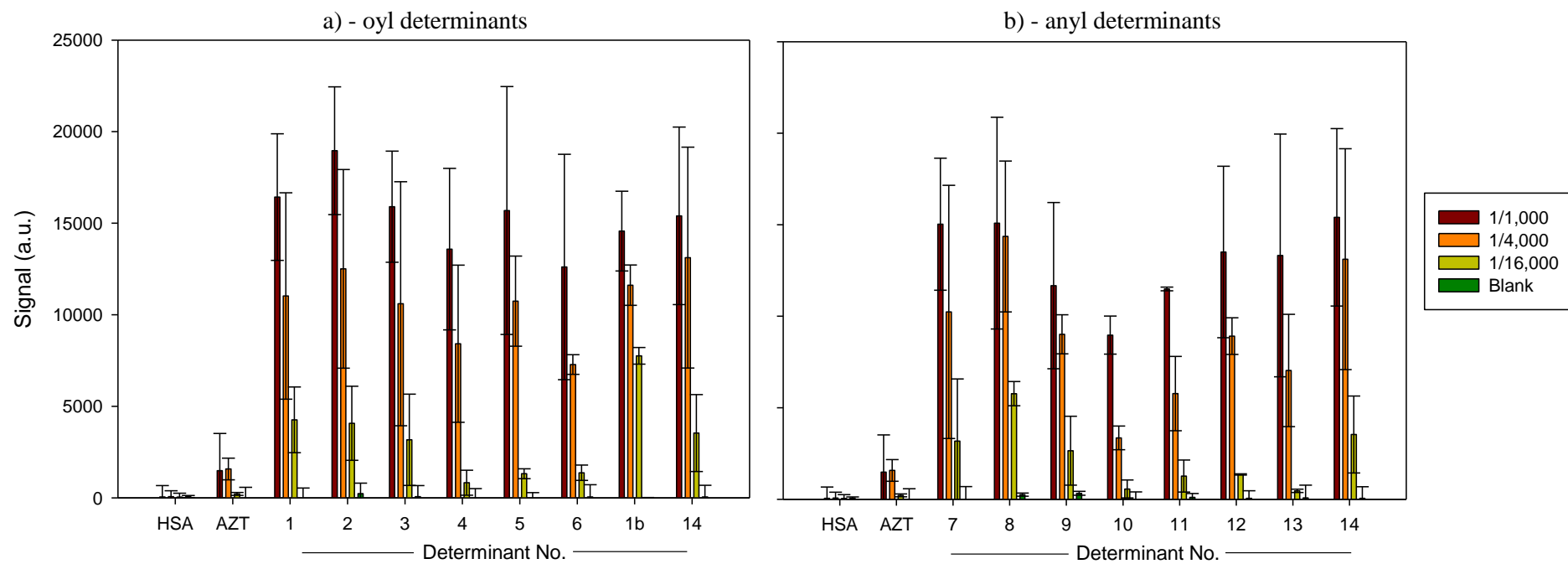
Using the results of a reference antigen **14**, three comparisons were made. First, polyclonal IgGs to PG were used. These specific IgGs were obtained by immunizing a rabbit with the reference -lloyl antigen linked to keyhole limpet haemocyanin (Scheme 6.1.S2, ESI<sup>†</sup>). Sera dilutions ranging from 1/1000 to 1/32 000 were tested and PBS-T was used as a blank control. The results were plotted and are shown in Figure 1.1.1. No recognition was observed for either HSA (< 500 arbitrary units) or the antigen prepared with aztreonam (AZT) (< 700 arbitrary units), used as negative controls. The term molar

recognition ratio (MRR) is used to compare antigenic-specific responses, representing the ratio between the signal given by the -oyl and/or -anyl antigen(s) to the reference signal (antigen **14**) and is expressed as a percentage. Overall, all synthesized antigens detected polyclonal IgG to PG and the assay signals were similar to those obtained for the reference antigen. For -oyl antigens (Figure 1.1.1a), PG-based antigens **1-3** showed higher MRRs (*ca.* 103-123 %), while for AMX-based antigens **4-6**, the MRRs ranged between 86 and 102 %.



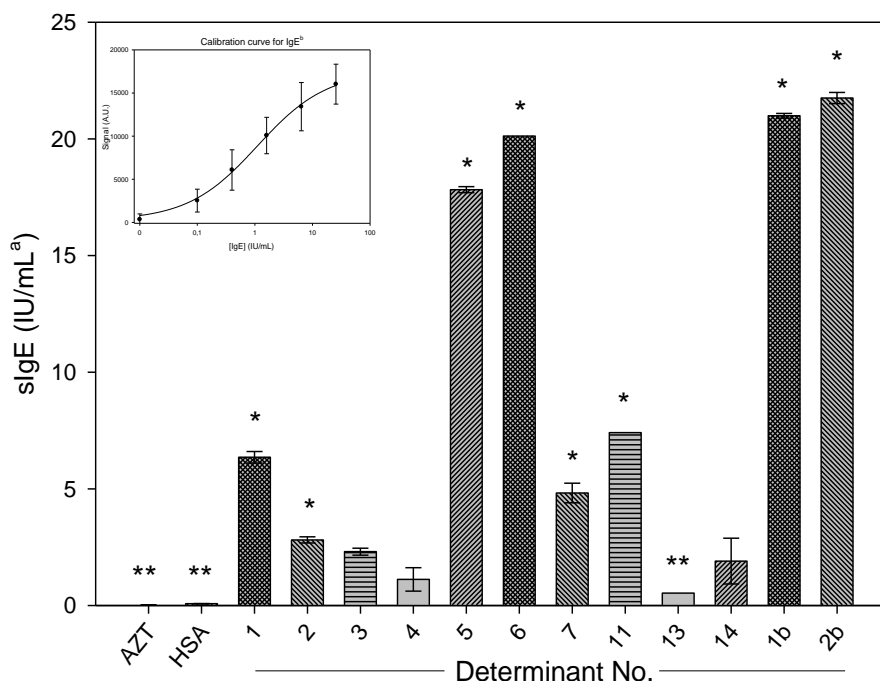
**Scheme 1.1.2.** Chemical structures of the antigens using diamine cationized proteins.  
Reaction conditions: -oyl haptens: sodium carbonate 0.5 M, pH 11.0.

Furthermore, the results revealed that -oyl antigens linked to 1,5-diaminopentane had the best responses, with MRR values of 123 % and 102 % for PG and AMX-based antigens, respectively. On the other hand, 1,7-diaminoheptane-based antigens had the lowest MRR values. For antigen **1b**, for which cationized carrier molecules were used, the results were similar, with an MRR of 95 %. Signals of -anyl antigens (Figure 1.1.1b) were lower than those of the reference. PG and AMX-based antigens **7-9** and **10-13** had MRRs between 76 % and 98 % and 58 % and 88 %, respectively. In terms of the protective effect of the free amine on AMX, antigen **13** achieved higher MRR values (*ca.* 86 %) than its corresponding non-protected antigen **10**, with an MRR value of 58 %. Contrarily, no general agreement was reached regarding diamine alkyl chain length. Indeed, antigen **10**, linked to 1,3-diaminopropane, showed the lowest serum response (*ca.* 58 %) for AMX, while PG antigen **9**, linked to 1,7-diaminopentane, reached a higher MRR value (76 %). In short, the recognition of PG-modified antigens was better than that of AMX.



**Figure 1.1.1.** Signals (in arbitrary units, a.u.) of the modified  $\beta$ -lactam antigens with specific rabbit IgG to benzylpenicillin ( $n = 5$ ; dilution factors represented: 1/1000, 1/4000 and 1/16 000). (a) -oyl antigens and (b) -anyl antigens.

Figure 1.1.2 shows the results of the evaluation of artificial human serum to PG. Variations were observed in PG-based antigens depending on the conjugation strategy. Antigens **1b** and **2b**, in which diamines were used to cationize the carrier, reached a specific IgE (sIgE) concentration of 23 IU mL<sup>-1</sup>, ten times higher than that of the reference, indicating good recognition of the sera for these antigens. Regarding AMX-antigens, the best results were observed for antigens **5** and **6** with an sIgE concentration of 20 IU mL<sup>-1</sup>.



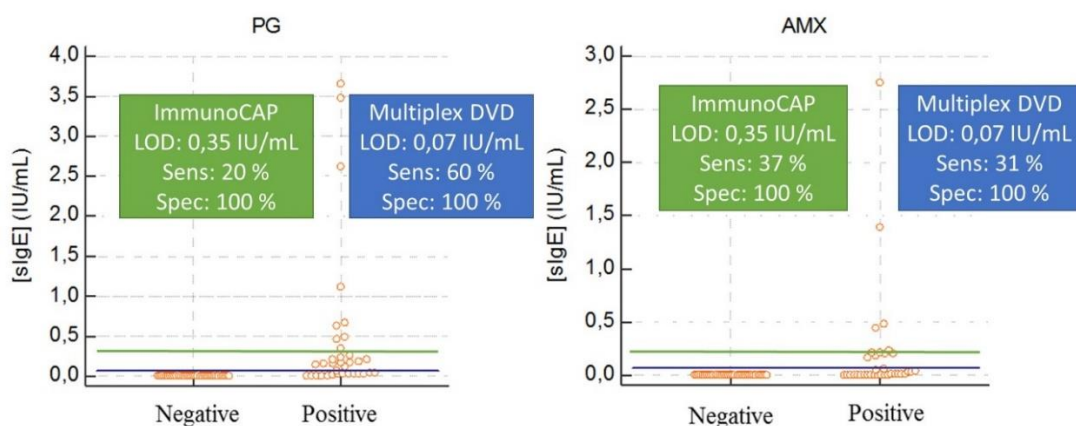
**Figure 1.1.2.** Evaluation of  $\beta$ -lactam antigens with artificial human serum specific to benzylpenicillin ([IgE] = 25 IU mL<sup>-1</sup>). 1 IU mL<sup>-1</sup> = 2.4 ng mL<sup>-1</sup>. The sensitivity, measured as 50 % effective concentration (EC<sub>50</sub>), was 2.08 ± 0.78 IU mL<sup>-1</sup> and the limit of detection, calculated as the concentration corresponding to the signal of the blank plus three times its standard deviation, was 0.07 IU mL<sup>-1</sup>. The inset shows the standard curve for total IgE. The limit of quantification was 0.92 IU mL<sup>-1</sup> and the relative standard deviation ranged from 3 to 15 %. \*indicates a statistically significant difference (p < 0.001) when compared to reference antigen **14**. \*\*indicates that the concentrations were below the limit of quantification.

No improvement of the results was seen when the free amine group of AMX was protected, as observed for antigens **7** and **13**, with sensitivities of 5 and 1 IU mL<sup>-1</sup>, respectively. This may explain why antigens **5** and **6** were promising for *in vitro* diagnosis of BLC allergies. The conjugation of AMX could have been directed not only towards the free amine of the diamine, but also towards the free amine in its lateral chain,

improving the molecular recognition yield. It may also explain the better response for AMX antigens, prepared by using diamines as spacer arms for the hapten synthesis in comparison to PG-based antigens, even in PG-specific artificial human serum. Furthermore, spacer arm length showed different patterns regarding the BLC antigen selected. This is in line with the results of a previous study in which molecular recognition differed on the selected hapten.<sup>24</sup>

A cohort of 70 subjects, 35 of whom developed an immediate reaction to  $\beta$ -lactams (Table 6.1.S1, ESI†), was tested. Results of the prick tests (*in vivo*), ImmunoCAP, and the multiplex immunoassay (*in vitro*) are shown in Table 6.1.S2 (ESI†). When diamines were used as spacer arms (both -oyl and -anyl antigens) the results were negative, with values below the limit of detection. Prick tests showed that four out of 31 PG patients (13 %) and 21 out of 31 AMX patients (68 %) were positive. The *in vitro* ImmunoCAP reference test showed that out of 35 allergic patients, seven PG (20 %) and thirteen AMX (37 %) patients were positive. The *in vitro* ImmunoCAP test allows sIgE to be quantified within a range of 0.01 to 100 IU mL<sup>-1</sup>, with a cut-off point of 0.35 for positive results and levels above 0.10 IU mL<sup>-1</sup> indicating sensitization. Furthermore, recent data indicate that the specificity of the ImmunoCAP system ranges from 85.7 % to 100 %, while the sensitivity ranges from 0 % to 25 %, depending on initial clinical symptoms.<sup>25</sup> The evaluation of our antigens with multiplex DVD-microimmunoassay in a cohort of 35 allergic patients resulted in 21 PG (60 %) and 11 AMX (31 %) positive subjects with antigen **3b**. The 35 control patients were negative. Figure 1.1.3 shows a dot plot diagram of the clinical performance of the developed multiplex microimmunoassay.

Evaluation of the different diamines used to modify the carrier proteins with AMX (antigens **3b**, **4b** and **5b**) revealed that the shorter diamine, ethylene diamine, provided the best results. Its short chain length ensures minimal steric effects and virtually no hydrophobic interactions, while the aromatic ring present in 1,4-phenylenediamine may induce extreme steric effects and become less accessible. For instance, patient 04 had a specific IgE value to AMX of 0.44 IU mL<sup>-1</sup> when ethylene diamine was used, and 0.02 IU mL<sup>-1</sup> when both 1,4-diaminobutane and 1,4-phenylenediamine were used. The same was observed with patient 09 with values of 2.75, 0.04, and 0.06 IU mL<sup>-1</sup>, respectively.



**Figure 1.1.3.** Dot plot of the specific IgE levels to PG and AMX determined using the multiplex DVD-assay. Positive and negative refer to samples from allergic patients and control subjects, respectively. The cut-off considered is the limit of detection of the developed multiplex DVD-assay and is represented as a blue line ( $> 0.07$ ). Sens and Spec stand for sensitivity and specificity using antigens **1b** and **3b** for PG and AMX, respectively.

### 3. Conclusions

In conclusion, the search for new antigens for allergy testing involves the synthesis and screening of chemical entities to delineate the selectivity and the strength of interaction between the antigens and the IgE targets. Given the need to consider the side-chain on individual  $\beta$ -lactam derived antigens and the heterogeneity of the target  $\beta$ -lactam antibiotics, this research contributes a new approach to synthesize structural haptens and generate specific antigens for immunoanalytical determination of IgEs, going beyond the state of the art. As we demonstrate, the synthesis of structurally new haptens to generate antigens for  $\beta$ -lactam allergy is key for developing highly sensitive *in vitro* tests to determine specific IgG and IgE antibodies. The purpose of our study was to synthesize a collection of haptens to produce a panel of major and minor antigens for PG and AMX to determine sIgE for these BLCs. We obtained a panel of 18 antigens, as well as a collection of different haptens (epitopes), using diamines of various length and different linkage strategies. To the best of our knowledge, this is the first report that describes the development of modified major and minor -oyl and -anyl antigens for benzylpenicillin and amoxicillin, demonstrating the potential for an improved serological diagnosis of IgE-mediated drug allergic reactions for commonly prescribed and consumed  $\beta$ -lactam antibiotics. We henceforth determine the presence of IgE antibodies in a defined allergic population in whom Augmentin and amoxicillin were the most



common inducers of immediate reactions. The results with patients showed that antigens for which diamines were used as spacer arms were not detected, although they recognized both specific IgG and IgE from immunized rabbits and artificial human sera to PG. We found a good correlation between our multiplex DVD immunoassay and ImmunoCAP for positive samples to both PG and AMX. Cationization of carrier molecules with diamines and their subsequent use to generate antigens is a suitable approach to detect specific IgE at low concentrations in allergic patients, improving the sensitivity of the existing *in vitro* tests for drug allergy. This result reveals that cationized antigens are a promising alternative to current -oyl derived antigens for boosting the sensitivity of the current *in vitro* test for antibiotic allergy. Synthesized amoxicillin -oyl haptens enhance drug-IgE molecular recognition, with a three-fold increase in sensitivity compared to the existing *in vitro* diagnostic testing for antibiotic allergy. The current lack of clinically standardized reagents makes the panel of synthesized structural haptens and the generated specific antigens a promising approach for mass detection of antibiotic allergies. In this way, better drug sensitization profiles could be defined, which will be of valuable support for allergy screening campaigns and antibiotic delabeling processes.

## ACKNOWLEDGMENTS

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### **3.1.2. Penicillin antigens based on metabolites for *in vitro* drug allergy testing**



## Penicillin antigens based on metabolites for *in vitro* drug allergy testing

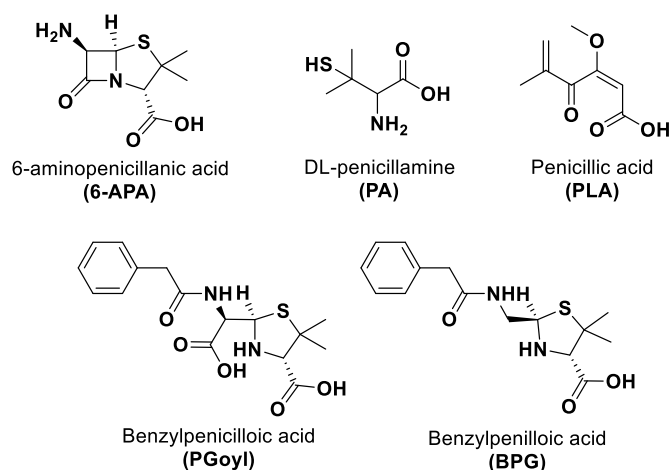
### 1. Introduction

Benzylpenicillin acid (PG) remains in equilibrium with pseudopenicillin under neutral conditions.<sup>1</sup> However, it quickly decomposes under several conditions which lead to  $\beta$ -lactam ring opening (major configuration) and the formation of several minor metabolites (see Figure 9, Section 1.6.4.).<sup>2,3</sup> Therefore, it is coherent to expect that these metabolites conjugated to carrier proteins could elicit an immunogenic response that could explain the diverse IgE recognition patterns observed in allergic patients.<sup>4</sup>

Several studies show the recognition patterns of penicillin metabolites by specific IgE antibodies from allergic patients.<sup>4-6</sup> However, the results obtained are still inconclusive and the molecular recognition pattern metabolite-IgE is unclear. For instance, penamaldic acid, formed from penicilloic acid (PGoyl), has been claimed to react *in vivo* with cystine residues from carrier proteins by formation of disulfide bonds. This would lead to the formation of several antigens, such as benzylpenamaldic acid cysteine mixed disulfide, penicillamine cysteine mixed disulfide and benzylpenilloaldehyde.<sup>7</sup> Although these metabolites have the functional groups needed to be linked to amino acid residues of carrier proteins to form antigens, they have been commonly used for skin testing in their native forms, as haptens.<sup>8</sup> For instance, the diagnostic sensitivity for haptens PGoyl, benzylpenilloic acid (BPG) and penicillamine (PA) ranged from 21 to 55 %, depending on the study.<sup>6,9,10</sup> Interestingly, the precursor of the penicillins, 6-aminopenicillanic acid (6-APA), has also been evaluated and not being immunogenic by its own.<sup>11</sup> By contrast, the metabolite penicillic acid (PLA), formed by acid degradation of penicillins, is considered a mycotoxin that has been thoroughly evaluated by the food industry due to the risk that it entails.<sup>12</sup> However, its recognition by specific IgE antibodies from allergic patients has not been studied yet.

In this study, several metabolites to penicillins (6-APA, PA, PLA, PGoyl and BPG, Figure 1.2.1) were tested after conjugation to the carrier proteins human serum

albumin (HSA) and histone from calf thymus (H1). The suitability of the PG antigens based on its metabolites was evaluated testing a cohort of 36 subjects, using a multiplex *in vitro* DVD-microimmunoassay. This study aims to get insight into the identification of metabolite-based antigens to penicillins with properties to be recognized by specific IgE antibodies.



**Figure 1.2.1.** Chemical structures of the studied penicillin metabolites

## 2. Experimental section

### General methods

Oxygen- and/or moisture-sensitive reactions were carried out in flame-dried glassware under nitrogen atmosphere. All reagents and solvents were purchased and used as received or synthesized according to cited procedures.

All yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Reaction progress was monitored by analytical thin-layer chromatography (TLC) and  $^1\text{H-NMR}$  spectroscopy. TLC analyses were performed using E. Merck silica gel 60 F254 pre-coated plates (250  $\mu\text{m}$ ). A handheld 254 nm UV lamp and potassium permanganate staining solution (with light heating) were used for detection.

NMR spectra were recorded on Bruker UltraShield 300 MHz and Ascend 400 MHz spectrometers. Compounds were characterized by  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$ .  $^1\text{H-}$  and  $^{13}\text{C-NMR}$  chemical shifts ( $\delta\text{H}$ ,  $\delta\text{C}$ ) are reported in parts per million (ppm) relative to the appropriate solvent—DMSO- $d_6$ : 2.50 ( $^1\text{H}$ ), 39.52 ( $^{13}\text{C}$ ) ppm—. Resonances are described using the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), quin.



(quintet), m (multiplet), br (broad), dd (doublet of doublets), etc. Coupling constants (J) are given in Hz and are rounded to the nearest 0.1 Hz. All NMR data were collected at 25 °C.

#### *Chemicals, Reagents and Buffers*

6-aminopenicillanic acid 96 %, penicillic acid  $\geq 98$  %, benzylpenicillin sodium salt, Tween 20, human serum albumin, *N,N'*- dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), histone from calf thymus are from Sigma-Aldrich (Madrid, Spain). DL-penicillamine 97 % is from VWR. Dimethylformamide (DMF), hydrochloric acid 37 % (HCl), sodium hydroxide (NaOH), isopropanol, ethanol, cupric sulphate and buffer salts are from Scharlau (Sentmenat, Spain) and used without further purification. Deuterated dimethyl sulfoxide (DMSO- $d_6$ ) was supplied by ACROS Organics (New Jersey, USA). Goat anti-rabbit antibody labeled with horseradish peroxidase (GAR-HRP) and goat anti-mouse antibody labelled with horseradish peroxidase (GAM-HRP) are from Abcam (San Francisco, USA). Tetramethylbenzidine (TMB) substrate is from SDT GmbH (Baesweiler, Germany) and Coomassie Brilliant Blue R-250 staining solution is from Bio-Rad (Madrid, Spain). Amicon Ultra 0.5 pre-concentrated 10 K MWCO filters and Dextran Desalting Columns, 5K MWCO, 5 mL are from Fisher Scientific (Madrid, Spain) and DVDs are from CD Rohling-up GmbH (Saarbrücken, Germany).

The employed buffers are (I) potassium carbonate 0.5 M, pH 11.0; (II) phosphate buffer saline (PBS 1X, 0.008 M sodium phosphate dibasic, 0.002 M sodium phosphate monobasic, 0.137 M sodium chloride, 0.003 M potassium chloride, pH 7.4); (III) PBS-T (PBS 1X containing 0.05 % Tween 20); (IV) sodium carbonate/bicarbonate buffer 0.1 M, pH 9.6; and (V) potassium phosphate dibasic 0.1 M. All buffers are filtered through a 0.45- $\mu$ m pore size nitrocellulose membrane from Thermo Fisher Scientific (Madrid, Spain) before used.

#### *Synthesis of metabolites*

6-APA, PA and PLA were used as supplied. PGoyl and BPG were synthesized from the commercial benzylpenicillin sodium salt as follows:<sup>13</sup>

## *Experimental results*

### - Benzylpenicilloic acid (PGoyl)

To a stirred solution of benzylpenicillin sodium salt (200.0 mg, 0.56 mmol, 1 eq) in H<sub>2</sub>O (1.0 mL) was added NaOH (100 mg/mL) at room temperature. The resulting solution was stirred at 40 °C for 2 h. Then, the solution was cooled down to 5-10 °C and the pH adjusted to 6 by slow addition of concentrated HCl (80 µL). Isopropanol (5 mL) was added slowly with rapid stirring and the solution was allowed to cool at 4 °C. Finally, the solution was filtered under vacuum, washed with isopropanol and the crystalized product dried over high vacuum to give a white solid in 97 % yield. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 7.29 – 7.18 (m, 5H), 4.95 (d, *J* = 2.9 Hz, 1H), 4.30 (dd, *J* = 8.3, 3.1 Hz, 1H), 3.53 (q, *J* = 14.7 Hz, 2H), 3.01 (s, 1H), 1.49 (s, 3H), 1.03 (s, 3H). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>): δ 172.54, 172.03, 170.32, 136.60, 129.83, 129.54, 128.69, 128.58, 126.78, 78.09, 59.48, 58.26, 58.10, 43.30, 29.65, 29.18.

### - Benzylpenilloic acid (BPG)

Benzylpenicillin sodium salt (300.0 mg, 0.84 mmol, 1 eq) was dissolved in H<sub>2</sub>O (1.5 mL) and NaOH (100 mg/mL) was added at room temperature and stirred at 40 °C for 2 h. Then, the solution was cooled down to 5-10 °C and the pH adjusted to 4 by slow addition of concentrated HCl (100 µL). Ethanol (1.5 mL) was added and the resulting solution stirred and heated at 80 °C until the effervescence ceased. The progress of the reaction was followed by TLC. Afterwards, the solution was filtered under vacuum and washed with H<sub>2</sub>O/ethanol (1/1 v/v). Finally, the desired product was allowed to crystallize at 4°C and dried over high vacuum to give a white solid in 79 % yield. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 8.28 (dt, *J* = 38.2, 5.9 Hz, 1H), 7.29 – 7.19 (m, 5H), 4.60 (t, *J* = 5.7 Hz, 1H), 3.42 (s, 2H), 3.40 (dt, *J* = 6.4 Hz, 1H), 3.37 (dt, *J* = 6.2 Hz, 1H), 3.09 (t, *J* = 6.4 Hz, 1H), 1.54 (s, 3H), 1.16 (s, 3H). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>): δ 171.41, 170.58, 137.00, 129.33, 128.61, 128.58, 126.74, 126.69, 73.12, 66.04, 59.67, 58.96, 42.60, 28.82, 27.91.

## *Preparation of the antigens*

The conjugation of the metabolite-based antigens was performed following the carbodiimide chemistry to obtain minor determinants. Briefly, the corresponding metabolite (6.0 mg) was reacted with DCC (1 equiv.) and NHS (1 equiv.) in DMF (0.1

M) for 4 h at room temperature. Afterwards, the mixture was centrifuged at 12,000 rpm to remove the acyl urea precipitate. Finally, the volume of the supernatant corresponding to 6.3-18.9  $\mu\text{mol}$  of the metabolite (depending on the carrier protein used) was mixed up with DMF to 100  $\mu\text{L}$  and added to 900  $\mu\text{L}$  of the carrier protein solution (2.0 mg) in PBS 1X, pH 7.4 overnight at 37 °C.

Furthermore, antigens 6-APA-2 and PGoyl-2, 6-APA and PGoyl were also linked to the carrier proteins by amidation between the carbonyl carbon of the  $\beta$ -lactam ring after  $\beta$ -lactam ring opening and lysine residues of the carrier proteins (considered as the major configuration). For that, 30-90  $\mu\text{mol}$  (depending on the carrier protein used) of 6-APA and PGoyl were conjugated to the carrier protein (2.0 mg) in basic media (potassium carbonate 0.5 M, pH 11.0) overnight at 37°C. PA-2 antigen was formed via disulphide bonds between the thiol group of the PA and cysteine residues of the carrier protein. For that, the carrier protein (2.0 mg) and PA (4.5 mg) were dissolved in potassium phosphate dibasic 0.1 M (1 mL) and stirred at 37°C for 2 h. Then, cupric sulphate (5 ppm) was added and the solution was allowed to oxidized at 4°C for 3 days.

All antigens were purified by gel filtration chromatography on Amicon Ultra 0.5 pre-concentrated 10 K filters using PBS 1X, pH 7.4, as elution buffer. Afterwards, antigens were diluted to 1.0 mg/mL and stored at -20 °C until used. The concentration of the antigens was determined by the Bradford protein assay.

#### *Protocol of the multiplex in vitro DVD-microimmunoassay*

Human samples from allergic patients and controls were analyzed as described in the Section 3.1.1.<sup>14</sup>, and the level of specific IgE was quantified following the heterologous total IgE calibration scheme (Section 3.2.1).<sup>15</sup>

### **3. Results and discussion**

The panel of antigens were evaluated, using a cohort of 36 subjects of which 18 developed an allergic reaction to one  $\beta$ -lactam antibiotic (Supplementary Table 6.2.S1). In this respect, the combination of amoxicillin (AMX) and the  $\beta$ -lactam inhibitor clavulanic acid, brand-named as Augmentin, was the culprit drug most commonly provoking the allergic episode, followed by AMX alone (13 and 3 subjects, respectively).

The remaining two subjects had an allergic reaction to cefuroxime and cefazolin. Regarding to the clinical data provided, 15 allergic reactions occurred by re-exposure to the culprit drug (immediate) and the other three were considered delayed allergic reactions provoked between 2 and 6 h after the exposure to the culprit drug.

The results of both *in vivo* (prick test) and *in vitro* (ImmunoCAP and multiplex DVD immunoassay) tests are shown in Table 1.2.1.

The analysis of the human samples by prick test showed that 10 out of 14 patients were positive to AMX (71 %), 8 out of 12 to ampicillin (AMP) (67 %) and 2 out of 14 to PG (14 %). Contrarily, none of the 13 patients tested for MDM and PPL were positive. On the other hand, the results of the reference *in vitro* test, ImmunoCAP, were less satisfactory. This is in agreement with previous studies that claimed that ImmunoCAP does not identify IgE to minor antigens or metabolites.<sup>16</sup> Therefore, the evaluation by ImmunoCAP showed that from the 18 allergic patients, 8 to AMX (44 %), 5 to phenoxymethylpenicillin (PV) (28 %) and 4 to PG (22 %) were positive. In the case of AMP, 5 out of 13 patients studied tested positive (38 %).

When the human samples were analyzed with the multiplex DVD-immunoassay, we observed that the pool of 18 control patients was negative to all the metabolite-based antigens, except to PLA (specific IgE = 0.11 IU/mL) antigen. The analysis of serum samples from the 18 allergic patients showed that all were positive to PLA (100 %), 17 to 6-APA (94 %), 16 to PGoyl (89 %), 12 to PA (67 %) and 6 to PGoyl-2 (33 %).

The detection of specific IgE by the penicillin based metabolite antigens revealed that minor antigens, prepared through the carbodiimide method, gave the best results. Therefore, it is important to maintain the  $\beta$ -lactam ring structure, common to all penicillin metabolites (except to BPG), unaltered in order to develop antigens which can be recognized by specific IgE antibodies.

Major antigens were not specifically recognized by IgE antibodies, as also observed by ImmunoCAP. Consequently, IgE antibodies from none of the selected allergic patients recognized antigen 6-APA-2. Although IgE antibodies from 6 allergic patients recognized antigen PGoyl-2, IgE concentration levels were generally lower (in 88 % of the cases) than those corresponding to minor antigen PGoyl. For instance, IgE

concentration was two fold increased when minor antigen PGoyl was used. What is more, 16 out of the 18 recruited allergic patients were positive to antigen PGoyl.

None of the allergic tested serum from allergic patients were detected using antigen PA-2. This antigen was prepared by forming disulphide bonds in physiological media (pH = 7). However, a study by Foster et al.<sup>17</sup> suggested that the protein has to be denaturalized with urea prior conjugation to PA, which can explain why antigen PA-2 did not recognize the selected allergic patients.

If the different tests (both *in vivo* and *in vitro* assays) are compared, at least one metabolite was recognized by IgE from those patients that were positive by prick test when the *in vitro* multiplex immunoassay was used. On the other hand, patients 5, 6, 8, 10, 13, 15, 16 and 18 tested negative (or tests were not realized) by prick test not only to MDM, PPL, PG, AMP (not patient 16), but also to AMX, even when the drugs involved in provoking these allergic episodes were AMX and augmentin. In particular, patients 5, 13 and 18 were also not detected by ImmunoCAP. Interestingly, these patients were positive to our prepared antigens PA and PGoyl (e.g., patient 18: [sIgE] = 0.11 and 0.62 IU/mL to antigens PA and PGoyl, respectively).

Finally, it is worth mentioning that, from all the tested allergic patients, none was positive by prick test to the minor determinant mixture (MDM) and the penicillin major determinant, PPL. In the past, MDM consisted of PG, the metabolites PGoyl (and its corresponding salt form, sodium benzylpenicilloate) and BPG. Nowadays, however, MDM only comprises sodium benzylpenicilloate (named as PGoyl in its acid form).<sup>18</sup> On the other hand, penicillin major determinant includes PG-antigen whose PG has been conjugated through the major configuration to poly-L-lysine, forming antigen PPL.

The fact that the selected patients tested negative by prick test to MDM and PPL but not to antigen PGoyl-2 (major configuration) can confuse the results obtained by *in vitro* assays. However, this is not the case and a report by Adkinson et al.<sup>4</sup> showed that the diagnostic sensitivity of *in vivo* testing with antigen PPL ranges from 36 to 90 %. A possible explanation for this, pointed out by Mayorga et al.,<sup>19</sup> suggests that the chemical stability of the antigens influences its recognition by IgE. To a large extent, PGoyl is better recognized in *in vitro* assays because otherwise it can epimerize in neutral conditions.

#### **4. Conclusions**

The incorporation of penicillin metabolites to the panel of in-use antigens is a promising approach to improve the detection of allergies to penicillins. In particular, minor metabolite-based antigens to penicillins have proven to be useful for the detection of allergic patients and discrimination of control subjects of a cohort of 36 human samples. As a result, a diagnostic specificity of 100 % was achieved (94 % when PLA was the antigen used) as well as improved diagnostic sensitivity. Therefore, the diagnostic sensitivities obtained ranged from 67 to 100 %, depending on the metabolite used. Interestingly, these values were higher than those corresponding to *in vivo* prick test or *in vitro* ImmunoCAP, analyzing the same serum samples. Herein, we demonstrated that multiplexing of metabolite-based antigens may tackle the difficulties still existing today regarding to the selectivity and the specificity of the current diagnostic tests.

**Table 1.2.1.** Results of the analysis of human serum samples

Patient No	Culprit drug	Prick-Test <sup>[a]</sup>					ImmunoCAP <sup>[b]</sup> BLC-specific IgE (IU/mL) <sup>[d]</sup>				Multiplex DVD Immunoassay <sup>[c]</sup> BLC-specific IgE (IU/mL)							
		MDM	PPL	PG	AMP	AMX	PG	PV	AMP	AMX	6-APA	6-APA 2	PA	PA 2	PLA	PGoyl	PGoyl 2	BPG
1	Augmentin <sup>[e]</sup>	N	N	N	N	P	0.04	0.01	NR	0.09	0.09	0.00	0.07	0.01	0.23	0.21	0.03	0.00
2	Cefuroxime	NR	NR	NR	NR	P	0.01	0.03	0.05	0.11	0.22	0.02	0.00	0.00	0.25	0.24	0.03	0.00
3	Augmentin	N	N	N	P	P	0.03	0.00	0.38	0.16	0.08	0.00	0.00	0.18	0.16	0.00	0.00	
4	Cefazolin	N	N	N	P	P	0.00	0.00	NR	0.82	0.16	0.04	0.00	0.00	0.08	0.00	0.00	0.00
5	Augmentin	NR	NR	NR	NR	NR	0.04	0.07	0.14	0.24	0.04	0.00	0.00	0.00	0.17	0.04	0.05	0.00
6	Augmentin	NR	NR	NR	NR	NR	1.83	2.48	1.86	1.52	0.19	0.00	0.07	0.00	0.24	0.16	0.03	0.00
7	Augmentin	N	N	N	P	P	1.83	0.97	1.91	0.86	0.17	0.00	0.20	0.00	0.38	0.24	0.05	0.00
8	Amoxicillin	NR	NR	NR	NR	NR	1.02	0.97	0.88	0.79	0.23	0.00	0.23	0.00	0.15	0.18	0.14	0.05
9	Augmentin	N	N	N	NR	P	0.08	0.06	0.07	0.13	0.11	0.00	0.06	0.00	0.20	0.10	0.02	0.02
10	Augmentin	N	N	N	N	N	0.00	0.00	0.03	0.95	0.14	0.00	0.00	0.00	0.24	0.16	0.00	0.00
11	Augmentin	N	N	N	P	P	0.12	0.37	0.10	0.29	0.47	0.00	0.44	0.00	0.40	0.14	0.37	0.00
12	Augmentin	N	N	N	P	P	0.30	0.27	0.16	0.24	0.27	0.00	0.03	0.00	0.48	0.13	0.05	0.00
13	Augmentin	NR	NR	P	NR	NR	0.03	0.03	0.09	0.08	0.06	0.00	0.22	0.02	0.10	0.41	0.05	0.02
14	Amoxicillin	N	N	N	P	P	0.17	0.30	0.43	0.46	0.67	0.00	0.17	0.00	0.85	0.62	0.07	0.00
15	Amoxicillin	N	N	N	N	N	0.43	0.69	NR	0.99	0.44	0.00	0.12	0.00	0.69	0.19	0.05	0.00
16	Augmentin	N	N	N	P	N	0.05	0.03	NR	0.41	0.43	0.01	0.07	0.00	0.30	0.18	0.06	0.00
17	Augmentin	N	N	P	P	P	0.09	0.06	NR	0.06	0.84	0.00	0.66	0.00	1.41	0.63	0.22	0.00
18	Augmentin	N	N	N	N	N	0.03	0.04	0.05	0.07	0.34	0.00	0.11	0.05	0.29	0.62	0.31	0.00
Pool -											0.00	0.01	0.02	0.02	0.11	0.00	0.05	0.00

MDM: minor determinant mixture (sodium benzylpenicilloate), PPL: penicillin major determinant (benzylpenicilloyl poly-L-lysine), PG: benzylpenicillin, AMP: ampicillin, AMX: amoxicillin, PV: phenoxymethylpenicillin, 6-APA: 6-aminopenicillanic acid, PA: penicillamine, PLA: penicillic acid, PGoyl: benzylpenicilloic acid and BPG: benzylpenilloic acid. [a] P= Positive, N= Negative, NR=Not Realized; [b] The range of quantification of ImmunoCAP is from 0.01 to 100 IU/mL, with levels above 0.10 and 0.35 IU/mL indicating sensitization and positivity, respectively; [c] Multiplex immunoassay values are the mean of three replicates using H1-based antigens. The limits of detection and quantification are 0.01 and 0.06 IU/mL, respectively. Relative standard deviation (RSD) ranged from 3 to 15 %; [d] IU/mL= 2.4 ng/mL; [e] Augmentin is a combination of amoxicillin and potassium clavulanate.

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*Experimental results*

Palacios, R.; Torres, M. J.; Vida, Y.; Perez-Inestrosa, E. The Role of Benzylpenicilloyl Epimers in Specific IgE Recognition. *Front. Pharmacol.* **2021**, *12*, 96.

In this chapter we addressed the preparation of antigens to penicillins and their evaluation by a multiplex *in vitro* colorimetric microimmunoassay.

On the one hand, 18 major and minor antigens based on amoxicillin and benzylpenicillin have been prepared, in the first part of this chapter (Section 3.1.1.), using aliphatic diamines of different alkyl chain lengths. Specifically, 13 of them were addressed using these diamines as spacer arms, while in the remaining five, diamines were utilized to generate cationizing proteins. The latter antigens were specifically recognized by IgE at low concentrations and the diagnostic sensitivity of the assay was three-fold improved in comparison with the current commercial *in vitro* diagnostic test for penicillin allergy.

On the other hand, the preparation of 8 major and minor antigens based on penicillin metabolites has been accomplished in the second part of this chapter (Section 3.1.2.). Their evaluation revealed that minor antigens permitted the detection of specific IgE as well as an improvement in terms of the diagnostic sensitivity and specificity of the developed *in vitro* assay obtained.



## *Chapter 2*

### **3.2. Production of antigens and identification of antigenic determinants to cephalosporin, carbapenem, monobactam and $\beta$ -lactam inhibitor antibiotics**



The quick development of resistance to the most commonly prescribed  $\beta$ -lactam antibiotics, AMX and PG, has gradually led to the use of less effective and broader spectrum antibiotics. In this respect, these families include new spectrum cephalosporins, carbapenems, monobactams and  $\beta$ -lactam inhibitors. However, the diagnostic of allergic reactions to these antibiotics is still an ongoing challenge. While these antibiotics have been used and included in *in vivo* tests, these tests still lack on diagnostic sensitivity ( $\leq 50\%$ ). Furthermore, there is any *in vitro* tests available on the market for the detection of allergies to these drugs. Only one cephalosporin, cephaclo, can be detected by using a commercial *in vitro* assay and, although a good diagnostic specificity is obtained, the reported diagnostic sensitivity can range from 25 to 80 %.<sup>1</sup>

The first section of this chapter (Section 3.2.1) describes the preparation of major and minor antigens for the antibiotics CFR, CFO, CFT, MRP and AZT. To evaluate the effect of the carrier protein used in the conjugation of the antibiotics, HSA and H1 are the carrier proteins used. Finally, these antigens will be evaluated by means of the *in vitro* multiplex immunoassay developed on the polycarbonate surface of DVDs, using serum samples from rabbits and from allergic patients and controls. This section highlights the relevance of both the carrier protein and the conjugation route to prepare the antigens, since the recognition patterns of IgE antibodies observed are significantly different.

On the other hand, the second section of this chapter (Section 3.2.2) addresses the production of IgG antibodies to clavulanic acid. To achieve that, three haptens derived from clavulanic acid are synthesized and conjugated to carrier proteins HSA and H1. The antibody sera raised in rabbits are also tested by immunoassay.

This chapter assesses the preparation of antigens to other families of  $\beta$ -lactam antibiotics in order to shed light on the the detection of specific IgG and IgE that might help to improve both the diagnostic sensitivity and specificity of the current *in vitro* tests.

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<sup>1</sup> Values obtained from the reference: Yoo H. S.; Kim S. H.; Kwon H. S.; Kim T. B.; Nam Y. H.; Ye Y. M.; Park H. S. Immunologic evaluation of immediate hypersensitivity to cefaclor. *Yonsei Med J.* **2014**; 55 (6), 1473-1483.





**3.2.1. Neo-antigens for the serological diagnosis of IgE-mediated drug allergic reactions to antibiotics cephalosporin, carbapenem and monobactam**



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## Neo-antigens for the serological diagnosis of IgE-mediated drug allergic reactions to antibiotics cephalosporin, carbapenem and monobactam

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### ABSTRACT

New antigens deriving from -lloyl and -llanyl, major and minor determinants, respectively, were produced for  $\beta$ -lactam antibiotics cefuroxime, cefotaxime, ceftriaxone, meropenem and aztreonam. Twenty  $\beta$ -lactam antigens were produced using human serum albumin and histone H1 as carrier proteins. Antigens were tested by multiplex *in vitro* immunoassays and evaluated based on the detection of specific IgG and IgE in the serum samples. Both major and minor determinants were appropriate antigens for detecting specific anti- $\beta$ -lactam IgG in immunized rabbit sera. In a cohort of 37 allergic patients, we observed that only the minor determinants (-llanyl antigens) were suitable for determining specific anti- $\beta$ -lactam IgE antibodies with high sensitivity ( $< 0.01$  IU/mL; 24 ng/L) and specificity (100 %). These findings reveal that not only the haptenization of  $\beta$ -lactam antibiotics renders improved molecular recognition events when the 4-member  $\beta$ -lactam ring remains unmodified, but also may contribute to develop promising minor antigens suitable for detecting specific IgE-mediated allergic reactions. This will facilitate the development of sensitive and selective multiplexed *in vitro* tests for drug-allergy diagnoses to antibiotics cephalosporin, carbapenem and monobactam.

## **1. Introduction**

Between 2000 and 2015, antibiotic consumption, expressed in defined daily doses (DDD), increased 65 % (21.1-34.8 billion DDDs) and its rate rose 39 % (11.3-15.7 DDDs per 1000 inhabitants/day). This increase was driven by low- and middle-income countries, where rising consumption has been correlated with gross domestic product per capita growth.<sup>1</sup> In fact,  $\beta$ -lactams (BLCs) are the most widely used antibiotics for treating bacterial infections and, consequently, the most frequent cause of allergic reactions.<sup>2-5</sup> They represent one of the world's major biotechnology markets with annual sales of around \$15 billion, and make up around 65 % of the total antibiotics market (world's antibiotic sales of  $3 \cdot 10^7$  kg/year of a total  $5 \cdot 10^7$  kg/year produced worldwide).<sup>6</sup> For many years, the most commonly prescribed and studied BLCs were penicillins<sup>4,7,8</sup> like benzylpenicillin (PG), amoxicillin (AMX) or ampicillin (AMP). Due to the increased allergies related to penicillins, antibiotic research rapidly led to the discovery and use of several other BLC families.<sup>9</sup> Indeed the global antibiotic consumption rate of cephalosporins, carbapenems and monobactams were 135.45, 11.26 and 6.23 DDDs, respectively, between 2016 and 2018.<sup>10</sup> In fact, the consumption of all these other BLCs in 2018 was 2.0 DDDs per 1000 inhabitants per day, which accounts for around 11 % of the total European intake.<sup>11</sup>

The chemical structure differences among BLC antibiotics promote the allergenic recognition of a broad spectrum of immune system specificities and may therefore help to provoke adverse reactions. BLCs generate -lloyl determinants after  $\beta$ -lactam ring opening under physiologic conditions. According to the bibliography, this -lloyl determinant is considered to be the 'major' determinant for penicillins because it is responsible for almost 95 % of all allergic reactions to penicillins.<sup>12</sup> The other determinants are classified as 'minor' or -llanyl determinants, and are associated with causing anaphylaxis in immediate immunoglobulin E (IgE)-mediated reactions in 95 % of cases,<sup>13,14</sup> but the explanation for the direct involvement of these determinants in provoking immediate hypersensitivity reactions still remains unclear.<sup>15</sup> To date, many studies have focused on major determinants.<sup>16</sup> However, BLC allergic reactions caused by minor determinants have been considered extremely important in penicillin allergies (e.g., skin tests for diagnosing  $\beta$ -lactam allergy),<sup>17</sup> and have been associated with specific systemic anaphylaxis.<sup>18</sup> For this reason, it is necessary to systematically and clinically study the relevance and relative importance of these -llanyl derivatives as a new range of

‘minor’ determinants for  $\beta$ -lactam allergy.<sup>13</sup> Several prospective studies are found in the literature that have evaluated the use of cephalosporins,<sup>4,5,19,20</sup> carbapenems<sup>21</sup> or monobactams in patients with documented penicillin allergy who specifically require BLC treatment, but very few *in vitro* assays are available for these studies.<sup>3</sup> In any case, many patients supposedly allergic to a BLC, or at least present allergic episode to them, are usually classified as allergic to a drug with no further investigation<sup>22</sup> or are not treated with BLCs because patients might be affected by them and at risk for anaphylaxis. The use of other antibiotics may contribute to the development of multiple drug-resistant bacteria, the emergence of other potentially dangerous side effects and a lead-in of higher healthcare costs.<sup>2</sup> Whatever the case, those patients self-reporting a penicillin allergy can be safely tested for the presence of a true allergy, normally by allergy diagnosis tests.

Allergy diagnoses must include a detailed clinical history, physical examination and *in vivo* tests (skin and/ or drug provocation tests). However, these tests pose life-threatening risks and can entail false-positive skin test results.<sup>23</sup> Currently, the gold standard *in vitro* test is based on ImmunoCap technology, but it is available only for five BLCs: PG, phenoxymethyl penicillin, AMP, AMX, cefaclor. Therefore, good antigenic determinants are lacking to develop accurate immunoassays with the potential for determining specific IgE to other BLCs in order to cover other subfamilies of BLCs to improve clinical diagnoses. A good diagnosis that relies on both *in vivo* and *in vitro* tests could allow a significant delabeling of the reported BLC allergy, and the majority of patients tolerate BLCs without incidents.<sup>24</sup> Furthermore, effective immunoassays for allergy diagnosis rely on the selection of appropriate haptens and metabolites to elicit an immune response. An examination of the specificities of BLC-reactive IgE antibodies in sera revealed a heterogeneous group of allergenic determinants, probably because the authors did not take into account the fine structural heterogeneity of the allergenic determinants of the complete range of BLCs.<sup>25</sup> When designing determinants, it is important to retain the functional groups that are unique to hapten.<sup>26</sup>

In this work, the main objective was to design and produce major and minor -lloyl- and -llanyl-derived antigens, respectively, for cefuroxime (CFR), cefotaxime (CFO), ceftriaxone (CFT), meropenem (MRP) and aztreonam (AZT). Compounds were evaluated by a multiplex direct microimmunoassay developed on the Digital Versatile Disk (DVD) using sera from immunized rabbits, and allergic patients and controls. To the best of our knowledge, this is the first report of major and minor -lloyl and -llanyl

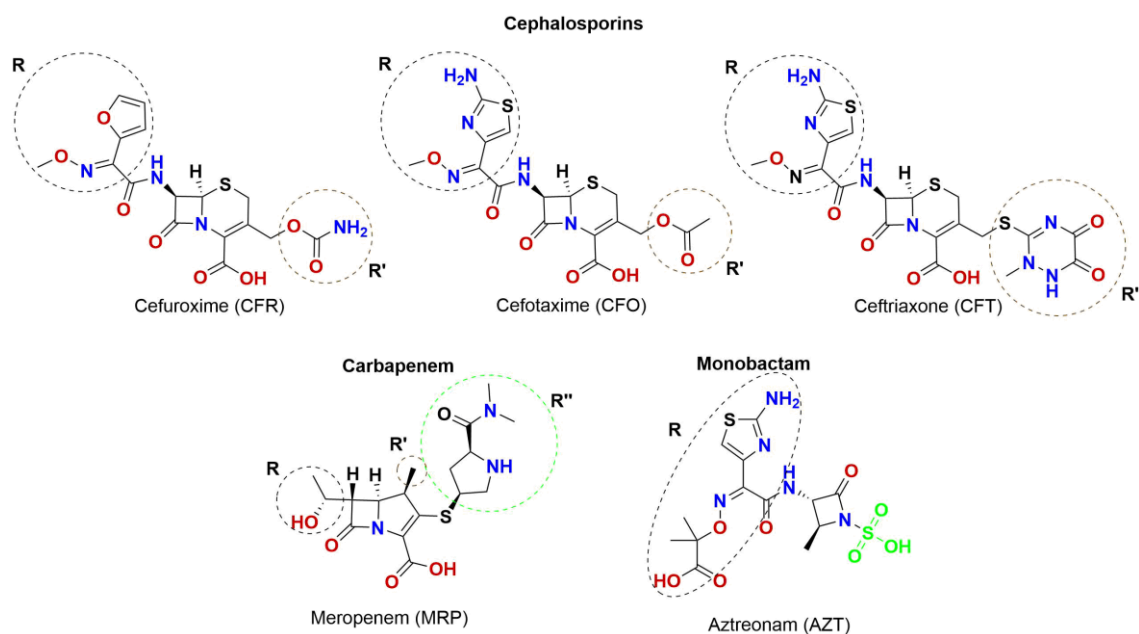
antigens for these antibiotics, and this work demonstrates the potential for an improved serological diagnosis of IgE-mediated drug allergic reactions for commonly prescribed and consumed  $\beta$ -lactam antibiotics.

## **2. Experimental section**

### *Chemicals, immunoreagents and buffers*

Benzyloxyphenoxymethyl penicillin sodium salt, meropenem trihydrate, aztreonam, cefuroxime sodium salt, cefotaxime sodium salt, ceftriaxone disodium salt hemi(heptahydrate), *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), Tween 20, human serum albumin (HSA), histone from calf thymus (H1) and keyhole limpet hemocyanin (KLH) came from Sigma-Aldrich (Madrid, Spain). The chemical structures of the studied BLCs are shown in Figure 2.1.1. Dichloromethane (DCM), dimethylformamide (DMF), hydrochloric acid 37 % (HCl) and buffer salts were purchased from Scharlau (Sentmenat, Spain) and used without further purification. Deuterated dimethyl sulfoxide (DMSO-d<sub>6</sub>) was supplied by ACROS Organics (New Jersey, USA). The anti-human IgE monoclonal antibody was purchased from Ingenasa, S.A. (Madrid, Spain). The goat anti-rabbit antibody labelled with horseradish peroxidase (GAR-HRP) and the goat anti-mouse antibody labelled with horseradish peroxidase (GAM-HRP) were supplied by Abcam (Cambridge, UK). IgE human serum (3<sup>rd</sup> WHO International Standard) was bought from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). The tetramethylbenzidine (TMB) substrate came from SDT GmbH (Baesweiler, Germany) and Coomassie Brilliant Blue R-250 staining solution was acquired from Bio-Rad (Madrid, Spain). Amicon Ultra 0.5 pre-concentrated 10K MWCO filters and Dextran Desalting Columns, 5K MWCO, 5 mL were from Fisher Scientific (Madrid, Spain).

Buffers were: (I) potassium carbonate 0.5 M, pH 11.0; (II) phosphate buffer saline (PBS 1X, 0.008 M sodium phosphate dibasic, 0.002 M sodium phosphate monobasic, 0.137 M sodium chloride, 0.003 M potassium chloride, pH 7.4); (III) PBS-T (PBS 1X containing 0.05 % Tween 20); (IV) sodium carbonate/bicarbonate buffer 0.1 M, pH 9.6, as the printing buffer. All the buffers were filtered through a 0.45- $\mu$ m pore size nitrocellulose membrane from Thermo Fisher Scientific (Madrid, Spain) before being used.



**Figure 2.1.1.** Chemical structures of the studied  $\beta$ -lactam antibiotics  
ChemDraw Professional, Version 17.0.0.206 (121)  
<https://www.perkinelmer.com/es/product/chemdraw-professional-chemdrawpro>.

### *Acidification of cephalosporin salts*

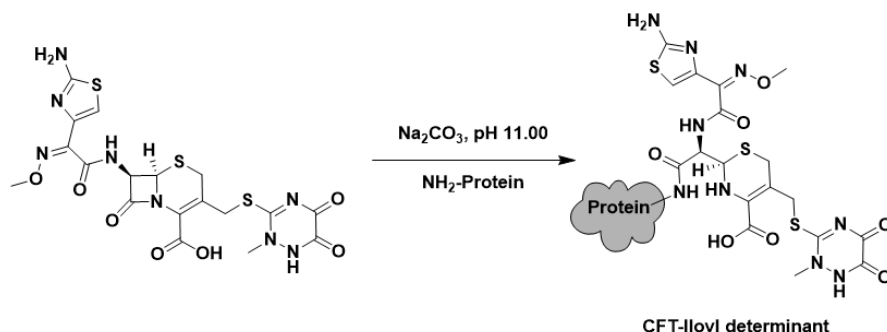
Firstly, cephalosporin salts were acidified as follows: a round-bottom flask was filled with a solution of the corresponding BLC salt dissolved in water. Then the solution was acidified with HCl 6 M followed by vacuum filtration. The compound was washed twice with acidified water and dried in a high vacuum to obtain the desired acid. The detailed procedures and data characterization of all the acidified cephalosporins are shown in the online Supplementary Information (SI).

### *Preparation of the structural antigens*

KLH was used to produce the immunogenic antigens (-lloyl and -llanyl), with which New Zealand white rabbits were immunized to raise specific IgG for CFT as a model of cephalosporin, MRP and AZT. Two carrier proteins, HSA and H1, were employed to produce the antigens. The preparation was as follows:

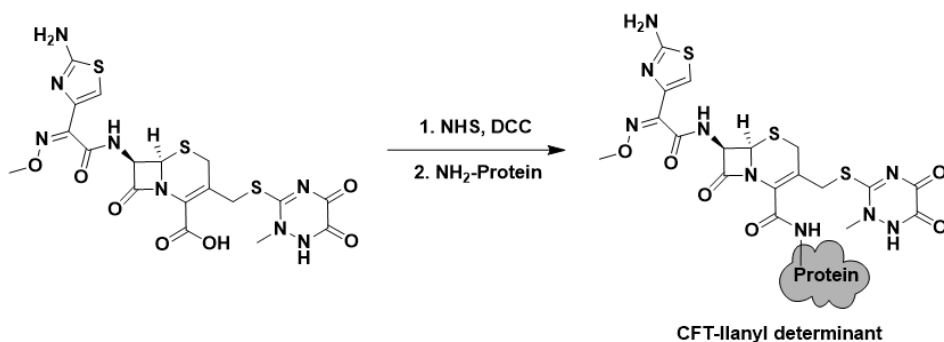
The  $\beta$ -lactam-lloyl determinants were linked through the lysine residues of the carrier proteins by  $\beta$ -lactam ring opening, through the amidation between the carbonyl carbon of the  $\beta$ -lactam ring and the amino group of the lysine residues, as previously

described<sup>27</sup> with few modifications (Figure 2.1.2). In short, the carrier protein (2.0 mg) dissolved in potassium carbonate 0.5 M, pH 11.0, was reacted with the corresponding BLC salt (22 – 30  $\mu\text{mol}$  depending on the used protein) overnight at room temperature.



**Figure 2.1.2.** Conjugation of protein CFT -lloyl determinant

The conjugation of the  $\beta$ -lactam -lanyl determinants was performed following the carbodiimide chemistry (Figure 2.1.3).<sup>28</sup> For that purpose, 22 – 30  $\mu\text{mol}$  (depending on the employed carrier protein) of the corresponding BLC as its free acid were reacted with 55  $\mu\text{mol}$  of NHS and DCC in DMF (500  $\mu\text{L}$ ) for 4 h at room temperature. Afterwards, the mixture was centrifuged at 12,000 rpm to remove the acyl urea precipitate. Finally, 250  $\mu\text{L}$  of the supernatant were added to 2.25 mL of the carrier protein solution (2.0 mg) in PBS 1X, pH 7.4, for 4 h at room temperature.



**Figure 2.1.3.** Conjugation of protein CFT -lanyl determinant

Instead of the chemical structure of AZT having a free carboxylic acid, it had a sulfonate moiety attached to the  $\beta$ -lactam ring (Figure 2.1.1). For this reason, the AZT-lanyl determinant was prepared with the free carboxylic acid presented in its side chain following the same procedure as described for the -lanyl determinants.

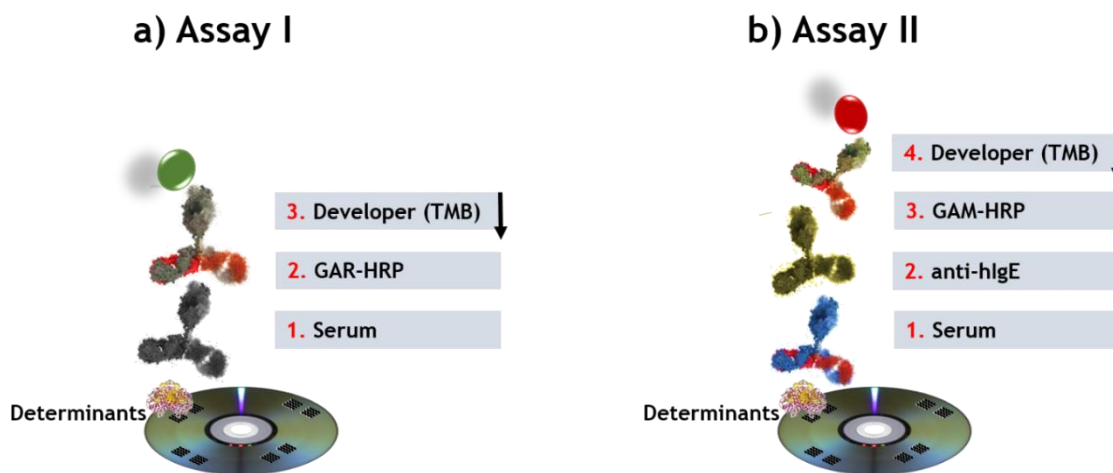


The HSA and H1 -lloyl and -llanyl determinants were purified by gel filtration chromatography using Amicon Ultra 0.5 pre-concentred 10 K filters and PBS 1X, pH 7.4, as the elution buffer. The KLH -lloyl and -llanyl determinants were purified by size exclusion chromatography on dextran desalting columns using PBS 1X, pH 7.4, as an elution buffer.

All the determinants were diluted to 1.0 mg/mL and stored at -20 °C until used. The concentration of the determinants was established by the Bradford protein assay.<sup>29</sup> The  $\beta$ -lactam/carrier protein molar ratio for the HSA determinants was established by MS-MALDI-TOF.<sup>30</sup> Histone H1 is an isolated lysine-rich fraction of mainly subfraction f1, with the other subfractions still present. The H1 antigens could not be analyzed by MS-MALDITOF. However, antigens HSA and H1 were prepared following the same experimental procedure and according to the formation of the major or minor determinants. As both proteins presented approximately 60 free lysine residues, the molar ratios of antigens H1 were estimated to be the same as those obtained for the respective HSA antigens. The KLH determinants were difficult to characterize because of the protein's high molecular weight. The selectivity of the raised rabbit IgGs was tested during the immunization by the dot blot technique<sup>31</sup> and the conjugation was considered positive when the sera obtained from the immunized rabbit specifically recognized the corresponding antigen.

#### *Assay protocol to evaluate antigens*

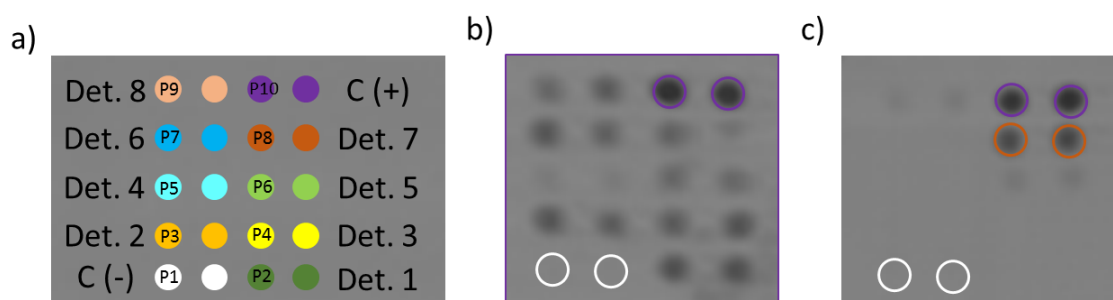
The assays consisted in detecting specific IgG (Figure 2.1.4a, assay I) and IgE (Figure 2.1.4b, assay II) on standard DVDs (CD Rohling-up GmbH, Saarbrücken, Germany). To this end, determinants (40  $\mu$ g/mL) and controls (negative and positive), prepared in printing buffer, were spotted in a microarray format (20 arrays per disk of 5  $\times$  4 spots) by dispensing 25 nL of each one using a non-contact printing device (AD 1500 BioDot, Inc., Irvine, CA, USA). Spots were 500  $\mu$ m in diameter with a centre-to-centre distance of 1.0 mm. In each microarray (Figure 2.1.5), the spots for the produced determinants (two replicates, position 2–9) and the negative (HSA, position 1) and positive (rabbit IgG or human IgE, position 10) controls were included. After printing, the DVD was incubated for 16 h at 37 °C.



**Figure 2.1.4.** Scheme of the multiplexed microimmunoassays I and II based on a direct format with colorimetric detection

To detect specific IgG against CFT, MRP and AZT, different dilutions (1/250-1/16,000) of the rabbit sera and control (PBS-T) (25  $\mu$ L per sample) were added to each array and incubated for 15 min. Then the DVD was washed with PBS-T and water before adding 25  $\mu$ L of polyclonal secondary antibody GAR-HRP in PBS-T buffer (dilution 1/400) for 15 min, followed by the washing step. To detect specific IgE, 25  $\mu$ L of the sample (allergic patients and controls) was added to each array and incubated for 30 min. After washing, 25  $\mu$ L of the mAb-IgE in PBS-T buffer (1  $\mu$ g/mL) were added and incubated for 15 min. After washing like before, 25  $\mu$ L of a 1/100 dilution of GAM-HRP were added for 15 min. Finally, an immunoreaction was run in both immunoassays by homogeneously dispensing 1.0 mL of TMB over the entire disc surface. The reaction was stopped by washing the disk with water after 15 min. Signals were read by a modified DVD drive and data were analysed as previously described.<sup>32,33</sup> All the experiments were repeated 3 times.

The affinity of the specific IgGs to CFT, MRP and AZT towards the antigens was calculated by measuring the apparent affinity constant ( $K_d^{app}$ ) in the saturation assays.  $K_d^{app}$  represents the apparent equilibrium dissociation constant between specific IgG and the corresponding antigenic determinant, and is related to the *in vitro* concentration (expressed as the dilution factor) of the specific IgG antibodies that reached half the maximum signal in the saturation assay. Binding curves were fitted by the SigmaPlot 11 software.



**Figure 2.1.5.** (a) Layout of the multiplexed microarray. Position 1: HSA, negative control, C(-); and Position 10: rIgG or hIgE, positive control, C(+); (b) Image of an array on the DVD after immunoassay I with  $\alpha$ -IgG to CFT, dilution factor 1/1,000 (Position P1: HSA, negative control; P2: CFT-HSA-llanyl determinant; P3: CFT-HSA-lloyl determinant; P4: CFT-H1-lloyl determinant; P5: CFR-HSA-llanyl determinant; P6: CFO-H1-llanyl determinant; P7: CFR-H1-lloyl determinant; P8: CFR-H1-llanyl determinant; P9: CFO-H1-llanyl determinant; P10: rIgG); (c) Representative result of the array with specific determinants and controls printed on the DVD after immunoassay II with patient 002 (determinants in the same positions as in (b), except for P10: hIgE).

The BLC-specific IgE levels expressed as units of specific IgE (IU/mL) were determined by the 3<sup>rd</sup> WHO standard for total serum IgE determinations, which involves heterologous interpolation as a calibration scheme. The calibration curve was built by performing a sandwich immunoassay where the 3<sup>rd</sup> WHO International Standard was used as a calibrator and Omalizumab<sup>34</sup> as the capture antibody. All the other immunoreagents were the same as those used for determining specific IgE (assay II). The standard data points, signal *versus* semilog concentration, were the mean of 10 curves performed on different disks on distinct days. A four-parameter logistic (4PL) curve was fitted (Supp. Fig. 6.3.S1) using the SigmaPlot 11 software. The specific IgE concentrations were calculated from the calibration curve for the total IgE.

### *Serum samples*

The study of the reactivity of the prepared determinants to the sera from the allergic patients included those patients (I) who had been diagnosed with an immediate allergic reaction to a BLC by prick tests; (II) whose culprit drug was one BLC (e.g., antibiotics amoxicillin, augmentin, benzympenicillin, cefuroxime and ceftriaxone); (III) with the total IgE (tIgE) values between 100 and 400 IU/mL (clinically considered the normal range for total IgE in a healthy population).<sup>35</sup> The clinical characteristics of the 37 patients included in this study are shown in Table 2.1.1. Thirty-seven subjects with

negative skin tests to BLCs and good tolerance to them were used as controls. Their clinical characteristics are shown in Table 6.3.S1. All the serum samples were kindly provided by the Hospital Universitari i Politènic La Fe in Valencia, Spain. All participants were enrolled after giving written informed consent according to protocols approved by the ethics review board at La Fe University Hospital (registry no. COBIOPHAD). The procedures followed were in accordance with the Helsinki Declaration of 1975 as revised in 2008. The patients were diagnosed following the procedure described in the European Network of Drug Allergy (ENDA) protocol based on skin testing, *in vitro* tests or drug provocation test, whenever necessary.

**Table 2.1.1.** Clinical characteristics of the cohort of allergic patients. The terms immediate and delayed refer to an allergic reaction provoked by re-exposure to a specific allergen and to late-phase allergic reactions that generally occur between 2 and 6 h after exposure to a specific allergen, respectively. <sup>a</sup>F female, *M* male; <sup>b</sup>NR not reported in the clinical history.

Patient Number	Sex <sup>a</sup>	Age (Years)	Culprit Drug	Clinical Manifestation	Route	Time
001	M	68	Augmentin	Anaphylaxis	Parenteral	Immediate
002	F	46	Cefuroxime	Anaphylaxis	Oral	Immediate
003	F	53	Cefuroxime	Cutaneous	Oral	Delayed
004	M	49	Augmentin	Cutaneous	Oral	Delayed
005	M	80	Augmentin	Anaphylaxis	Parenteral	Immediate
006	F	37	Cefuroxime	Cutaneous	Oral	Immediate
007	M	40	Augmentin	Cutaneous	Oral	Immediate
008	F	24	Amoxicillin	Cutaneous	Oral	Immediate
009	M	27	Ceftriaxone	Cutaneous	Parenteral	Immediate
010	F	68	Augmentin	Anaphylaxis	Parenteral	Immediate
011	F	49	Augmentin	Anaphylaxis	NR <sup>b</sup>	Immediate
012	F	52	Augmentin	Anaphylaxis	Oral	Immediate
013	M	61	Augmentin	Cutaneous	Oral	Delayed
014	F	34	Cefuroxime	Anaphylaxis	Oral	Immediate
015	M	54	Amoxicillin	Anaphylaxis	Oral	Immediate
016	M	10	Amoxicillin	Unspecific	Oral	NR
017	F	41	Amoxicillin	Cutaneous	Oral	Immediate
018	M	45	Augmentin	Cutaneous	Oral	Delayed
019	F	53	Augmentin	Cutaneous	Oral	Immediate
020	M	39	Amoxicillin	Cutaneous	Oral	NR
021	F	56	Augmentin	Cutaneous	Oral	Immediate

Patient Number	Sex <sup>a</sup>	Age (Years)	Culprit Drug	Clinical Manifestation	Route	Time
022	F	68	Amoxicillin	Unspecific	Oral	Immediate
023	M	40	Penicillin G	Cutaneous	NR	Delayed
024	M	53	Augmentin	Anaphylaxis	Oral	Immediate
025	F	49	Ceftriaxone	Anaphylaxis	Parenteral	Immediate
026	F	53	Augmentin	Cutaneous	Oral	Immediate
027	M	35	Amoxicillin	Anaphylaxis	Oral	Immediate
028	F	36	Augmentin	Cutaneous	Oral	Immediate
029	F	54	Augmentin	Cutaneous	Oral	NR
030	F	46	Augmentin	Anaphylaxis	Oral	Immediate
031	F	71	Augmentin	Cutaneous	Oral	Immediate
032	F	32	Cefuroxime	Anaphylaxis	Oral	NR
033	F	55	Cefuroxime	Cutaneous	Oral	Delayed
034	F	39	Cefuroxime	Cutaneous	Oral	Delayed
035	M	46	Augmentin	Anaphylaxis	Oral	Immediate
036	F	80	Cefuroxime	Anaphylaxis	NR	Immediate
037	F	72	Cefuroxime	Cutaneous	NR	Immediate

### 3. Results and discussion

#### *Production of $\beta$ -lactam antigens*

Firstly, acidification of cephalosporin salt (CFR, CFO and CFT) was attempted to obtain the corresponding carboxylic acid. The reaction proceeded easily with a high yield (ca. 90 %). The purity of the acidified BLC was high (see the NMR spectra in the Supplementary Information). The production of antigens relied on the conjugation of the acidified BLC (CFR, CFO, CFT, MRP and AZT) to the carrier proteins.<sup>36,37</sup> In this study, human serum albumin and histone H1 were used, and both presented approximately 60 free lysine residues to approach two different conjugation routes in order to obtain the corresponding major and minor determinants for each  $\beta$ -lactam antibiotic. The initial molar ratio ( $\beta$ -lactam/carrier protein) was set at 1,000/1 and 240/1 for HSA and H1, respectively, to ensure the conjugation of antibiotics. No elucidation method was followed for the characterization of the determinants. However, according to the MS-MALDI-TOF results (see the online Supplementary MS-MALDI-TOF spectra), the molar ratio of the HSA antigens ranged from 1 to 8. In particular, the molar ratios of both the aztreonam and ceftriaxone major and minor antigens were similar, whereas the molar

## *Experimental results*

ratios were 1 and 8, respectively, for the meropenem antigens. In general, the conjugation yield was very low for the HSA antigens (< 0.8 %). This could be due to the protecting-group-free production of the -lanyl antigens. Most of the studied BLCs bear a primary amine group in their R substituent, which could compete with that amino from Lys to form some kind of oligomers of  $\beta$ -lactam by amidation.

## *Evaluation of antigens*

The antigens were firstly evaluated as a proof of concept using sera raised to both the -loyl and -lanyl antigens for CFT, MRP and AZT. As observed in Supplementary Figures 6.3.S2-6.3.S4, the sera obtained from the immunized rabbit with the -lloyl-based determinants specifically recognized the corresponding antigen in the dot-blot assay. Conversely, this was not the case with the -lanyl determinants, which showed no specific recognition. For instance, as seen in online Supplementary Figure 6.3.S4, the AZT sera obtained from the immunized rabbit with the -lloyl-based determinant specifically recognized both the -lloyl and -lanyl determinant antigens at a dilution factor of 1/100 (v/v), but only recognized the AZT -lloyl antigen when the dilution factor was 1/500 (v/v). However, the AZT sera obtained with the -lanyl determinant did not recognize any determinant at any of the studied sera dilutions.

In order to evaluate the effect of the conjugation on assay sensitivity, each -loyl and -lanyl antigen was screened by a direct multiplex immunoassay with the -lloyl immunized sera to CFT, MRP and AZT. The tested sera dilutions were 1/250, 1/1,000, 1/4,000 and 1/16,000, while PBS-T was used as a blank. The obtained results are plotted in Figures 2.1.6 and 2.1.7 for the -lloyl and -lanyl determinants, respectively. The values represented the mean of the signals ( $n = 3$ ) obtained in each assay, together with the standard deviation (SD) expressed as error bars. As shown in the figures, the signals corresponding to the positive control (rIgG) were around 15,000 a.u. ( $\pm 8\%$ ) for all studied sera with no recognition observed for HSA (< 500 a.u) used as a negative. The dilution of the different sera allowed immunoassay sensitivity to be estimated. As we can see in Figure 2.1.7b, using sera raised to MRP ( $\alpha$ -IgG-MRP) detected the meropenem -lanyl antigen up to the 1/16,000 dilution, and gave a signal value of 1,500 a.u, which corresponded to the limit of detection.

When focusing on the 1/1,000 dilution, the response and cross-reactivity between the determinants for the different rabbit sera were compared. As we can see in Figure 2.1.6, the -lloyl determinants for cephalosporin (CFR, CFO, CFT) were specifically identified mostly by the serum raised for CFT and no cross-reactivity was observed for AZT and MRP. A similar pattern was detected when using the AZT determinants and the serum raised for it. Indeed, as expected, no other produced determinants were recognized, which indicates that recognition was specific for monobactams. Conversely, the MRP-derived determinants were recognized mostly by the corresponding serum, but values were similar to those obtained for the cephalosporin antigens, which suggests that the selectivity of the serum raised in rabbit was not high.

As far as cross-reactivity was concerned, there was evidence for different patterns with the case -llanyl determinants (Figure 2.1.7), depending on the used carrier protein. As we can see, the serum raised for CFT mostly detected the antigens produced for CFO and no cross-reactivity to AZT and MRP was identified. However, the results were the opposite for CFR due to the poor recognition of these determinants in any studied immunization sera. With CFT, the serum raised for it actually detected the HSA-antigen and cross-reactivity against other sera. In general, the sera increased for MRP and AZT detected the -llanyl MRP and AZT antigens, respectively, with no cross-reactivity to other BLCs, although AZT responses were higher than those observed with MRP.

In order to study the affinity of the structural determinants to the serum raised for the different  $\beta$ -lactam families, the binding values were analyzed. The results are provided in online Supplementary Figures 6.3.S5 and 2.1.S6. The affinity values (as  $K_d^{app}$  and coefficient of determination,  $R^2$ ) for all the rabbit IgG antibodies to each antigenic determinant are shown in online Supplementary Tables 6.3.S2 and 6.3.S3. The  $K_d^{app}$  values ranged from  $10^{-4}$  to  $10^{-2}$  (dilution factors from  $\sim 1/1000$  to  $\sim 1/20$ ), whereas  $R^2$  ranged between 0.9605 and 0.9994 and 0.6111 and 0.9984 for the -lloyl and -llanyl determinants, respectively.

It can be concluded that the HSA -lloyl determinants for CFR and AZT showed the best affinity (higher dilution) to the sera raised against CFT and AZT with  $K_d^{app}$  values corresponding to a dilution factor of 1/1,011 and 1/5,587, respectively. However, the H1-lloyl determinants for CFT and CFO showed the best recognition to the sera raised against ceftriaxone at dilution factors of 1/1,157 and 1/2,271, respectively.

### *Experimental results*

For the HSA -lanyl determinants, AZT showed the best affinity to the sera raised against it with a  $K_d^{app}$  value corresponding to a dilution factor of 1/2,228. However, the H1 -lanyl CFT and MRP determinants indicated the best recognition to the sera raised against them with  $K_d^{app}$  values corresponding to dilution factors of 1/2,168 and 1/1,437, respectively.

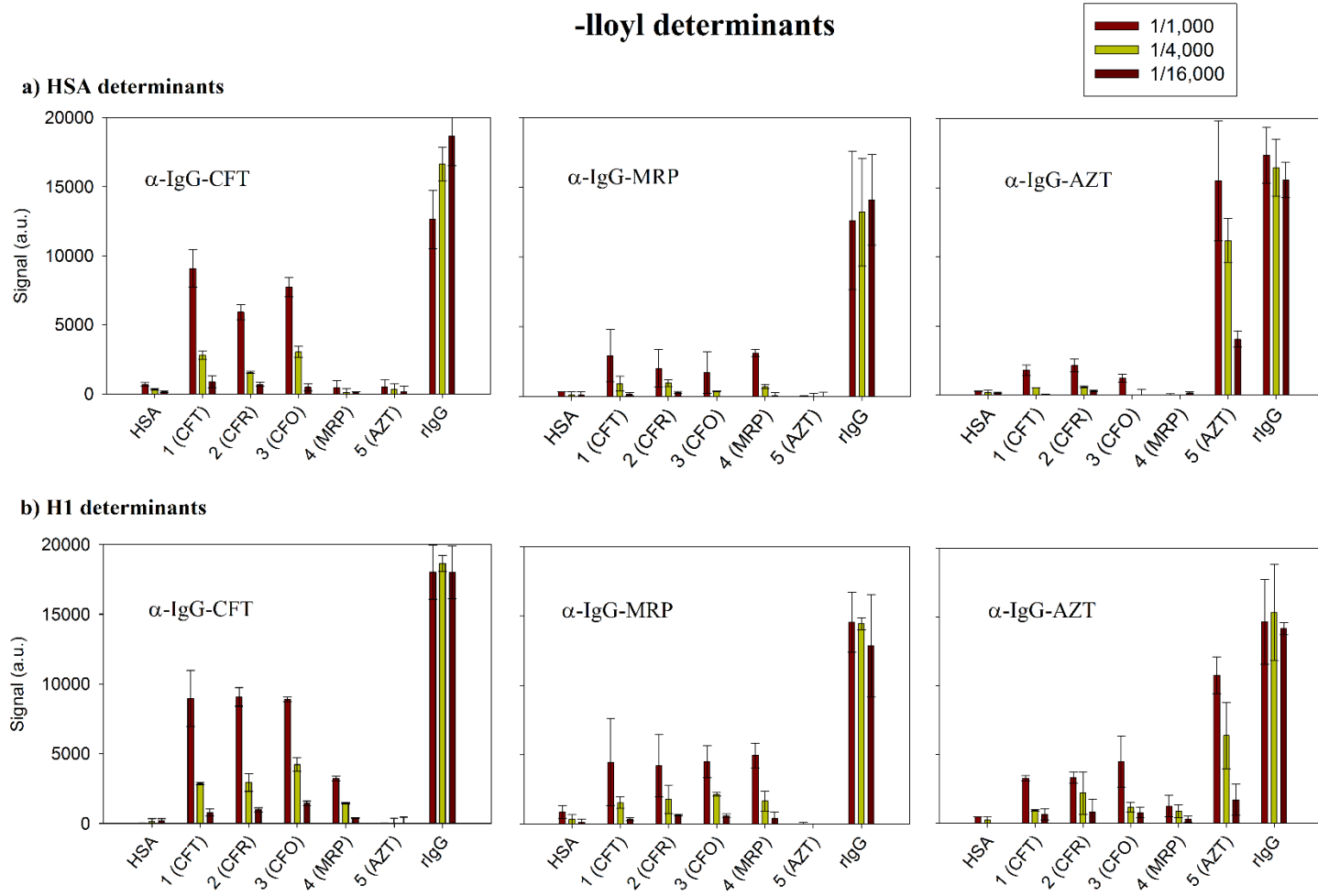
Even though some determinants had an affinity to serum, this did not necessarily mean that they gave a good response;<sup>38</sup> e.g., this is the case with H1 -lanyl antigens CFT and CFO to the sera raised against AZT. It was generally concluded that the prepared major and minor determinants were selective to the recognized specific IgGs, with significant differences between the employed carrier proteins.

### *Evaluation of the structural determinants with human serum samples*

A cohort of 74 subjects was studied, of whom 37 positively developed an immediate reaction to BLC. According to the clinical data available for the allergic patients, 14 were men and 23 were women aged 10 and 80 years. The most frequent culprit drug was augmentin, a combination of AMX and potassium clavulanate (12 subjects), followed by CFR (9), AMX (7), CFT (2) and PG in one subject (Table 2.1.1).

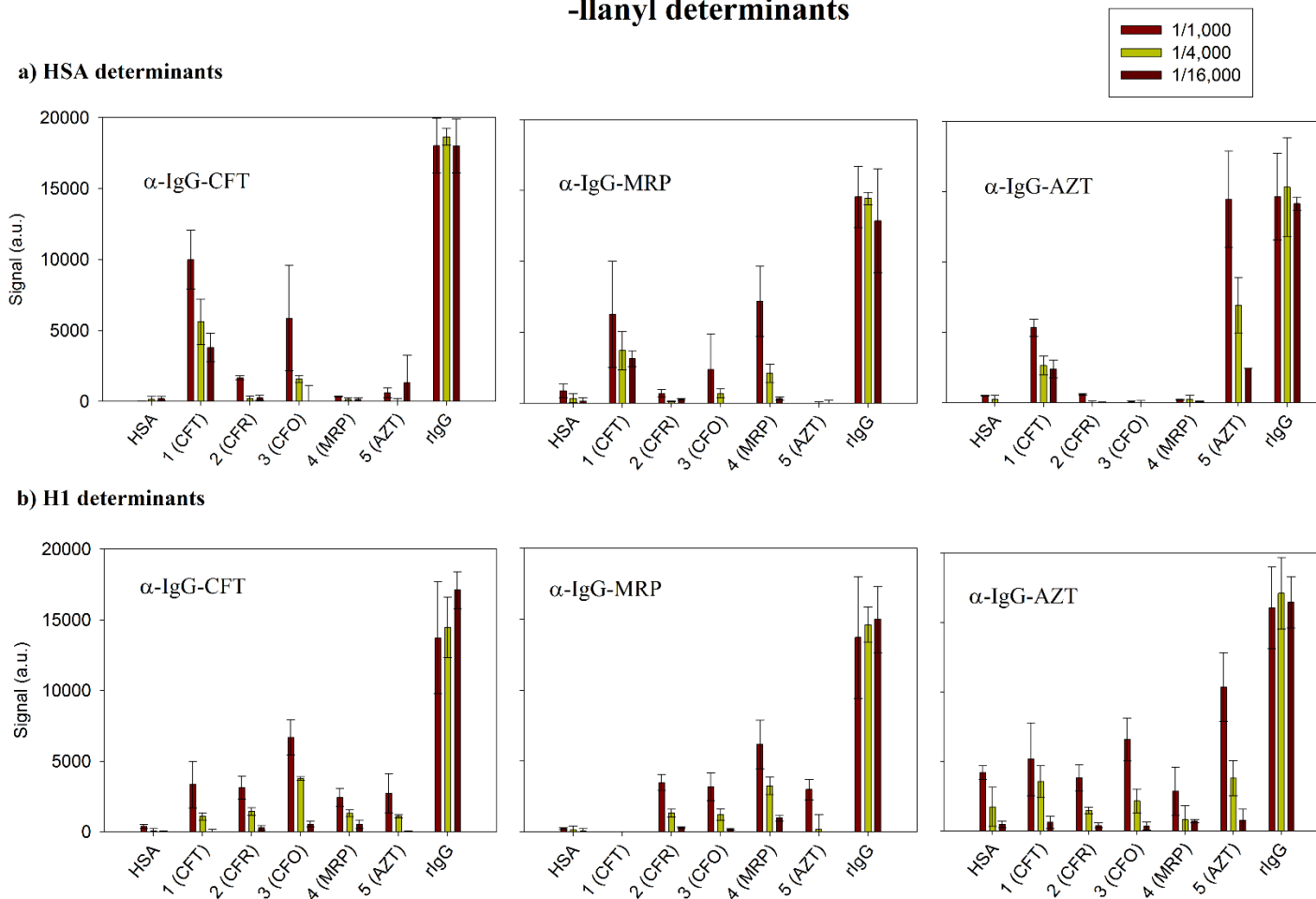
The results of the prick test and the multiplex immunoassay are described in Table 2.1.2. It is worth mentioning that all the -lloyl and HSA -lanyl determinants did not render any positive results, with values below the LOD (< 0.01 IU/mL). Only the values given by the H1 -lanyl determinants are included in Table 2.1.2.





**Figure 2.1.6.** Signals obtained for the -Iloyl determinants with polyclonal rabbit IgG. Dilution factors: 1/1,000, 1/4,000 and 1/16,000). **(a)** HSA determinants; **(b)** H1 determinants.

## -lanyl determinants



**Figure 2.1.7.** Signals obtained for the -lanyl determinants with polyclonal rabbit IgG. Dilution factors: 1/1,000, 1/4,000 and 1/16,000). (a) HSA determinants; (b) H1 determinants.

Firstly, it is important to highlight the lack of clinical information available for skin prick-tests for most of the studied antibiotics. Skin tests were performed only for CFR, and sometimes for CFT. In order to assess tests' clinical sensitivity and selectivity, a gold standard is needed, defined as the diagnostic method that can discriminate patients with an allergic reaction to BLCs from those without one. It is difficult to calculate the sensitivity of skin tests because drug provocation tests cannot be performed as a gold standard to classify subjects as allergic or not allergic because of the high risk they entail.<sup>39</sup>

The skin test results showed that 6 of the 33 patients for CFR (18 %), 3 of the 19 for CFT (29 %) and 0 of the 2 for CFO were positive. As far as MRP and AZT were concerned, only 2 and 1 of 7 and 3 patients were skin tested, which corresponded to 29 % and 33 %, respectively. It is noteworthy that the skin-tested patients to CFO, MRP and AZT obtained low percentage values (in terms of being positive to skin tests *versus* all the skin-tested patients), which must not be considered a general statement.

In order to determine the LOD and BLC-specific IgE levels, a sandwich immunoassay for total serum IgE determinations was performed as a calibration scheme. As the LOD of the multiplex immunoassay was 0.01 IU/mL (online Suppl. Fig. 6.3.S2), which corresponded to 24 ng/L of specific IgE, we chose this LOD as the cut-off threshold for evaluation purposes. When the determinants with the sera of the 37 recruited patients were evaluated, 13 for CFR (35 %), 6 for CFT (16 %), 19 for CFO (51 %), 18 for MRP (49 %) and 1 for AZT (3 %) were positive. The mean IgE values  $\pm$  SD of the studied BLCs were  $0.27 \pm 0.77$ ,  $0.02 \pm 0.01$ ,  $0.05 \pm 0.05$ ,  $0.23 \pm 0.46$  and  $0.05 \pm 0$  IU/mL to CFR, CFT, CFO, MRP and AZT, respectively. All the 37 control patients recruited in this study tested negative. Finally, no correlation was found between the total IgE levels from patients and the BLC-specific IgE levels.

Regarding the culprit drug that caused the allergic reaction, for CFR only five patients tested positive by prick tests, two were negative and two were not tested (see Table 2.1.2). The H1 CFR -Ilanyl determinant detected five patients diagnosed as positive for CFR with values ranging from 0.03 to 2.80 IU/mL. Interestingly, this antigen detected patient 006 that tested negative by prick tests. The IgE values for CFR were 0.37 and 2.80 IU/mL for patients 002 and 014, respectively. Fortunately, no cross-reactivity was observed for patient 014 to CFR, CFO, CFT, MRP or AZT. However, patient 002 was tested positive by skin tests for CFT and MRP. As the results of the skin tests and BLC-

specific IgE quantifications differed, special attention was paid to these patients owing to the high health risk for CFR administration and the possibility of cross-reactivity. With patients 009 and 025, for whom CFT provoked an allergic reaction, our determinant was not selective enough to detect it, but both patients were skin-tested positive.

To CFO, MRP and AZT, none of the studied patients had an allergic episode. Of the 17 patient samples with specific IgE values to MRP, four had specific IgE values above 0.35 IU/mL (patients 006, 012, 016 and 017) and two between 0.10 and 0.20 IU/mL (patients 018 and 019). With CFO, of the 15 positive patients detected, four had specific IgE values between 0.10 and 0.20 IU/mL (patients 004, 005, 006 and 011). It is emphasized that none of these patients was skin-tested for MRP nor CFO. This fact could have potential effects because patients can be affected by them or be at-risk for anaphylaxis.

The purpose of our study was to produce a panel of major and minor determinants for CFR, CFO, CFT, MRP and AZT to develop a multiplex *in vitro* assay to detect specific IgE for these BLCs. We not only produced a panel of determinants, but also worked on developing a panel of possible epitopes using different carrier proteins. Firstly, our results with patients showed that no major (-Iloyl) determinants were detected in any of the selected subjects, nor in the HSA-based determinants. These results reveal the importance of the conjugation route and the selection of the appropriate carrier protein. However, it cannot exclude HSA as a carrier protein candidate with immunogenic properties or -Iloyl determinants as antigenic determinants to be used in further studies as this study followed a specific conjugation methodology. Besides, a good correlation was also found between positive *in vitro* immunoassay responses to CFR and skin test results, which reveals that the produced determinant is specific and can be used for *in vitro* diagnosis, especially if we consider that both assays (specific IgE quantification and prick test) confirmed five positive patients. Moreover, the multiplex assay with the appropriate antigens was able to detect specific IgE values to CFR in one patient with a negative prick-test result (patient 006) and in two patients in whom prick tests were not performed (patients 002 and 014). Therefore, IgE assays might be suitable when skin tests have not been done, when using non-soluble BLCs, with severe reactions or with high-risk patients. In fact, for MRP for which no skin tests were performed, four patients were positive according to the specific IgE amount determined with values above 0.35 IU/mL

(patients 006, 012, 016 and 017) with the meropenem determinant. This confirmed the utility of the multiplexed assay for drug allergy diagnoses.

The use of *in vivo* and *in vitro* tests to diagnose allergic patients to BLCs needs to be adapted to the current scenario in which the consumption pattern of these antibiotics has changed, and penicillins are being progressively replaced mainly with other antibiotics like cephalosporins, carbapenems, monobactams, quinolones and tetracyclines.<sup>40</sup> Some authors suggest that skin testing with antigenic determinants of penicillins is no longer sufficient for evaluating patients for BLC allergy and that cross-reactivity between penicillins and cephalosporins<sup>41</sup> or carbapenems<sup>42</sup> can exist. For instance, cephalosporins with identical R side chains should be avoided for patients believed to be selectively allergic to aminopenicillins (e.g., cefatrizine and cefadroxil for amoxicillin or cefaclor for ampicillin).<sup>42</sup> However, these patients may receive other cephalosporins that can also promote an allergic episode,<sup>43</sup> which could be avoided by analyzing these sera by a multiplex *in vitro* assay.

In our study, specific IgE levels to CFT and MRP were observed in the patients whose culprit drug was augmentin or AMX. This was the case, for instance, of patients 012, 016 and 017 whose specific MRP IgE values ranged from 0.35 to 1.91 IU/mL. Cross-reactivity between CFR and MRP was noted in patient 006 whose culprit drug was CFR and was positive to MRP (IgE = 0.64 IU/mL). Data about related to cross-reactivity between cephalosporins and carbapenems is insufficient, but some authors suggest that carbapenems should be considered potentially cross-reactive with cephalosporins because of similarities to the  $\beta$ -lactam ring.<sup>44</sup> In patient 006, IgE antibodies were probably directed against the  $\beta$ -lactam ring, a common nuclear determinant shared by all BLCs, but no recognition was observed for CFT. The acetyl and carbamoyl groups of the R' lateral chain of cefotaxime and cefuroxime, respectively, can be easily hydrolyzed<sup>45</sup> when producing antigens. However, the thiotriazinedione moiety of CFT is stable and may take part in the epitope of the antigen, which was probably the reason for the negative response to CFT.

Consequently, it is necessary to use a panel of BLCs because there are no clinically approved validated reagents. A well-established multiplex *in vitro* method was herein followed to evaluate the presence of IgE antibodies in a defined allergic population in which augmentin, CFR and AMX were the drugs that most often induced immediate reactions. As some authors suggest that anaphylaxis predominates the cutaneous

condition<sup>46</sup> (presented as urticaria) in allergic reactions to BLCs, using a multiplex *in vitro* test is a good alternative to *in vivo* assays to detect positive patients in short times and to study a conjunction of BLCs together and instantaneously given this multiplex capacity. The problem with false-negatives is also noteworthy. For instance, Demoly and Romano indicate a positive provocation test for 8 – 17 % of patients with negative skin tests to penicillin.<sup>22</sup> Therefore, in those patients who are shown negative by skin and/or drug provocation tests, the combination of both *in vivo* and *in vitro* tests is a useful approach to detect all the potential allergic patients.

#### **4. Conclusions**

The production and evaluation of structural determinants for  $\beta$ -lactam allergy is key for developing sensitive and selective *in vitro* tests to determine specific IgE that can complement *in vivo* tests, data and clinical history, whenever possible. The conjunction of both techniques can allow better diagnoses and allow significant delabeling that should allow improvements in future antibiotic treatments of reported BLC allergy. Unlike all the studies related to penicillins reactivity, in which the chemical structures of their antigenic determinants are already established, the chemistry of other  $\beta$ -lactam antibiotics lacks this information.

Allergic reactions to these families may occur by sensitization to them or to determinants that share similar chemical chains to those of penicillins. Different epitopes have not yet been identified. This study develops a multiplex sensitive immunoassay for the *in vitro* determination of both specific IgG and IgE levels by using major and minor determinants of a wide variety of less-studied  $\beta$ -lactam antibiotics, such as cefuroxime, cefotaxime, ceftriaxone, meropenem and aztreonam. It demonstrates that all the produced antigenic determinants are selective for detecting specific anti- $\beta$ -lactam IgG in rabbit sera, but only minor determinants are able to detect specific anti- $\beta$ -lactam IgE in human serum samples from allergic patients because the -lloyl determinants formed with this protocol are not recognized. This work also demonstrates that the carrier protein plays an important role in molecular recognition pattern terms as only the prepared H1-derived determinants were specifically recognized using allergic patient samples. Thus in patients with a history of a serious and potentially IgE-mediated reaction to a cephalosporin, it is critical to avoid re-exposure to the same cephalosporin, a cephalosporin compound that shares

**Table 2.1.2.** Results of the analysis of human serum samples by *in vivo* (prick test) and *in vitro* (multiplex DVD assay) tests

Patient Number	Culprit Drug	ImmunoCAP	Prick-Test <sup>a</sup>										BLC-specific IgE <sup>b</sup> (IU/mL)				
		tIgE (IU/mL <sup>c</sup> )	MDM	PPL	PG	AMP	AMX	CFR	CFT	CFO	MRP	AZT	CFR	CFT	CFO	MRP	AZT
001	Augmentin <sup>d</sup>	265	N	N	N	N	P	P	N	NR	NR	N	<LOD <sup>e</sup>	<LOD	<LOD	<LOD	<LOD
002	Cefuroxime	129	NR	NR	NR	NR	P	NR	P	NR	P	NR	0.37	0.01	0.02	<LOD	<LOD
003	Cefuroxime	27	N	N	N	N	N	P	N	NR	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
004	Augmentin	14	N	N	N	P	P	N	NR	NR	NR	NR	<LOD	<LOD	0.16	<LOD	<LOD
005	Augmentin	7,686	N	N	N	P	P	N	N	NR	NR	NR	<LOD	0.02	0.13	0.03	<LOD
006	Cefuroxime	4	N	N	N	N	N	N	NR	NR	NR	NR	0.03	<LOD	0.16	0.64	<LOD
007	Augmentin	146	N	N	P	P	P	N	N	NR	N	NR	<LOD	<LOD	<LOD	<LOD	<LOD
008	Amoxicillin	95	N	N	N	N	P	N	NR	NR	NR	NR	<LOD	<LOD	0.04	<LOD	<LOD
009	Ceftriaxone	401	N	N	N	N	N	N	P	NR	NR	NR	<LOD	<LOD	0.06	0.04	<LOD
010	Augmentin	300	N	N	N	P	P	N	N	NR	N	NR	<LOD	0.02	0.03	0.02	<LOD
011	Augmentin	36	NR	NR	NR	NR	NR	N	N	NR	N	NR	0.02	0.04	0.11	0.03	<LOD
012	Augmentin	30	N	N	N	NR	P	N	N	NR	NR	NR	<LOD	<LOD	<LOD	1.91	<LOD
013	Augmentin	124	N	N	N	N	N	N	NR	NR	NR	NR	0.02	0.01	0.02	0.05	0.05
014	Cefuroxime	1,847	N	N	N	N	P	NR	NR	NR	N	NR	2.80	<LOD	0.01	0.01	<LOD
015	Amoxicillin	206	N	N	N	N	P	N	NR	NR	NR	NR	0.01	<LOD	<LOD	<LOD	<LOD
016	Amoxicillin	152	P	N	N	N	N	N	NR	NR	NR	NR	<LOD	<LOD	<LOD	0.49	<LOD
017	Amoxicillin	114	N	N	N	N	P	N	NR	NR	NR	NR	<LOD	<LOD	<LOD	0.35	<LOD
018	Augmentin	42	N	N	N	N	P	N	NR	NR	NR	NR	0.01	<LOD	0.02	0.16	<LOD
019	Augmentin	126	N	N	N	P	P	N	NR	NR	NR	NR	0.01	<LOD	0.01	0.14	<LOD
020	Amoxicillin	353	N	N	N	N	P	N	NR	NR	NR	NR	<LOD	<LOD	0.02	0.07	<LOD
021	Augmentin	287	N	N	N	P	P	N	N	NR	NR	NR	0.01	<LOD	0.01	<LOD	<LOD
022	Amoxicillin	273	N	N	N	N	N	N	N	NR	N	N	<LOD	<LOD	0.02	0.03	<LOD

**Table 2.1.2. (continuation).** Results of the analysis of human serum samples by *in vivo* (prick test) and *in vitro* (multiplex DVD assay) tests

		ImmunoCAP	Prick-Test <sup>a</sup>										BLC-specific IgE <sup>b</sup> (IU/mL)				
Patient Number	Culprit Drug	tIgE (IU/mL <sup>c</sup> )	MDM	PPL	PG	AMP	AMX	CFR	CFT	CFO	MRP	AZT	CFR	CFT	CFO	MRP	AZT
023	Benzylpenicillin	286	P	P	N	P	N	N	N	N	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
024	Augmentin	228	NR	NR	P	NR	NR	NR	N	NR	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
025	Ceftriaxone	8	N	N	N	N	N	N	P	N	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
026	Augmentin	187	N	N	N	N	N	N	NR	NR	NR	NR	<LOD	<LOD	<LOD	0.04	<LOD
027	Amoxicillin	135	N	N	N	N	N	N	NR	NR	NR	P	0.01	0.01	0.07	0.02	<LOD
028	Augmentin	434	NR	NR	NR	NR	NR	N	N	NR	P	NR	0.01	<LOD	<LOD	0.02	<LOD
029	Augmentin	130	N	N	N	N	P	N	N	NR	NR	NR	<LOD	<LOD	0.01	<LOD	<LOD
030	Augmentin	267	N	N	P	NR	P	N	N	NR	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
031	Augmentin	241	N	N	N	N	P	N	N	NR	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
032	Cefuroxime	245	N	N	N	N	N	P	NR	NR	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
033	Cefuroxime	9	N	N	N	N	N	N	NR	NR	NR	NR	<LOD	<LOD	0.03	0.05	<LOD
034	Cefuroxime	47	N	N	N	N	N	P	P	P	NR	NR	0.08	<LOD	<LOD	<LOD	<LOD
035	Augmentin	348	N	N	N	N	N	N	NR	NR	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
036	Cefuroxime	177	N	N	N	N	N	P	N	NR	NR	NR	<LOD	<LOD	<LOD	<LOD	<LOD
037	Cefuroxime	457	N	N	N	N	N	P	P	NR	N	NR	0.08	<LOD	0.04	<LOD	<LOD
Controls	–	–						–	–	–	–	–	<LOD	<LOD	<LOD	<LOD	<LOD

MDM: minor determinants mixture (benzylpenicillin, sodium benzylpenicilloate and benzylpenicilloic acid), PPL: penicillin major determinant (benzylpenicilloyl poly-L-lysine), PG: benzylpenicillin, AMP: ampicillin, AMX: amoxicillin, CFR: cefuroxime, CFT: ceftriaxone, CFO: cefotaxime, MRP: meropenem and AZT: aztreonam. Immunoassay values are the mean of three replicates. Relative standard deviation (RSD) ranged from 4 to 13 %. <sup>a</sup>P= Positive, N= Negative, NR=Not Realized; <sup>b</sup>Multiplex-DVD assay, using the H1-llanyl determinants; <sup>c</sup>IU/mL= 2.4 ng/mL; <sup>d</sup>Augmentin is a combination of amoxicillin and potassium clavulanate; <sup>e</sup><LOD= Value below the limit of detection (LOD).



the same side chain, and even to other BLC sharing the same side chain, such as ceftazidime and cefadroxil and aztreonam.

These findings reveal that haptization of  $\beta$ -lactam antibiotics renders enhanced and interesting molecular recognition events, even when the  $\beta$ -lactam ring remains unmodified. This contribution may improve the current clinical diagnostics of allergies to antibiotics cephalosporin, carbapenem and monobactam together with drug provocation tests used as the gold standard. Indeed, future studies are needed to corroborate these *in vitro* findings with oral provocation tests.

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### **3.2.2. Hapten synthesis for clavulanic acid**





## Hapten synthesis for clavulanic acid

### ABSTRACT

Three derivatives of the  $\beta$ -lactam inhibitor clavulanic acid have been synthesized and conjugated to carrier proteins and further used to raise polyclonal antibodies. Two of the three antigens generated IgG antibodies highly specific to clavulanic acid, highlighting the importance of the chemical structure. Thus, esterification of the potassium carboxylate and oxidation of the alcohol at the C-6 side chain have proven to be valuable in the production of specific IgG. Therefore, the prepared antigens could be incorporated in the panel of the selected antigens employed to commercially test allergies to clavulanic acid as well as applied to developed serum prepared *ad hoc*.

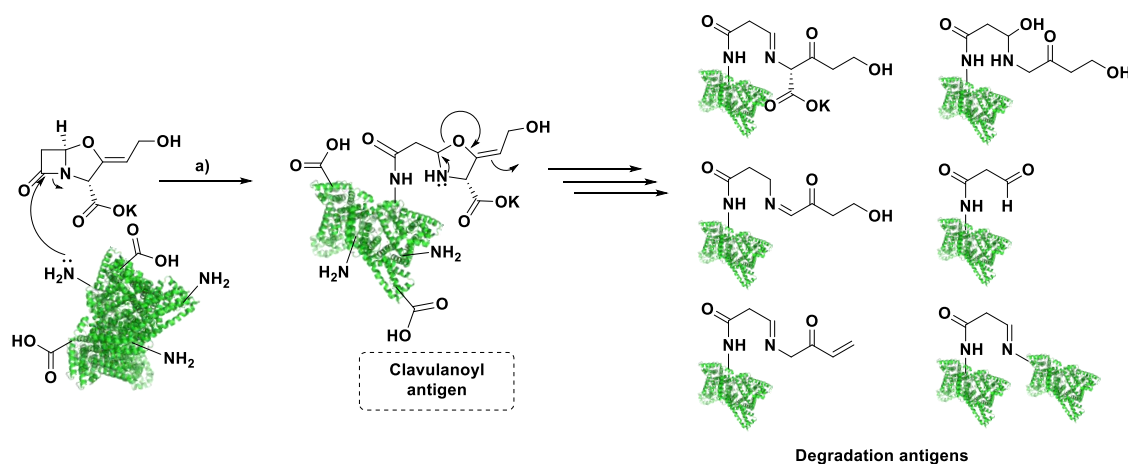
### 1. Introduction

Clavulanic acid is considered a  $\beta$ -lactam inhibitor as it forms covalent adducts with the  $\beta$ -lactamase *Mycobacterium tuberculosis*.<sup>1</sup> Although it is not effective enough on its own as an antibiotic, its use is widely widespread.<sup>2</sup> The potassium salt, potassium clavulanate (CVL), is administered in conjunction with amoxicillin (AMX), forming a combination commonly brand-named as Augmentin (AMX-CVL), or co-amoxiclav.<sup>3</sup> It is referred as one of the most administrated drugs, together with AMX alone.<sup>4</sup> According to a report from the World Health Organization (WHO),<sup>5</sup> AMX and AMX-CVL were the most frequently consumed oral antibiotics to treat and prevent infections in the European Union (EU), between 2016 and 2018. They accounted for a median proportional use of 16 and 15 %, respectively. Moreover, AMX-CVL generated sales exceeding \$100 million only in the United States (US) in 2003.<sup>6</sup> It is not surprising, therefore, that AMX and AMX-CVL are the major cause of drug hypersensitivity reactions (DHRs) in the last decades.<sup>7</sup> According to a survey by the European Academy of Allergy and Clinical Immunology (EAACI),<sup>8</sup> allergies to AMX-CVL accounted for 76 % of a total 54 cases of DHRs in the EU during 2016. However, the true incidence of AMX-CVL allergy is still unknown. For instance, a study by Heng et al.<sup>9</sup> reported that from 75 (43.1 %) up to

173 allergic episodes to AMX-CVL, only 8.1 % tested positive to AMX-CVL after skin testing.

Initially, it was thought that AMX-CVL allergy was only due to AMX, since the antigens or metabolites formed by CVL were considered to be non-immunogenic.<sup>10</sup> However, the presence of IgE antibodies to CVL was observed in allergic patients to this drug.<sup>11,12</sup> From that point on, several studies focused on the allergenicity of CVL antigens.<sup>13,14</sup> For instance, a study by Torres et al.<sup>15</sup> indicated that 16 (29 %) up to 55 allergic patients to AMX-CVL were due to CVL and Sánchez-Morillas et al.<sup>16</sup> reported nine allergic episodes to CVL after intradermal skin testing. However, the number of true allergic patients to CVL is still very low and more studies with a larger number of samples are needed. Thus, the need to work on the quantification of clavulanic acid and the elucidation of the mechanisms and antigens involved in its immunological recognition still remains challenging.

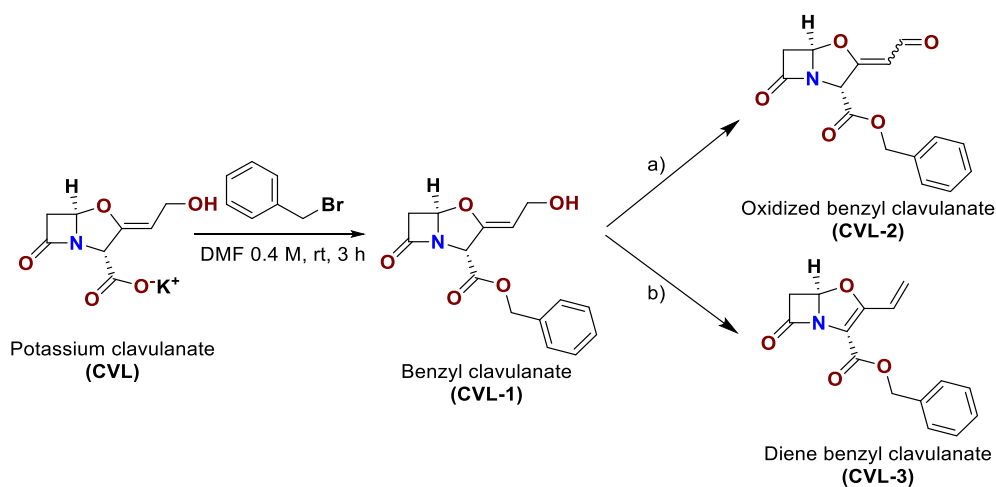
CVL belongs to clavams. Its chemical structure consists of an oxazolidine ring fused to the  $\beta$ -lactam ring, which is characteristic of all the  $\beta$ -lactam antibiotics (BLCs).<sup>17</sup> The most abundant antigen, known as clavulanoyl antigen, is formed by amidation between amine groups of lysine residues of the carrier proteins and the resulting carbonyl carbon after  $\beta$ -lactam ring opening.<sup>18</sup> However, this antigen has been observed to be an unstable oxazolidine-like intermediate that tautomerizes and decomposes to form other antigens without the oxazolidine moiety (Figure 2.2.1).<sup>19,20</sup> These structures have been observed *in vitro* at high concentrations in four patients exposed to clavulanic acid.<sup>19</sup> However, the immunogenicity of these degradation antigens still remains unclear and they have not been fully tested for diagnostic purposes.



**Figure 2.2.1.** Scheme of the antigens formed during CVL conjugation to carrier proteins

Instead of studying the immune response of the mentioned degradation antigens, in this work the preparation of major antigens based on potassium clavulanate has been evaluated through the synthesis of three haptens that maintain the characteristic structure of the clavulanate intact (Figure 2.2.2). The chemical entities incorporated may facilitate the stabilization of the conjugates formed, allowing the identification of possible epitopes that can be recognized by specific IgE to clavulanic acid. The first derivative, **CVL-1**, has a similar structure to CVL, but the potassium carboxylate has been esterificated with benzyl bromide. Oxidation of the primary alcohol to aldehyde at the C-6 substituent generated the second hapten, **CVL-2**, modifying the  $\beta$ -hydroxyethylidene side chain to  $\beta$ -oxoethylidene. Finally, a diene has been formed in **CVL-3** and the oxazolidine ring characteristic of oxapenamams modified into a 2,3-dihydrooxazoline ring linked to a vinyl as a side chain. These haptens have been conjugated to the carrier proteins human serum albumin (HSA) and histone (H1), and the immunogenic protein keyhole limpet hemocyanin (KLH).

This study aims to get insight into the identification of CVL-antigens with the immunological properties needed to produce rabbit IgG antibodies. For that, the evaluation of the produced sera has been carried out by means of two direct immunoassays, the enzyme linked immunosorbent assay (ELISA) and an *in vitro* microimmunoassay based on digital versatile disc (DVD) technology. The production of IgG antibodies will not only allow for the construction of rabbit antibody libraries highly specific and selective, but also will permit their use as standards for drug allergy diagnostic immunoassays.



**Figure 2.2.2.** Scheme of the synthesized clavulanate derivatives. a) DMP, DMC 0.05 M, 0 °C, 30 min; b) MsCl, Et<sub>3</sub>N. DCM 0.10 M, 0 °C, 30 min; then rt, 3 h.

## **2. Experimental section**

### *General methods*

Oxygen- and/or moisture-sensitive reactions were carried out in flame-dried glassware under nitrogen atmosphere. All reagents and solvents were purchased and used as received from commercial vendors or synthesized according to cited procedures.

All yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Reaction progress was monitored by analytical thin-layer chromatography (TLC) and <sup>1</sup>H-NMR spectroscopy. TLC analyses were performed using E. Merck silica gel 60 F254 pre-coated plates (250 μm). A handheld 254 nm UV lamp and potassium permanganate staining solution (with light heating) were used for detection. Flash column chromatography was performed using a Teledyne ISCO CombiFlash Rf+ purification system with RediSep Rf Gold Normal-Phase Silica columns.

NMR spectra were recorded on Bruker UltraShield 300 MHz and Ascend 400 MHz spectrometers. Known compounds were characterized by, at minimum, <sup>1</sup>H-NMR. Further NMR experiments were performed as needed to confirm structural assignment. <sup>1</sup>H- and <sup>13</sup>C-NMR chemical shifts (δH, δC) are reported in parts per million (ppm) relative to the appropriate solvent–CDCl<sub>3</sub>: 7.26 (<sup>1</sup>H), 77.16 (<sup>13</sup>C) ppm. Resonances are described using the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), quin. (quintet), m (multiplet), br (broad), dd (doublet of doublets), etc. Coupling constants (J) are given in Hz and are rounded to the nearest 0.1 Hz. All NMR data were collected at 25 °C.

Low-resolution mass spectra (LRMS) were acquired on a Waters 2795 separations module coupled to a 3100 mass detector operating in ESI+ mode.

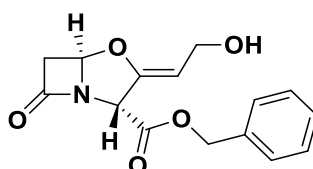
### *Chemicals, Reagents and Buffers*

Potassium clavulanate is from Combi-Blocks. Benzylpenicillin sodium salt, ceftriaxone disodium salt hemi(heptahydrate), aztreonam, meropenem trihydrate, benzyl bromide, Dess Martin periodinane, mesyl chloride, trimethylamine, o-phenylenediamine (OPD), Tween 20, human serum albumin (HSA), histone from calf thymus (H1) and

keyhole limpet hemocyanin (KLH) are from Sigma-Aldrich (Madrid, Spain). Dimethylformamide (DMF), dichloromethane (DCM) and buffer salts are from Fisher Scientific (MA, USA) and used without further purification. Hydrogen peroxide, solution 30 % w/w, and sulfuric acid, 95-97 %, are from Scharlau (Sentmenat, Spain). Deuterated chloroform ( $\text{CDCl}_3$ ) is from Cambridge Isotope Laboratories (MA, USA). Goat anti-rabbit antibody labeled with horseradish peroxidase (GAR-HRP) and the goat anti-mouse antibody labelled with horseradish peroxidase (GAM-HRP) are purchased from Abcam (San Francisco, USA). IgE human serum (3<sup>rd</sup> WHO International Standard) was bought from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). Tetramethylbenzidine (TMB) substrate is from SDT GmbH (Baesweiler, Germany) and Coomassie Brilliant Blue R-250 staining solution is from Bio-Rad (Madrid, Spain). Amicon Ultra 0.5 pre-concentred 10 K MWCO filters and Dextran Desalting Columns, 5K MWCO, 5 mL are from Fisher Scientific (Madrid, Spain). ELISA plates (96-well polystyrene, high-binding) are from Costar (NY, USA) and DVDs are from CD Rohling-up GmbH (Saarbrücken, Germany).

The employed buffers are (I) potassium carbonate 0.5 M, pH 11.0; (II) phosphate buffer saline (PBS 1X, 0.008 M sodium phosphate dibasic, 0.002 M sodium phosphate monobasic, 0.137 M sodium chloride, 0.003 M potassium chloride, pH 7.4); (III) PBS-T (PBS 1X containing 0.05 % Tween 20); (IV) sodium carbonate/bicarbonate buffer 0.1 M, pH 9.6; and (V) substrate buffer (0.03 M citric acid, 0.06 M sodium phosphate dibasic, pH 5.5). All buffers are filtered through a 0.45- $\mu\text{m}$  pore size nitrocellulose membrane from Thermo Fisher Scientific (Madrid, Spain) before used.

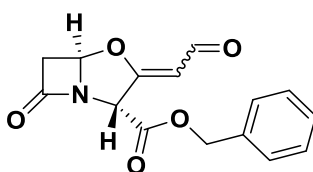
#### *Synthesis of modified haptens*



#### **Benzyl clavulanate (CVL-1)**

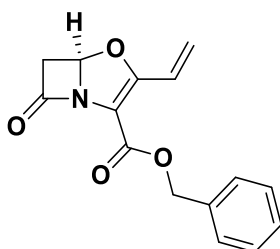
Potassium clavulanate (200 mg, 0.81 mmol) in dry DMF (2.03 mL, 0.4 M) was treated with benzyl bromide (0.58 mL, 4.85 mmol, 6 equiv). The solution was kept at room temperature (approx. 17-18 °C) for 3 h under anhydrous conditions. The solution was

concentrated by rotary evaporation and the residue was purified by flash column chromatography (ISCO) to give **CVL-1** as a colorless oil (124 mg, 52 % yield).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.45 – 7.32 (m, 5H), 5.72 (d,  $J = 2.8$  Hz, 1H), 5.23 (s, 2H), 5.11 (s, 1H), 4.90 (dt,  $J = 7.0, 1.5$  Hz, 1H), 4.24 (dd,  $J = 7.1, 3.3$  Hz, 2H), 3.51 (dd,  $J = 16.7, 2.8$  Hz, 1H), 3.10 (d,  $J = 16.7$  Hz, 1H), 1.39 (br, 1H). LRMS ( $\text{ESI}^+$ )  $m/z$  calculated for  $\text{C}_{15}\text{H}_{15}\text{NO}_5$  ( $[\text{M}+\text{H}]^+$ ): 290.10, found: 290.10.



### Oxidized benzyl clavulanate (CVL-2)

Benzyl clavulanate (400 mg, 1.38 mmol) in 10.00 mL of DCM was added at 0 °C to a solution of Dess Martin periodinane (1.17 g, 2.77 mmol, 2 equiv.) in 17.60 mL of DCM. The mixture was stirred for 1 h at room temperature, and filtered through a pad of  $\text{Na}_2\text{SO}_4$ . The solution was concentrated by rotary evaporation and the residue was purified by flash column chromatography (ISCO) to give the yellow oil **CVL-2** as a 1:1.2 mixture of the *E*- and *Z*- isomers (176 mg, 40 % yield).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  (*E*-isomer): 9.63 (d,  $J = 5.3$  Hz, 1H), 7.45-7.29 (m, 5H), 5.95 (d,  $J = 2.8$  Hz, 1H), 5.92 (d,  $J = 5.2$  Hz, 1H), 5.71 (s, 1H), 5.21 (s, 2H), 3.61 (dd,  $J = 3.4, 2.5$  Hz, 1H), 3.20 (d,  $J = 16.9$  Hz, 1H); (*Z*-isomer): 9.92 (d,  $J = 7.7$  Hz, 1H), 7.45-7.29 (m, 5H), 5.84 (d,  $J = 2.9$  Hz, 1H), 5.41 (d,  $J = 7.7$  Hz, 1H), 5.30 (s, 1H), 5.22 (s, 2H), 3.67 (dd,  $J = 2.9, 2.5$  Hz, 1H), 3.27 (d,  $J = 17.0$  Hz, 1H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  (*E*-isomer): 189.1, 176.7, 173.1, 164.4, 134.4, 129.0, 128.9, 128.9, 128.8, 128.4, 103.7, 91.8, 68.4, 62.5, 46.9; (*Z*-isomer): 188.2, 176.7, 173.1, 164.4, 134.4, 129.0, 128.9, 128.9, 128.8, 128.4, 103.5, 89.2, 68.7, 65.3, 46.9. LRMS ( $\text{ESI}^+$ )  $m/z$  calculated for  $\text{C}_{15}\text{H}_{13}\text{NO}_5$  ( $[\text{M}+\text{H}]^+$ ): 288.08, found: 288.27.



**Diene benzyl clavulanate (CVL-3)**

This compound was obtained when benzyl clavulanate was reacted with mesyl chloride. Benzyl clavulanate (80 mg, 0.28 mmol) was dissolved in anhydrous DCM (2.77 mL) at 0°C. Then, mesyl chloride (26  $\mu$ L, 0.33 mmol, 1.2 equiv.) was added dropwise via syringe under a N<sub>2</sub> atmosphere followed by triethylamine (48  $\mu$ L, 0.36 mmol, 1.3 equiv.). The solution was kept at 0 °C and stirred for 30 min and at room temperature (approx. 17-18 °C) for 3 h under anhydrous conditions. The resulting mixture was extracted with dichloromethane (3x4 mL) and water (4 mL), and combined organic layers were washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. The solution was concentrated by rotary evaporation and the residue was purified by flash column chromatography (ISCO) to give diene **CVL-3** as a colorless oil (6 mg, 8 % yield). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$ : 7.41 – 7.31 (m, 5H), 6.61 (dd,  $J$  = 17.0, 10.8 Hz, 1H), 6.32 (d,  $J$  = 16.8 Hz, 1H), 5.77 (d,  $J$  = 10.9 Hz, 1H), 5.66 (dd,  $J$  = 4.1, 2.1 Hz, 1H), 5.33 – 5.25 (m, 2H), 3.54 (dd,  $J$  = 15.9, 4.2 Hz, 1H), 3.24 (dd,  $J$  = 15.6, 2.0 Hz, 1H). LRMS (ESI<sup>+</sup>)  $m/z$  calculated for C<sub>15</sub>H<sub>13</sub>NO<sub>4</sub> ([M+H]<sup>+</sup>): 272.09, found: 271.84.

*Antigen preparation*

0.5 mg of the carrier protein (HSA or H1) were added to a solution of the desired compound **CVL-1**, **CVL-2** and **CVL-3** (7  $\mu$ mol, 1,000 and 240 equiv. for HSA and H1, respectively) in potassium carbonate 0.5 M, pH 11.0, in 10 % DMF and stirred overnight at room temperature. Moreover, CVL, PG, CFR, AZT and MRP antigens were prepared and used as models for the clavam, penicillin, cephalosporin, monobactam, and carbapenem families, respectively. To prepare them, the corresponding BLC was dissolved, as commercially supplied, in potassium carbonate 0.5 M, pH 11.0 and stirred overnight. Antigens were purified by gel filtration chromatography on Amicon Ultra 0.5 pre-concentrated 10 K filters using PBS 1X, pH 7.4, as elution buffer.

Two New Zealand white rabbits per antigen were immunized with **CVL-1**, **CVL-2** and **CVL-3** and boosted every 15 days for 3 months. To prepare the immunogenic antigens, the same protocol as for the preparation of the coating antigens was followed, but KLH (5 mg, 5,000 equiv.) was used as the immunogenic protein. All immunogens were purified by size exclusion chromatography on dextran desalting columns using PBS 1X, pH 7.4, as elution buffer.

## *Experimental results*

All antigens were diluted to 1.0 mg/mL and stored at -20 °C until used. The concentration of the antigens was determined by UV-Vis absorbance and the Bradford protein assay and the molar ratio  $\beta$ -lactam/carrier protein for HSA antigens was established by MS-MALDI-TOF. The selectivity was tested measuring the sera antibody titers.

### *ELISA procedure*

ELISA plates were coated with 50  $\mu$ L of each antigen (5  $\mu$ g/mL) in sodium carbonate/bicarbonate buffer 0.1 M, pH 9.6, and incubated overnight at 37 °C. Then, the plates were washed five times thoroughly with PBS-T using a manual microplate washer. Sera incubation was done for 60 min at 37 °C by adding 50  $\mu$ L per well of the corresponding antibody solutions diluted two-fold down columns in PBS-T at dilution factors of 1/500-1/512,000, and the blank, PBS-T.

After incubation, the plates were again washed five times thoroughly with PBS-T. Then, they were incubated with 50  $\mu$ L per well of GAR-HRP (1/2,000 dilution in PBS-T) for 60 min at 37 °C. The plates were washed as before, and the reaction was developed by adding 50  $\mu$ L per well of a freshly prepared OPD solution, containing 2 mg/mL of OPD and 1  $\mu$ L/mL of H<sub>2</sub>O<sub>2</sub> 30 % v/v in substrate buffer.

The reaction was stopped by the addition of 50  $\mu$ L per well of H<sub>2</sub>SO<sub>4</sub> 2,5 M. Absorbance at 490 nm was measured by a dual wavelength automated plate reader (Wallac VICTOR, Perkin Elmer) with the reference wavelength set at 650 nm. All experiments were repeated three times.

### *Multiplexed in vitro microimmunoassay protocol*

To detect specific IgE, the protocol explained in Peña-Mendizabal et al. was followed.<sup>21</sup> Briefly, reagents (25 nL, 40  $\mu$ g/mL) were spotted in microarray format, using a noncontact printing device (AD 1500 BioDot, Inc., CA, USA), in DVDs that were later incubated overnight at 37 °C. HSA and human IgE (hIgE) were used as positive and negative controls of the assay. Finally, signals were read and data was analyzed by a modified DVD drive.<sup>22</sup> The level of specific IgE was quantified following the heterologous total IgE calibration scheme (Section 3.2.1).<sup>21</sup>



All experiments were repeated three times. Statistical significances between the prepared antigens and the reference were determined by the Holm-Sidak method using the SigmaPlot 11 software and P values of <0.001 were considered significant.

#### *Serum samples*

All samples from allergic patients and controls were kindly provided by the Hospital Universitari i Politènic La Fe, Valencia, Spain, and informed consent for the diagnostic procedures was obtained from all patients. Participants were enrolled after giving written informed consent according to protocols in accordance with the Helsinki Declaration of 1975 as revised in 2008 and approved by the Hospital Universitari i Politènic La Fe.

A cohort of 12 human samples from allergic patients and a pool of negative controls were tested. Clinical characteristics from the selected allergic patients is detailed in Section 3.3, Table S2.

### **3. Results and discussion**

#### *Preparation of the antigens*

Three derivatives of clavulanic acid have been obtained with yields ranging from 8 to 52 %. The syntheses consisted of benzyl esterification (**CVL-1**),<sup>23</sup> alcohol oxidation (**CVL-2**) and formation of a diene derivative (**CVL-3**). These syntheses have been fast, simple and did not require complex instrumentation. Formation of **CVL-2** derivative allow for the modification of the CVL-side chain to  $\beta$ -oxoethylidene. In the case of the synthesis of **CVL-3**, the initial envisaged idea was the azidation of benzyl clavulanate. For that, the reaction proceeded in two steps: (I) mesylation of the alcohol by using mesyl chloride and (II) azide incorporation with sodium azide. Characterization by <sup>1</sup>H-NMR of the crude after the first step showed the formation of a diene derivative of clavulanic acid (as well as the presence of trimethylamine) in front of the mesylation of the alcohol. This, modified the oxazolidine ring characteristic of CVL to a 2,3-dihydrooxazoline ring. Then, it was decided to study its immunological capacities and raise specific polyclonal IgG also using this compound, **CVL-3**. Finally, compounds **CVL-1**, **CVL-2** and **CVL-3** were obtained and selected for rabbit immunization.

## *Experimental results*

Antigen generation via amidation in basic media proceeded as expected. In this work, coating antigens were prepared through the use of two different carrier proteins: HSA and H1. This is important since the nature of the carrier protein and the antigen formed are critical for generating antibodies, as observed in previous studies which shown that epitope recognition of specific IgG and IgE in allergic patients varies according to the carrier protein used.<sup>21</sup> In the case of immunogenic antigens, the immunogenic protein used was KLH, different from the proteins used for antigen conjugation, to avoid non-specific interactions and/or false positives. Protein quantification by the Bradford colorimetric assay showed recovery yields ranging from 54 to 85 % for coating antigens and from 32 to 48 % in the case of immunogenic antigens. The conjugation yield was low, as observed by the determination of the molar ratios (synthesized compound/carrier protein) by MS-MALDI-TOF, ranging from 2 to 13.

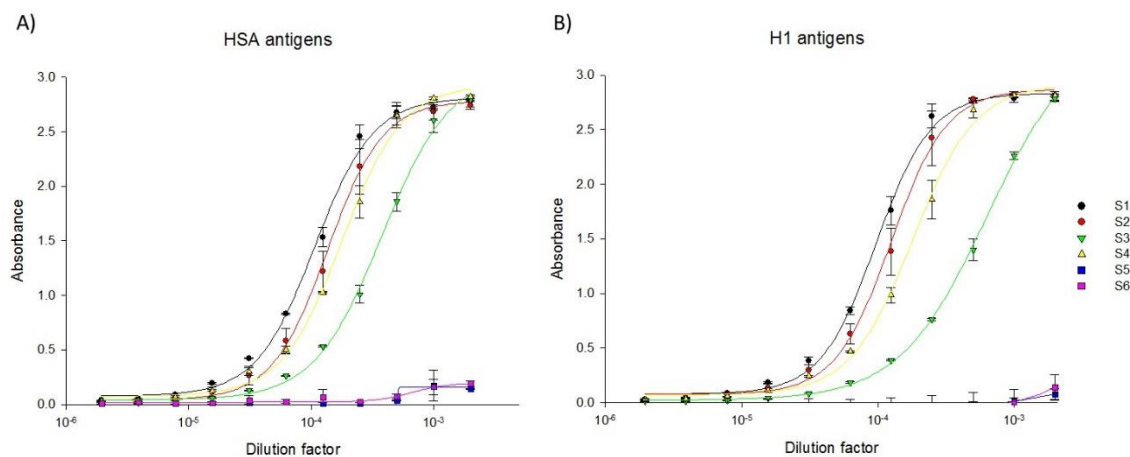
### *Sera titration*

Six rabbits were immunized with the CVL-based antigens to produce allergen-polyclonal antibodies. The sera antibody titers obtained were depicted as S1-S6. S1 and S2 sera correspond to immunized antigen **CVL-1**, S3 and S4 to immunized antigen **CVL-2**, and S5 and S6 to immunized antigen **CVL-3**.

All antigens were tested for their immunogenicity by studying the antibody titers of each serum after immunization by direct homogeneous and heterogeneous ELISA assays. The results of the antibody titers are presented in Figure 2.2.3 (homogeneous assays). (3a and 3b indicate HSA and H1-antigens, respectively). Absorbance (A) values are the mean of all the signals ( $n = 3$ ) obtained in each assay together with the standard deviation (SD) expressed as error bars. Dilutions assayed ranged from 1/500 ( $2.00 \cdot 10^{-3}$ ) to 1/512,000 ( $2.00 \cdot 10^{-6}$ ) with respect to the IgG antibody titer, together with the blank, PBS-T.

Firstly, sera S5 and S6, corresponding to rabbits immunized with antigen **CVL-3**, showed weak recognition in the homogeneous ELISA assay, as observed in Figure 2.2.3. A maximum absorbance of 0.2 was obtained for serum S5 at a dilution factor 1/1,000 when it was tested with the HSA coating antigen, and of 0.1 when the antigen tested was the H1-based. For serum S6, the maximum absorbances were obtained at a dilution factor 1/500 ( $A_{\text{HSA-antigen}} = 0.2$  and  $A_{\text{H1-antigen}} = 0.1$ ). As noted, these absorbance

values are very low and can be considered negligible. This indicates that the antigen prepared with KLH is not immunogenic enough. Therefore, the modification of the oxazolidine ring, characteristic of the CVL, to a 2,3-dihydrooxazoline ring seems to be not of great value for raising IgG antibodies for the detection of clavulanic acid.



**Figure 2.2.3.** Dose-response curves obtained with polyclonal IgG by homologous ELISA assays. A) HSA antigens, B) H1 antigens

On the other hand, high titers of anti-IgG antibodies to CVL were obtained for the other S1-S4 sera, which specifically recognized **CVL-1** and **CVL-2** coating antigens. A maximum absorbance of 2.7 - 2.8 was reached at a dilution factor 1/500 that was mostly maintained up to a dilution factor 1/2,000.

The titer of the sera was evaluated by determining the maximum dilution of serum that achieved the arbitrary value of  $A = 1.0$ . As for **CVL-1**, the serum titer was 1/8,000 in all cases (both HSA and H1-based antigens screened against S1 and S2 sera) with absorbance values between 1.2 (HSA-based antigen screened against S2) and 1.8 (S1 and H1-based antigen). When it comes to **CVL-2**, results were not as general. As HSA coating antigens reached a serum titer of 1/8,000 and 1/4,000 for S4 and S3, the serum titer obtained for H1 antigens was 1/4,000 ( $A = 1.9$ ) and 1/2,000 ( $A = 1.4$ ) for S4 and S3, respectively.

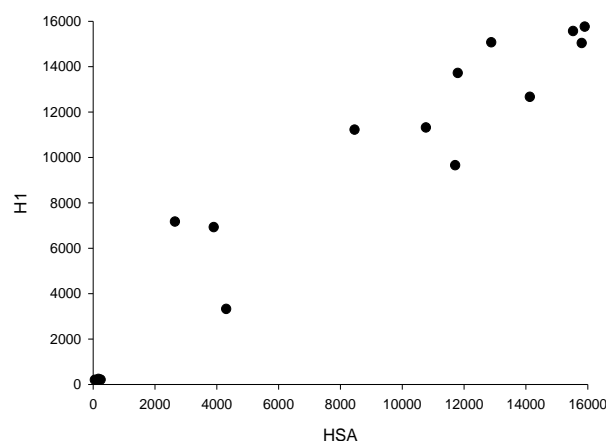
The limit of detection (LOD) and the sensitivity ( $EC_{50}$ ) were established and are summarized in Supplementary Table 6.4.S1 (together with the coefficient of determination,  $R^2$ ). LOD ranged from dilution factor 1/50,000 to 1/20,000 (S5 and S6 were not considered), whereas  $EC_{50}$  ranged from ca. 1/10,000 to 1/1,667.  $R^2$  ranged from 0.99715 to 0.99934. In particular, **CVL-1** antigen showed the best affinity to S1 and S2

## Experimental results

sera with EC<sub>50</sub> values corresponding to a dilution factor of 1/9,346 and 1/7,407, respectively (1/10,320 and 1/8,000 for H1-based antigen). As for **CVL-2**, EC<sub>50</sub> values were ca. 1/2,500 and 1/5,000 for sera S3 and S4 (1/1,580 and 1/5,556 for H1-based antigen). In general, the behavior of both HSA and H1-based antigens for the different sera is similar, indicating that the carrier protein used to prepared the antigens did not affect in the selectivity of the assay.

### Results of the direct immunoassay

The development of routine evaluation immunoassays was carried out with our multiplex *in vitro* microimmunoassay based on DVDs due to the multiplexed capacity offered by the DVD.<sup>24</sup> For this, polyclonal IgG antibodies S1-S6 against CVL-based antigens were used, as in the previous section. Dilutions tested were 1/250, 1/1,000, 1/4,000 and 1/16,000, with respect to the IgG antibody titer, and the blank, PBS-T. All values obtained for all dilutions and all antigens were considered, as shown in Supplementary Table 6.4.S2. The results (Figure 2.2.4) showed that there is a good correlation between HSA- and H1-based antigens ( $R^2 = 0.9079$ ). Although this difference was not statistically significant, only H1-based antigens were considered in the following studies.

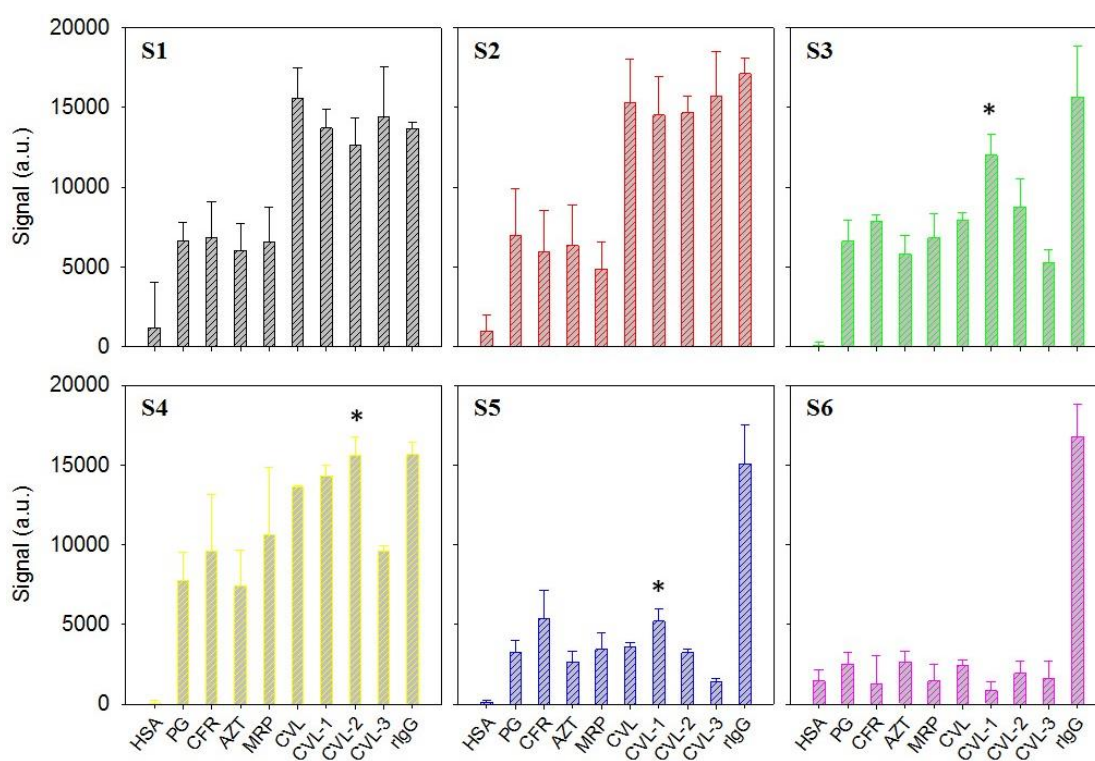


**Figure 2.2.4.** Representation of signals obtained (in arbitrary units, a.u.) for coating antigens in serum S1. X and Y axes represent HSA and H1-based antigen values. Values from all dilutions were considered.

The selectivity of the coating antigens to polyclonal IgG antibodies S1-S6 was tested and the results shown in Figure 2.2.5. Apart from H1 antigens, reference antigens

(PG, CFR, AZT, MRP, CVL), together with both positive (rIgG) and negative (HSA) controls are included. Values are expressed in arbitrary units (a.u.) and are the mean of all the signals ( $n=3$ ) obtained in each immunoassay, with SD expressed as error bars. Only values corresponding to the dilution factor 1/1,000 are shown for clarification.

Values for the positive and negative controls were as expected and those corresponding to rIgG oscillated around 15,000 a.u. for all sera and HSA was not recognized by any sera. The specificity of the immunoassay and the cross reactivity of the antigens was studied with antigens corresponding to other BLC families, concretely PG, CFR, AZT and MRP antigens. In general, the recognition of specific IgG antibodies to clavulanic acid with these antigens was not as high as with clavulanic-based ones. Maximum values of ca. 6,500 a.u. were obtained at a dilution factor 1/1,000. However, IgG antibodies from S1 and S2 have shown to be the most specific to clavulanic acid when compared to antigens from other families ( $> 50\%$ ).



**Figure 2.2.5.** Signals obtained (in arbitrary units, a.u.) for controls and H1-based antigens with polyclonal IgG at a dilution factor 1/1,000. \* indicates statistically significant difference as compared to the reference CVL ( $P < 0.001$ ).

## *Experimental results*

In general, it has been observed that responses obtained by all S1-S6 sera regarding to CVL-based antigens match with those corresponding to the ELISA assay (Figure 2.2.3). In this respect, the results are shown in the Supplementary Figure 6.4.S1. Dilution factors tested were 1/2,000, 1/4,000 and 1/8,000, but only the dilution factor 1/4,000 was represented for clarification (proportional results were obtained with the other dilutions). In general, the response of the antigens was identical to that corresponding to the colorimetric immunoassay. For this reason, immunoassay responses would be considered and compared in the manuscript.

CVL, used as reference antigen, is generally detected by specific IgG generated in sera with good selectivity. Concretely, signals were almost equal to that offered by the other antigens, as shown in S1 and S2, reaching signals around 15,000 a.u. for all CVL-based antigens. However, statistical differences were sometimes observed. In this respect, specific IgG generated in S4 is capable of recognizing the **CVL-2** antigen with significant difference (signal ca. 15,700 a.u.). Moreover, antigen **CVL-1** is recognized by specific IgG generated in serum S3 and S5 with statistically significant difference as compared to the reference CVL ( $P < 0.001$ ), with values of ca. 12,000 and 5,200 a.u., respectively. This highlights that not only homogeneous assays reached good recognition profiles, but also heterogeneous assays are capable of detect specific IgG with great sensitivity and good specificity, as observed with antigen **CVL-1**.

We have therefore proven that the prepared CVL-based antigens are recognized by specific IgG raised in immunized rabbit to the aforementioned antigens, sometimes in values better to that corresponding to the clavulanic reference antigen.

## *Analysis of serum samples*

A cohort of 12 patients was evaluated who developed an immediate reaction to AMX or AMX-CVL. Furthermore, a negative pool from 18 control patients was used to screened the diagnostic specificity of the immunoassay. The results obtained by *in vivo* and *in vitro* assays are described in Table 2.2.1.

The *in vivo* evaluation was carried out by skin tests. These results showed that 7 out of 11 patients for AMX (64 %), 8 out of 9 for AMX-CVL (89 %) and 3 out of 4 for CVL (75 %) were positive. To this respect, it is worth nothing the lack of clinical information available for prick tests to CVL, in particular. As far as CVL was concerned,

only 4 patients were evaluated by prick test, corresponding to 33 % of the entire selected cohort.

On the other hand, no commercial *in vitro* assay to diagnose allergies to CVL is available, difficulting the quantification of specific IgE. To this aim, the proposed DVD *in vitro* microimmunoassay permitted the evaluation of these antigens. The diagnostic specificity of the assay was 100 %, since the negative pool was not detected by any of the antigens. However, when the antigens were tested with the sera of the 12 recruited allergic patients, only antigen **CVL-1** detected specific IgE. Therefore, 6 out of 12 patients (diagnostic selectivity = 50 %) were detected with antigen **CVL-1** linked to HSA.

Concretely, even when the levels of specific IgE were low, the culprit drug of all the patients who tested positive to antigen **CVL-1** was AMX-CVL. To mention some figures, patients 10 and 11, positive by prick test, were not detected by any of the prepared antigens. Interestingly, however, antigen **CVL-1** linked to HSA detected patient 007 that tested negative by prick test to CVL and AMX.

**Table 2.2.1.** Results of the analysis of human serum samples by *in vivo* (prick test) and *in vitro* (DVD microimmunoassay) tests

No	Culprit drug	Prick test <sup>[a]</sup>			Multiplex DVD immunoassay <sup>[b]</sup>					
		AMX	AMX-CVL	CVL	CVL-1 HSA	CVL-1 H1	CVL-2 HSA	CVL-2 H1	CVL-3 HSA	CVL-3 H1
1	Augmentin	P	P	P	0.06	< LQ	< LQ	< LQ	< LQ	< LQ
2	Amoxicillin	NR	NR	NR	< LQ <sup>[c]</sup>	< LQ	< LQ	< LQ	< LQ	< LQ
3	Augmentin	P	P	NR	0.10	< LQ	< LQ	< LQ	< LQ	< LQ
4	Augmentin	P	P	NR	0.11	< LQ	< LQ	< LQ	< LQ	< LQ
5	Augmentin	P	P	NR	0.12	< LQ	< LQ	< LQ	< LQ	< LQ
6	Augmentin	N	N	NR	0.12	< LQ	< LQ	< LQ	< LQ	< LQ
7	Augmentin	N	NR	N	0.09	< LQ	< LQ	< LQ	< LQ	< LQ
8	Amoxicillin	P	P	NR	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ
9	Augmentin	N	P	P	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ
10	Augmentin	N	NR	P	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ
11	Unspecific	P	P	NR	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ
12	Amoxicillin	P	P	NR	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ
Pool	-	-	-	-	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ

AMX: amoxicillin, AMX-CVL: augmentin, CVL: clavulanic acid. Immunoassay values are the mean of three replicates. Relative standard deviation (RSD) ranged from 4 to 13 %. <sup>[a]</sup>P= Positive, N= Negative, NR=Not Realized; <sup>[b]</sup>Values correspond to IgE concentration expressed in IU/mL= 2.4 ng/mL; <sup>[c]</sup>< LQ= Value below the limit of quantification.

In conclusion, the combination of both techniques (*in vivo* and *in vitro* assays) is an appropriate tool to diagnose allergies to CVL. Concretely, results revealed that the antigen **CVL-1** linked to HSA is selective and can be used for *in vitro* diagnosis, especially if we consider that the assay confirmed six patients as positive, versus the three patients confirmed by prick test as positive to CVL.

#### **4. Conclusion and future trends**

The high consumption of clavulanic acid, which associates with a large number of DHRs, made it of great interest to identify the chemical structures involved in the generation of specific antigens to clavulanic acid. In this respect, three haptens derived from clavulanic acid were synthesized, **CVL-1**, **CVL-2** and **CVL-3**, and their respective antigens tested by an ELISA and a colorimetric microimmunoassay based on DVDs.

The results highlighted the importance of the chemical structure for drug allergic diagnosis. On the one hand, modification of the characteristic oxazolidine ring of oxapenamams to a 2,3-dihydrooxazoline ring (**CVL-3**) was neither recognized by IgG nor IgE antibodies. This emphasizes that the antigen recognition by specific antibodies has to maintain the general structure characteristic of this  $\beta$ -lactam family, which correlates with why *in vitro* diagnosis of clavulanic acid allergies tests negative when other antigens from other families are involved. However, on the other hand, the selectivity of **CVL-1** and **CVL-2** to detect polyclonal IgG antibodies raised to CVL has been demonstrated. The detection was three-fold higher than those to other families of BLCs when these antigens were tested with IgG antibodies. Moreover, it has been therefore proven that esterification of the potassium carboxylate to produce **CVL-1** is of great value in the production of specific IgE antibodies to clavulanic acid when tested with a cohort of 12 allergic patients.



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In this chapter we addressed the preparation of antigens to other families of  $\beta$ -lactam antibiotics and their evaluation by a multiplex *in vitro* colorimetric microimmunoassay.

On the one hand, the preparation of 20 major and minor antigens based on cephalosporin, carbapenem and monobactam antibiotics has been described in the first part of this chapter (Section 3.2.1.). Their evaluation by the developed assay revealed that only those minor antigens permitted the detection of specific IgE, improving the clinical performances of the *in vitro* assays to other families of  $\beta$ -lactam antibiotics.

On the other hand, three haptens derived from clavulanic acid have been synthesized in the second part of this chapter (Section 3.2.2.). They were conjugated to proteins to prepare 6 antigens as well as to produce specific IgG antibodies to clavulanic acid. Furthermore, antigen CVL-1, prepared after esterification of potassium clavulanate, was recognized by specific IgE from a set of allergic patients.



*Chapter 3*

**3.3. Synthesis of skeletally diverse  $\beta$ -lactam  
haptens for the *in vitro* diagnosis of IgE-mediated  
drug allergy**





The preparation of antigens for the diagnosis of allergies has been commonly based on the direct and covalent attachment of the hapten to carrier proteins, which confer the immunogenic capacity. When this attachment is not possible, for example when the hapten does not have the functional groups needed, the solution usually comes from incorporating spacer arms or crosslinkers, as has been demonstrated in Chapter 1. But even then, it has been stated that IgE antibodies from some allergic patients still do not recognize all this set of antigenic determinants.

Nowadays, new and modern methodologies related to the synthesis of compounds are emerging that seek a broader approach, in which it is suggested that the search for antigens in libraries with functionally diverse compounds increases the chances of finding biologically active molecules. However, until now, these techniques have been oriented in the search for new and promising drugs, without accounting for the high potential that they confer. In this respect, DOS provides the spatial diversity and the structural complexity needed to cover a wide spectrum of possible epitopes responsible for allergic episodes.

In this chapter, a cohort of skeletally-diverse compounds will be synthesized for the  $\beta$ -lactam antibiotics AMX and AMP and the precursors of the penicillins and cephalosporins, 6-APA and 7-ACDA, that will be later linked to carrier proteins to generate immunogenic antigens. The evaluation of the most promising antigens will be performed using a multiplex *in vitro* colorimetric immunoassay and by detecting specific IgG from rabbits and IgE from a cohort of 30 samples from allergic patients to these  $\beta$ -lactam antibiotics and controls.



## Synthesis of skeletally diverse $\beta$ -lactam haptens for the *in vitro* diagnosis of IgE-mediated drug allergy

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### ABSTRACT

We present the first synthesis of  $\beta$ -lactam-derived haptens, leveraging the principles of diversity-oriented synthesis to discover compounds for drug allergy *in vitro* testing. We designed, synthesized, and performed *in vitro* immunological evaluation on 18 structurally diverse haptens derived from  $\beta$ -lactam antibiotics. The obtained haptens—conjugated to carrier proteins—allow for the detection of specific anti- $\beta$ -lactam immunoglobulins G and E. Excellent diagnostic sensitivity (79 %) and specificity (100 %) were achieved when the panel of antigens were tested against a cohort of 38 human serum samples using a CD technology-based *in vitro* testing tool.

## 1. Introduction

Currently over 6.5 % of the globally studied population carries a penicillin or  $\beta$ -lactam allergy label.<sup>1</sup> The overdiagnosis of  $\beta$ -lactam allergy has several clinical consequences. Inpatients with such a label often receive second-line treatments, which are less effective, often more toxic, and associated with a higher risk of antibiotic-resistant infections. To reduce the risk of near-fatal reactions from *in vivo* allergy tests, the clinical guidelines advise that—for subjects who have a history of severe immediate reactions—the *in vivo* allergy tests should be preceded by *in vitro* IgE tests.<sup>2</sup> However, current *in vitro* tests provide only a gross prediction of drug allergy, and negative results (< 0.35 IU/mL) do not necessarily exclude the possibility of  $\beta$ -lactam allergy, with the mean clinical sensitivity at only ~25 %. As such, there is a need for additional reagents (haptens and antigens) to enable the development of accurate *in vitro* assays to discriminate selectively and with high sensitivity allergic from non-allergic subjects. Experimental studies on the synthesis of  $\beta$ -lactam-derived haptens are limited.<sup>3</sup> Recent studies have described the synthesis of new haptens that incorporate spacers to create distance between the antibiotic molecule and the carrier protein, revealing that the inclusion of different drug-derived conjugates during sIgE evaluation can improve the accuracy of diagnosis.<sup>4</sup> Other study reports pyrazinone derivatives as novel antigenic determinants of  $\alpha$ -aminocephalosporins showing low percentage of positivity.<sup>5</sup> Indeed, to meet the challenge of the accumulated burden and increasing demand, allergy specialists need to be supported with innovative, evidence-based approaches for drug-allergy assessment and delabeling. In this respect, diversity-oriented synthesis (DOS) provides an appealing route to access compounds that have both structural complexity and diversity, and incorporate chemical features common to natural products, such as  $sp^3$ -hybridized carbon atoms, basic nitrogen atoms, stereogenic elements, and novel skeletons.<sup>6</sup> To this aim, we synthesized using DOS a collection of 22 haptens using different  $\beta$ -lactam antibiotics containing greater structural diversity than typically observed for these antibiotics.

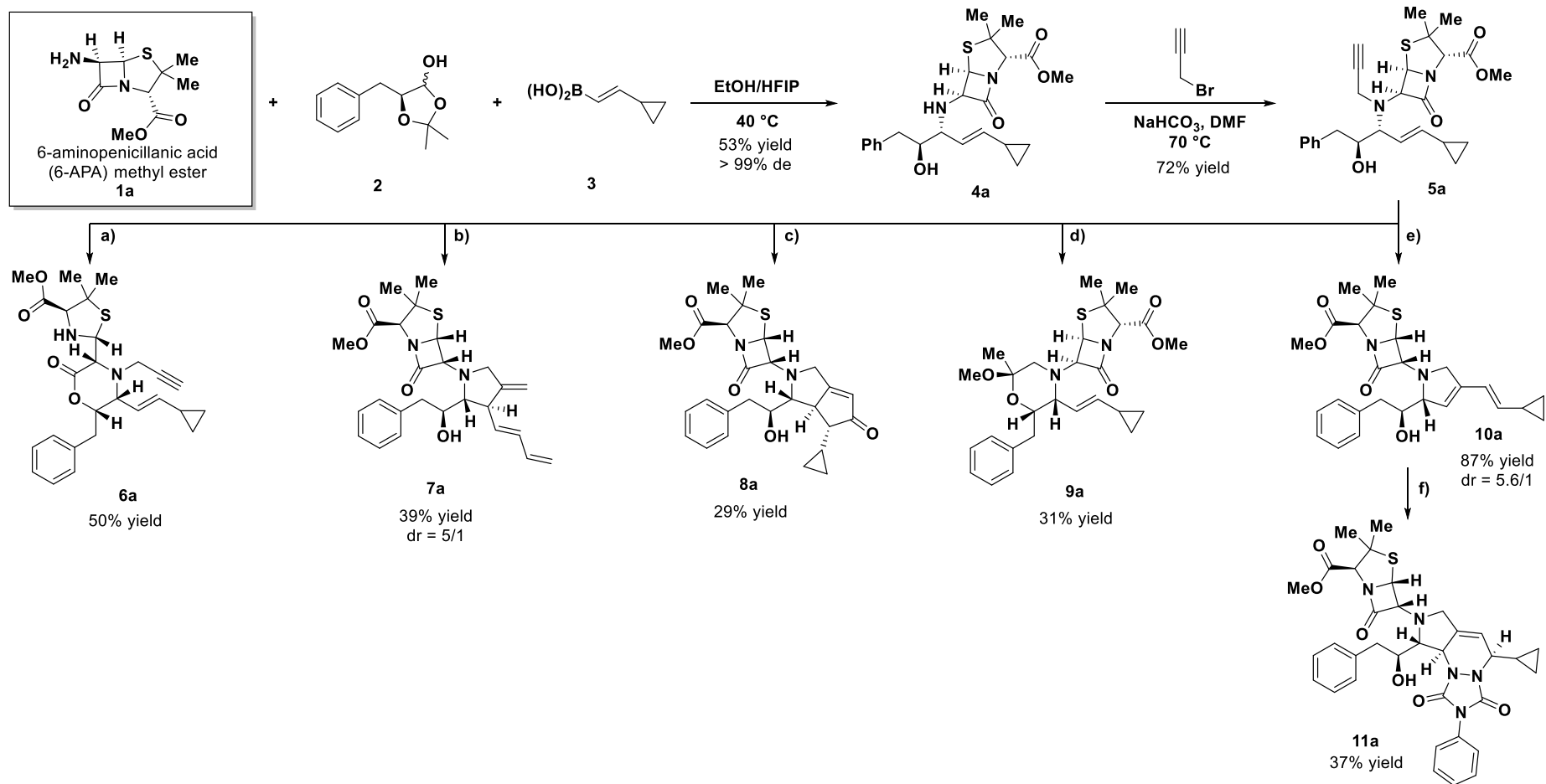
## 2. Results and discussion

Using 6-aminopenicillanic acid (6-APA)—the precursor of all semi-synthetic penicillins—we performed a Petasis three-component, boronic-acid Mannich reaction followed by amine propargylation to yield  $\beta$ -amino alcohols, which were subjected to an array of skeletal diversification reactions.<sup>7</sup> Herein, we improve the yield of the three-

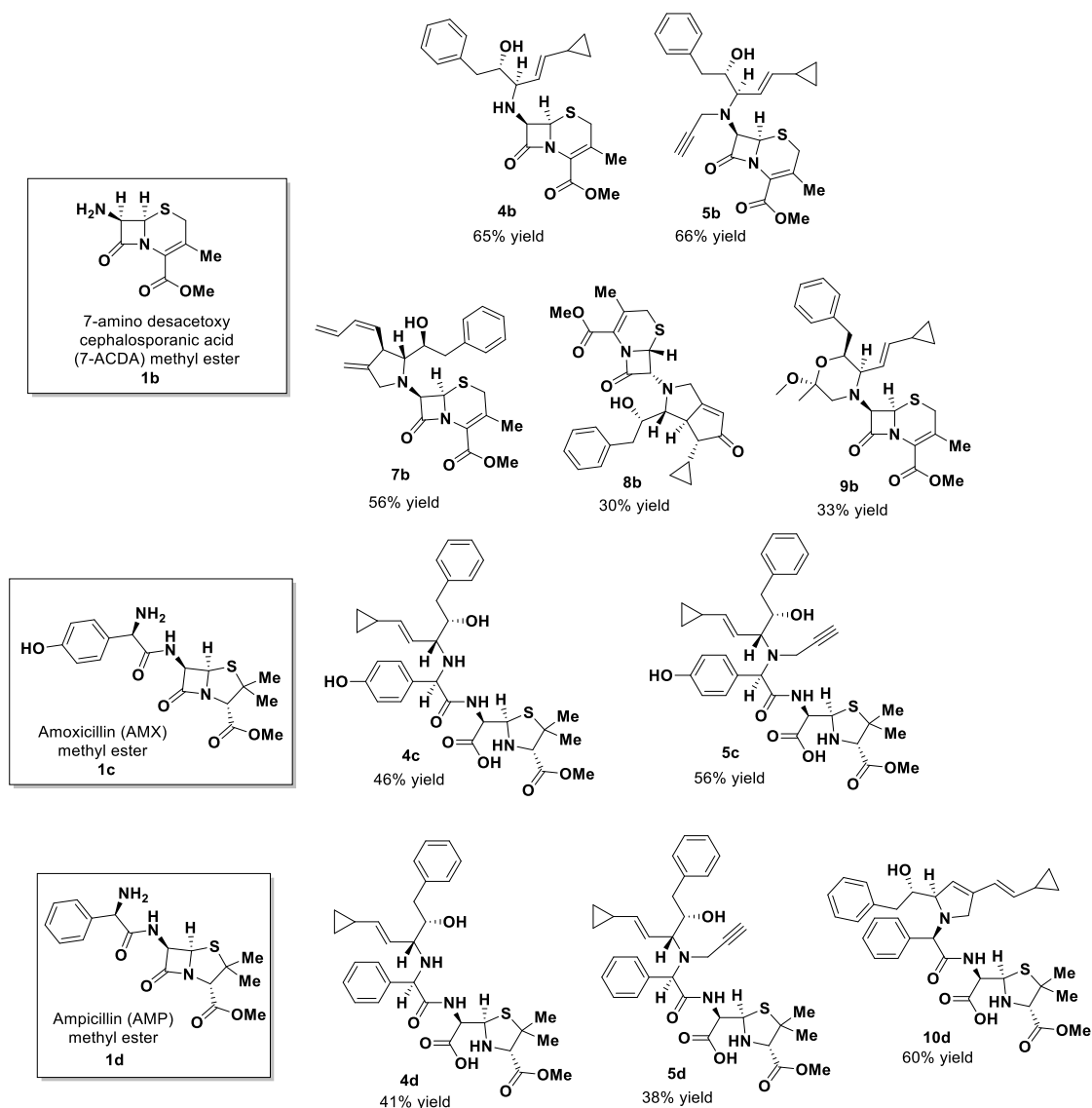
component reaction involving 6-APA using different organic solvents and conditions (Table 6.5.S1, ESI†). Using these improved conditions, we began with 6-APA methyl ester (**1a**) to gain access to compounds **4a-11a** (Scheme 3.1). The best conditions comprised a solvent system of 9:1 EtOH/HFIP (v/v) at 40 °C for 72 h, using an excess of both the antibiotic-based ester **1a** and (E)-2-cyclopropylvinylboronic acid **3**, and 3 Å molecular sieves as a water scavenger.<sup>8</sup> As for L-3-phenyllactic acid, it was protected as the acetonide using 2,2-dimethoxypropane and p-toluenesulfonic acid to obtain the (S)-lactone in 96 % yield, and subsequently reduced with DIBAL-H to afford the corresponding (S)-lactol **2** in 84% yield.<sup>9</sup> The Petasis reaction with **1a** afforded the anti-diastereomer **4a** exclusively in 53 % yield, again demonstrating the diastereoselectivity of this transformation. In order to prepare a probe for the diversification reactions, N-alkylation of **4a** with propargyl bromide was performed to provide the desired compound **5a** in 72 % yield. Next, skeleton-diversifying reactions were performed using different catalysts to provide five 6-APA-based compounds (**6a-11a**).

The conditions of the Petasis reaction and metal-catalyzed cyclizations were also used with other antibiotic cores (Figure 3.1). This pathway allowed for the preparation of a set of haptens derived from 7-amino-deacetoxycephalosporanic acid (7-ACDA)—the precursor of almost one-third of all commercial cephalosporins—, amoxicillin (AMX), and ampicillin (AMP). As for 6-APA, 7-ACDA and the antibiotics amoxicillin and ampicillin were esterified with TMS-diazomethane to obtain the methyl esters **1b-1d**. Compound **1b**, the methyl ester of 7-ACDA, was thus incorporated into the synthetic pathway using the same conditions as for 6-APA.  $\beta$ -amino alcohols **4b** and **5b** were generated and were subjected to the Pd-catalyzed cycloisomerization with [Pd(PPh<sub>3</sub>)<sub>2</sub>(OAc)<sub>2</sub>], the Pauson–Khand reaction with [Co<sub>2</sub>(CO)<sub>8</sub>], and the Au-catalyzed alkyne activation and cyclization to afford structurally diverse compounds **7b-9b**, respectively. In the process, we observed the displacement of the double ring in the 3,6-dihydro-2H-1,3-thiazine ring, forming a byproduct that compromised the yield of the reactions.

AMX and AMP, two of the most prescribed  $\beta$ -lactam antibiotics and having an additional free amine group on their side chains, were also selected as building blocks and incorporated into the synthetic pathway. We observed that heating at 70 °C for 72 h led to decomposition of AMX methyl ester (**1c**) and AMP methyl ester (**1d**). Mild conditions (room temperature for 72 h) were sufficient to obtain the desired compounds



**Scheme 3.1.** Scheme of the synthesized 6-APA-derived haptens. a) NaH, toluene 0.1 M, rt, 16 h; b)  $[\text{Pd}(\text{PPh}_3)_2(\text{OAc})_2]$  (10 mol %), benzene 0.05 M, 80 °C, 1 h; c)  $[\text{Co}_2(\text{CO})_8]$ , TMANO, benzene 0.1 M, rt, 4 h; d)  $\text{NaAuCl}_4$  (10 mol %), MeOH 0.05 M, rt, 1 h; e) Grubbs second-generation catalyst (10 mol %), DCM 0.05 M, reflux, 1 h; f) 4-phenyl-1,2,4-triazoline-3,5-dione, DCM 0.1 M, rt, 1 h.



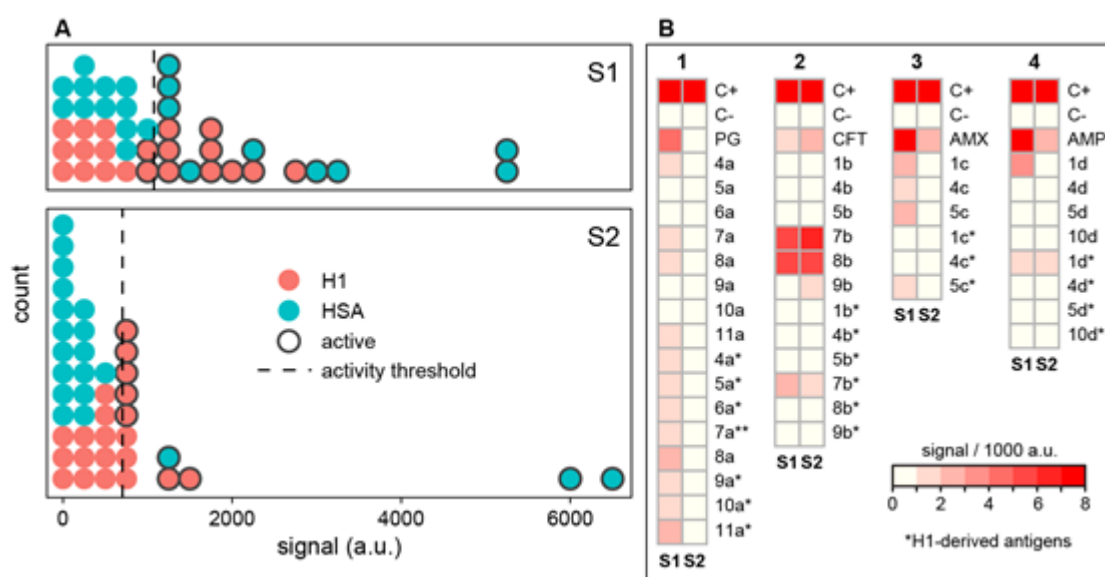
**Figure 3.1.** Chemical structures of the synthesized  $\beta$ -lactam-derived haptens

**4c** and **4d** in reasonable yields (ca. 45 %), but 2D-NMR studies confirmed that the  $\beta$ -lactam ring opens during the Petasis reaction. After amine propargylation to afford the desired amino alcohols **5c** and **5d** in a reasonable yield (56 and 38 %, respectively), cycloadditions were attempted with AMX without success. To assess the feasibility of this pathway, enyne metathesis was chosen as a proof-of-concept in the synthesis of an AMP-based compound, and the desired diene **10d** was obtained in 60 % yield.

The synthesized  $\beta$ -lactam-derived haptens were used to prepare structural antigens following the ‘major’ configuration. Two carrier proteins were selected—human serum albumin (HSA) and histone (H1)—each of which contains approximately 60 free lysine residues. The antigens were prepared via amide-bond linkage between the lysine

residues and the carbonyl carbon atoms of the open  $\beta$ -lactam ring. A collection of 44 antigens were prepared, 22 for each carrier protein.

The antigens were first evaluated using a CD technology-based *in vitro* testing tool<sup>10</sup>, heron CD test, with rabbit sera raised for benzylpenicillin (PG) and ceftriaxone (CFT). The results are depicted in Figure 3.2. We determined which antigens could be considered ‘active’ by identifying those that exhibited signals significantly different from that produced by the negative control. The histogram depicting the ability of all antigens to elicit activity in the immunoassay is shown in Figure 3.2A and comprises assay data for compounds against sera raised against PG (S1) and CFT (S2). Compounds with assay responses greater than the threshold—corresponding to the mean of the negative control plus three standard deviations—were considered ‘active.’ Twenty-two (50 %) of the antigens were found to be ‘active’ with either serum S1 (PG) or S2 (CFT). Four of the 44 antigens (7b-HSA, 8b-HSA, 7b-H1, and 1d-H1) were found ‘active’ across both sera, and can therefore be considered generic for the detection of IgG (Figure 2B).



**Figure 3.2.** A) Histogram of the ‘activity’ of the antigens. B) Heatmaps for the cohort of the HSA- and H1-derived antigens representing the signals obtained in the immunoassays for each immunized sera (S1 and S2). 1) 6-APA, 2) 7-ACDA, 3) AMX, and 4) AMP-derived antigens.

We next explored whether this activity was serum-specific with respect to each  $\beta$ -lactam antibiotic derivative. As is depicted in Figure 3.2B, 6-APA-derived antigens were selectively identified by the IgGs of serum S1 with very negligible cross-reactivity



with S2, with **4a**-, **7a**-, **8a**- and **11a**-derived antigens showing the highest responses independent of the carrier protein used.

A similar pattern was detected for AMX- and AMP-derived antigens. These antigens were also highly selective except **1d**-H1. This is in good agreement with expectations since 6-APA, AMX, and AMP haptens share a common structure ( $\beta$ -lactam fused to a saturated five-membered ring) and serum S1 was raised against benzylpenicillin. On the other hand, **9b**-HSA was the only antigen that was selective for serum S2, although haptens **7b** and **8b** were strongly recognized by the specific IgGs.

Furthermore, we tested a cohort of 38 subjects, of which 15 developed an immediate reaction, four suffered from a delayed reaction to  $\beta$ -lactams (Table 6.5.S2, ESI†), and 19 were control individuals. The results of the prick test and the *in vitro* immunoassays (ImmunoCAP and CD test) are shown in Table 3.1. Also, notable is the observation that for PG, the 16 tested patients were diagnosed as negative by the prick test. In contrast, ten and nine were diagnosed as positive for AMX and AMP, respectively. Only one was positive for CFT among the 13 patients tested for CFT. Drug provocation tests were not performed for ethical reasons since they are potentially dangerous and must be performed at specialized centres.<sup>11</sup>

Sera from these patients were also tested using two *in vitro* immunoassays: ImmunoCAP and CD test.<sup>10</sup> None of the 19 control subjects tested positive using either *in vitro* immunoassay, achieving a specificity of 100 %. On one hand, four allergic patients tested positive for AMX (21 %) and one for PG and AMP (5 % each) using ImmunoCAP. This is consistent with previous reports that show high variability in the sensitivity for ImmunoCAP, especially with respect to the  $\beta$ -lactam antibiotics involved (sensitivity values range from 0 to 50 %).<sup>12</sup> On the other hand, when we evaluated the sera of allergic patients using the synthesized haptens with the CD test, we found that nine antigens were recognized by specific IgE. Notably, 15 patients tested positive using at least one of the nine antigens. Specifically, we obtained positive results for 6 patients for **1d** (32 %), 4 for **10a** (21 %), 3 for **1c** (16 %), 2 for **10d** and **8a** (10.5 %), and 1 for **1b**, **4b**, **7b**, and **9b** (5 % each). We found that—of the four best haptens—two (**1c** and **1d**) were structurally similar to amoxicillin, being the methyl esters of amoxicillin and ampicillin, respectively. This is perhaps unsurprising given the patients' known allergies to amoxicillin and augmentin (a combination of amoxicillin and clavulanate). However,

we also found that haptens based on **10a** and **10d** were specifically recognized by IgE of allergic patients, with these compounds exhibiting more substantial structural differences from their respective antibiotic precursors. Interestingly, both compounds share the same 3-vinyl-2,5-dihydropyrrole moiety arising from enyne metathesis, and are the only two compounds tested that contain this group. While anecdotal, this observation suggests that chemically diverse antigens can successfully mimic the epitopes responsible for provoking an allergic episode, and demonstrates that even when the chemical structures of the antibiotics have been considerably modified and the level of specific IgE in serum is very low (< 0.5 IU/mL), patients can be accurately diagnosed as allergic.

### **3. Conclusions**

Overall, we have shown that, in contrast to other standard antigens, those prepared using DOS-derived haptens were good candidate reagents for detecting even low levels of specific IgE in the serum of allergic patients, representing a significant improvement upon the diagnostic sensitivity of *in vitro* allergy tests, in particular the reference assay ImmunoCAP.

In fact, for the cohort of serum samples analyzed, while only four allergic patients (21 %) tested positive for amoxicillin with ImmunoCAP, 15 tested positive using the DOS-derived antigens (79 %) in a multiplex configuration, considering the set of the nine selected antigens as a whole what represents an almost four-fold increase over the reference test. Of the 10 patients who tested positive using the skin prick test, eight were identified using the DOS hapten-derived antigen collection, double that identified using the reference method ImmunoCAP. Lastly, the four patients detected as positive by ImmunoCAP also tested positive using the DOS derived antigens, although the concentration values were significantly different. In summary, we designed, synthesized, and performed *in vitro* immunological evaluation on 18 structurally diverse haptens derived from  $\beta$ -lactam antibiotics. The synthesis of these new haptens and the preparation of the corresponding antigens is key for accurate  $\beta$ -lactam allergy testing. Furthermore, the elucidation of new antigenic determinants for  $\beta$ -lactam antibiotics might provide new insights about the mechanism involved in allergy and improve the clinical performances of the current *in vitro* tests. Taken together, these results suggest that the incorporation of structural diversity into  $\beta$ -lactam antibiotics can drive molecular IgE recognition—even

when the chemical structures of the antibiotics are highly modified—and, therefore, the allergic episode, demonstrating the potential of DOS for the preparation of structurally diverse  $\beta$ -lactam-derived haptens. We posit that adoption of this strategy could help inform decision-making by allergists, define allergen-avoidance regimes, aid  $\beta$ -lactam delabeling initiatives and improve the quality of life of allergic people.

**Table 3.1.** Assaying human serum samples using *in vivo* (prick test) and *in vitro* (ImmunoCAP and multiplex DVD assay) tests

Sample	Culprit drug	Prick test <sup>a</sup>				ImmunoCAP <sup>b</sup>			Multiplex DVD assay <sup>b,c</sup>								
		PG	CFT	AMX	AMP	PG	AMX	AMP	8a	10a	1b	4b	7b	9b	1c	1d	10d
1	Augmentin	N	N	P	N	< LD <sup>d</sup>	< LD	< LD	< LD	0.33	< LD	< LD	< LD	< LD	< LD	< LD	< LD
2	Amoxicillin	NP	NP	NP	NP	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	0.11
3	Augmentin	N	P	P	P	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	0.23	0.15	< LD
4	Augmentin	N	NP	P	P	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	0.50	0.16	< LD
5	Augmentin	N	N	P	P	2.16	1.64	1.64	< LD	< LD	< LD	< LD	< LD	< LD	0.22	0.16	0.35
6	Augmentin	N	NP	N	N	< LD	< LD	< LD	< LD	0.19	< LD	< LD	< LD	< LD	< LD	< LD	< LD
7	Augmentin	N	N	N	N	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
8	Amoxicillin	N	NP	P	P	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	0.37	< LD	< LD	< LD
9	Augmentin	N	N	N	N	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	0.25	< LD
10	Augmentin	NP	NP	N	NP	< LD	< LD	< LD	< LD	0.20	< LD	< LD	< LD	< LD	< LD	< LD	< LD
11	Amoxicillin	N	N	P	P	< LD	0.69	< LD	0.33	0.31	< LD	< LD	< LD	< LD	< LD	0.13	< LD
12	Augmentin	N	N	P	P	1.83	0.86	1.91	< LD	< LD	0.22	< LD	< LD	< LD	< LD	< LD	< LD
13	Cefuroxime	N	N	N	N	< LD	< LD	< LD	< LD	< LD	< LD	< LD	0.14	< LD	< LD	< LD	< LD
14	Cefuroxime	N	N	N	N	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	0.15	< LD
15	Amoxicillin	NP	NP	N	NP	< LD	< LD	< LD	0.12	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
16	Augmentin	N	N	P	P	0.40	0.54	0.89	< LD	< LD	< LD	0.11	< LD	< LD	< LD	< LD	< LD
17	Amoxicillin	N	N	N	N	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
18	Augmentin	N	N	P	P	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
19	Augmentin	N	N	P	P	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD

<sup>a</sup>P= Positive, N= Negative, NP=Not performed; <sup>b</sup>Values correspond to IgE concentration (1 IU/mL= 2.4 ng/mL) and are the mean of three replicates. RSD ranged from 4 to 13 %. <sup>c</sup>Histone-derived antigens; <sup>d</sup>LD= limit of detection.

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## **4. GENERAL DISCUSSION**





## 4. GENERAL DISCUSSION

As it is well known, to dispose of suitable reagents, mainly haptens or determinants and antigens, is key to determine specifically IgE to  $\beta$ -lactam antibiotics. Apart from the synthesis of novel structures, the carrier protein used, the chemical reactive group taking part in the conjugation and the conjugation route itself are also fundamental factors in the molecular recognition of specific IgE. Therefore, this thesis aims to gain insight in the determination of specific IgE by working on the synthesis of novel haptens, which have been chemically modified from the respective antibiotics, in order to generate selective antigens to  $\beta$ -lactam antibiotics.

In this respect, the generation of promising antigens will help to set up reliable drug allergy tests with proper diagnostic sensitivity and specificity. So far, *in vivo* skin tests are the quintessential assays for the detection of drug allergies. These tests, although fast and specific (specificity > 98 %), generate a large percentage of false positives (ca. 66 %), and are invasive and risky.<sup>205</sup> This is problematic, since there is a risk of not prescribing drugs to patients who really need and, most importantly, tolerate them. On the other hand, the *in vitro* reference assay, ImmunoCAP detects only 5 BLCs, 4 of which correspond to the most prescribed penicillins, PG and AMX. In addition, it is established that ImmunoCAP can provide an extremely high percentage of false negatives (negative predictive value<sup>2</sup> ca. 53 %),<sup>207</sup> which correlates with the low diagnostic sensitivity (ca. 50 %) achieved.<sup>207</sup> In particular, according to the scientific literature, the diagnostic specificity of this test for these antibiotics ranged from 83 to 100 % and the sensitivity from 32 % for PG to 43 % for AMX.

This dissertation reports the synthesis of novel AMX- and PG-based haptens to prepare antigens that may help to overcome the deficiencies, in terms of diagnostic sensitivity and specificity, in the currently available tests. To do this, first, aliphatic diamines of different lengths have been used as spacer arms. According to the scientific literature, the incorporation of spacer arms can separate the hapten from the carrier

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<sup>2</sup> Negative predictive value considers the predicted percentage of individuals with a negative test result who are actually not allergic.

protein, reducing steric hindrances and leaving the hapten more accessible. Therefore, 13 diamine-based major and minor haptens have been synthesized and characterized. These haptens were used to generate minor antigens, with HSA as the carrier protein, following the carbodiimide crosslinker chemistry. The evaluation of all the prepared antigens by the in-house *in vitro* multiplex colorimetric assay showed that all of them were immunogenic and were able to recognize specific IgG from immunized rabbits. Nonetheless, the effect of the spacer arm length showed different profiles depending on the antibiotic used, without a clear pattern. Furthermore, differences were observed depending on the conjugation strategy followed. In short, major antigens were better recognized by specific IgG than the corresponding minor antigens. When artificial human serum was used, AMX antigens led to detect specific IgE concentration of ca. 20 IU/mL, ten-fold increase compared to the reference antigen BPO. Unfortunately, antigens prepared through this strategy did not detect specific IgE from a cohort of 35 allergic patients to AMX and/or PG, probably because they did not recognize the specific epitopes provoking the allergic episode.

On the other hand, the next strategy considered in this dissertation was the cationization of carrier proteins, since the rise of the amino groups available in the protein were expected to have an effect in antibody recognition. In this respect, five AMX- and PG-based antigens using HSA and H1 as cationized carrier proteins were prepared. To modify and cationize the carrier proteins, aliphatic and aromatic diamines of different length were also used. When artificial human serum was used, these cationized antigens reached values of a sIgE concentration of 23 IU/mL. However, it is even more of concern the fact that these antigens detected specific IgE from a cohort of 35 allergic patients. Specifically, the limit of detection was reduced to 0.07 IU/mL compared to the reference assay, which only allows the detection of concentrations equal to or greater than 0.35 IU/mL. Even better, the sensitivity of the *in vitro* diagnostic test for PG was three-fold improved when cationized determinants were used. Furthermore, none of the 35 controls patients tested positive (specificity = 100 %). Regarding to the diamine-length for the generation of the cationized proteins, it has been proven that the use of the antigen prepared with the shorter diamine achieves the best sensitivity. The short chain of the diamine helps by reducing steric and hydrophobic effects. The selection of the carrier protein was also proven to be key for the discrimination of allergic patients and controls. In fact, H1 antigens were able to detect more allergic patients than HSA antigens did.

Furthermore, it is well known that penicillins can decompose *in vivo*, leading to the formation of multiple metabolites after the  $\beta$ -lactam ring opening. In this respect, several studies aim to identify metabolite-based determinants by specific IgE antibodies. Herein, it has been reported the evaluation of several penicillin metabolites by the *in vitro* microimmunoassay. To this aim, penicillin based-metabolites PGoyl and BPG were synthesized using sodium benzylpenicillin and the other metabolites (6-APA, PA and PLA) used as commercially supplied. First, all the metabolites were conjugated to the carrier proteins HSA and H1 by the carbodiimide crosslinker method. Furthermore, major antigens of 6-APA and PGoyl were prepared as well as a minor antigen of PA by the formation of disulphide bonds between cysteine residues of the carrier protein and the thiol group of the metabolite. The former antigens were linked only to the carrier protein H1 as a proof of concept since the previous strategy showed that H1 antigens were better recognized by IgE antibodies than those referring to HSA.

In a set of 36 allergic patients and controls, H1-based antigens worked better than the corresponding HSA ones: a pattern that is in agreement with what was observed previously. Secondly, the selected 18 control patients tested negative to 4 out of 5 metabolite-based antigens. Concretely, the diagnostic specificity of the assay with antigen PLA was 94 %. When these antigens were screened against sera from allergic patients, only the antigen to BPG was not able to detect allergic patients. This may be due to the loss of immunological activity after the decarboxylation of the carboxylic acid after opening of the  $\beta$ -lactam ring, characteristic of penicillins. Having said this, IgE antibodies from most of the 18 recruited allergic patients to  $\beta$ -lactam antibiotics (mostly to penicillins) recognized all the metabolite antigens prepared through the carbodiimide crosslinker chemistry. On the other hand, major antigens as well as the minor antigen to PA linked to cysteine residues did not detect allergic patients, highlighting the importance of the conjugation route in the preparation of antigens. In this case, the great recognition by IgE antibodies of antigens prepared through the carbodiimide chemistry may come from the preservation of the  $\beta$ -lactam ring intact. In conclusion, the *in vitro* microimmunoassay allows for the quantification of sIgE in allergic patients that were not detected by other *in vivo* and *in vitro* tests. In particular, the diagnostic specificity of the microimmunoassay was of 100 % (94 % to PLA) and the diagnostic sensitivity ranged from 67 to 100 %, depending on the metabolite used.

Other families of  $\beta$ -lactam antibiotics, such as cephalosporins, carbapenems, monobactams or  $\beta$ -lactam inhibitors are not as beneficial as penicillins and lack an *in vitro* immunoassay for the detection of allergies to them. In fact, the consumption of these other families stands at 11 % of the total consumption of antibiotics in the EU between 2016 and 2018, which highlights their importance. In this respect, only one cephalosporin, cefaclor, can be tested for positivity using ImmunoCAP. Thus, this dissertation has also investigated the preparation of antigens of other less known and prescribed families. To this end, the major and minor antigens generated for several cephalosporin (CFT, CFR and CFO), carbapenem (MRP) and monobactam (AZT) antibiotics, using, again, HSA and H1 as carrier proteins show a great potential. In this respect, the preparation of minor antigens following the carbodiimide chemistry has been previously based on the acidification of those antibiotics in their salt forms (CFT, CFR and CFO). Major and minor antigens were, first, tested, as a proof of concept, with polyclonal IgG for each of these families but that were conjugated using KLH as the immunogen protein. Their evaluation yielded similar results regardless of the carrier protein used. Furthermore, in general, no cross-reactivity has been observed between these families, especially in the case of the major antigens. Interesting results have been observed when testing allergic patients and controls. Only those minor antigens prepared with H1 as the carrier protein were able to detect allergic patients. The sensitivity obtained in the immunoassay was high (LOD < 0.01 IU/mL). Furthermore, a diagnostic specificity of 100 % was achieved when the antigens were tested in the immunoassay with 37 control patients. Specifically, 35, 51 and 49 % of a set of 37 patients were recognized and gave positive results when they were tested with the minor antigens of CFR, CFO and MRP. This is an important point because routine diagnostic tests were not performed for most of these patients, except in some cases for the detection of allergies to CFR. These results are valuable to highlight both the importance of the different conjugation routes, as well as the carrier protein used, when generating antigens.

In the case of clavulanic acid, *in vitro* diagnostic test to quantify sIgE to this drug has been developed. To this aim, three derivatives of CVL were synthesized, since the combination of CVL and AMX is the antibiotic most frequently administered and provokes the major cases of DHRs. In this respect, from the three derivatives prepared, it has been observed that the antigen CVL-3 has weak recognition towards clavulanic acid. To generate this antigen, the characteristic ring of CVL was modified to a 2,3-

dihydrooxazoline ring. On the other hand, it has been proven that CVL-1, with a similar structure to CVL, but in which the potassium carboxylate has been esterified, and CVL-2, with the alcohol at the C-6 side chain oxidized, are important and valuable because after conjugation to proteins as immunogens develop specific IgG antibodies to clavulanic acid. However, when these antigens were tested with a set of 12 allergic patients, only antigen CVL-1 linked to HSA recognize the epitopes causing allergic reactions, being able to detect specific IgE to clavulanic acid, at low levels, in 6 out of 12 allergic patients (diagnostic sensitivity = 50 %). Finally, the diagnostic specificity of the assay was 100 % when the antigens were tested with a pool of 18 controls patients.

The classical way of generating determinants for allergy diagnosis has commonly been based on maintaining the original structure of the antibiotic intact. To this aim, the covalent attachment of the carrier protein is usually accomplished either through direct attachment with the functional and reactive groups of the antibiotic or by incorporating spacer arms, dendrimers, or previously chemically modified proteins. However, the chemical modification of the antibiotic itself to obtain structurally different and/or more accessible compounds from a spatial point of view had not been addressed. The hypothesis was that combinatorial techniques, which aim to discover clinically effective drugs, could also be applied to explore the generation of antigens to improve the clinical performances of the *in vitro* tests. Moreover, these approaches may facilitate the discovery of the allergy-causing epitopes, which might make it possible to be an alternative to the "classical" antigens.

For that purpose, DOS has been the technique proposed to generate novel  $\beta$ -lactam determinants in this dissertation. This technique provides the necessary structural diversity and complexity by incorporating  $sp^3$ -hybridized carbon atoms, basic nitrogen atoms, stereogenic elements, and novel skeletons. In this respect, 20 haptens derived from the  $\beta$ -lactam antibiotics AMX, AMP, MRP and AZT antibiotics, and the precursors of the penicillins and cephalosporins, 6-APA and 7-ACDA, have been synthesized using the Petasis-three component reaction, amine propargylation and metal-catalyzed cyclizations. Subsequently, major antigens were prepared using the HSA and H1 as carrier proteins and were evaluated using the *in vitro* multiplex-immunoassay. Interestingly, all the antigens prepared by this technique, except those 7-ACDA-derived, have been specific to detect IgG antibodies when tested with rabbit sera for PG and CFT. Those antigens to 7-ACDA proved to be highly inspecific, which may be due to a bad

recognition of these antigens by the IgG antibodies from rabbits specific to the antibiotic CFT. In this respect, ceftriaxone has a metabolically stable thiotriazinedione moiety instead of the easily hydrolyzed acetyl group present in 7-ACDA.

Thereafter, the suitability of the antigens was evaluated, testing human serum samples from a cohort of 19 allergic patients and the specificity when testing a set of 19 control human samples (diagnostic specificity = 100 %). In this respect, although a high correlation between HSA and H1 antigens was observed when these antigens were tested with sIgG from immunized rabbits, H1 antigens did work better for the discrimination of allergic patients. Regarding antigen recognition, 15 patients gave a positive result using the DOS-derived antigens, while with ImmunoCAP only four patients tested positive. Although more work related to the preparation of antigens by combinatorial techniques needed to be done, this study set unprecedented results. Therefore, a diagnostic sensitivity of 79 % was obtained when considering the set of the nine selected antigens as a whole, indicating that sIgE from allergic patients recognized the  $\beta$ -lactam ring. The results demonstrate the potential of DOS as well as the Petasis three-component reaction as a suitable antigen preparation strategy.

To sum up, Table 5 shows the main features of the haptens and antigens developed throughout this dissertation. From the results, it is concluded that 41 novel haptens have been synthesized in order to prepare 100 antigens. Therefore, the approaches assessed in this dissertation covered from the study of well known metabolites to penicillins to the exploration of combinatorial techniques in the production of haptens to prepare antigens.

Both the diagnostic sensitivity and specificity of the developed *in vitro* immunoassay were improved by using the prepared antigens presented in this dissertation, apart from the antigens based on spacer arms. Concretely, the diagnostic specificity of the immunoassay was improved compared with the *in vivo* tests currently in use. Therefore, the diagnostic specificity ranged from 94 % for PLA to 100 % for all the other prepared antigens. In the case of the diagnostic sensitivity, values varied according to the antibiotic used in the haptenization process, and ranged from 16 to 100 %. In this respect, those strategies using cephalosporin and AMX-based antigens achieved the lowest diagnostic sensitivities in the assay. However, the results obtained

with the AMX-based antigens correlate well with those obtained by the reference ImmunoCAP.

On the other hand, however, the preparation of novel antigens to less studied  $\beta$ -lactam families, such as cephalosporins, carbapenems and monobactams, permitted the quantification of specific IgE for the first time. Furthermore, values obtained for diagnostic sensitivities are even better than those corresponding to the prick test.

According to the scientific literature, multiple studies have been focused on the detection of specific IgE from allergic patients to  $\beta$ -lactam antibiotics. Both the diagnostic sensitivity and specificity of the proposed *in vitro* immunoassays render positive results.

In this respect, the multiplexed capacity of the microimmunoassay allows for the simultaneous *in vitro* detection of sIgE to several antibiotics in the same assay. This platform would permit the identification of the epitopes provoking the allergic episodes to up to ten different well and less known antibiotics, improving the currently commercial *in vitro* assays. Nevertheless, the combination of all the different approaches is necessary and the complementation of all of them will permit a better diagnosis of drug allergies. Furthermore, the *in vitro* immunoassay using the prepared antigens presented in this dissertation allows the detection of cross-reactivity between different  $\beta$ -lactam antibiotics. This may enable the avoidance of incorrect delabelings and the prevention of prescribing medications to patients who cannot tolerate them. In conclusion, the antigens generated from the synthesized haptens might be suitable to be used to diagnose allergies to  $\beta$ -lactam antibiotics when used supporting the current diagnostic methods.

**Table 5.** Summary of the antigens prepared in this dissertation

Antigens based on:	Spacer arms	Cationized proteins	Metabolites	Neo-antigens	Clavulanic acid	DOS
BLC involved	Penicillins (AMX and PG)	Penicillins (AMX and PG)	Penicillins (PLA, 6-APA, PGoyl, PA and BPG)	Cephalosporins (CFT, CFR, CFO) Carbapenems (MRP) Monobactams (AZT)	Clavulanic acid (CVL)	Penicillins (AMX, AMP, 6-APA) Cephalosporins (7-ACDA)
Haptens synthesized (antigens generated)	13 (13)	0 (5)	2 (16)	3 (20)	3 (6)	20 (40)
N <sub>o</sub> human samples (N <sub>o</sub> allergic patients)	70 (35)	70 (35)	36 (18)	74 (37)	30 (12)	38 (19)
Positive results with allergic patients (conjugation conditions)	No	Yes (only H1 and carbodiimide chemistry)	Yes (better H1 and carbodiimide chemistry)	Yes (only H1 and carbodiimide chemistry)	Yes (only antigen CVL-1 HSA)	Yes (only H1)
Diagnostic sensitivity DVD immunoassay (%)	-	60 (PG) 31 (AMX)	100 (PLA) 94 (6-APA) 89 (PGoyl) 67 (PA)	35 (CFR) 51 (CFO) 49 (MRP)	50 (CVL)	26 (6-APA) 16 (AMX) 37 (AMP) 21 (7-ACDA)
Diagnostic specificity DVD immunoassay (%)	-	100	100 94 (PLA)	100	100	100
LOD (IU/mL)	-	0.07	0.06	0.01	0.06	0.06
Diagnostic sensitivity ImmunoCAP (%)	20 (PG) 37 (AMX)	20 (PG) 37 (AMX)	22 (PG) 44 (AMX) 38 (AMP)	-	-	8 (PG) 17 (AMX) 8 (AMP)
Diagnostic sensitivity Prick test (%)	13 (PG) 68 (AMX)	13 (PG) 68 (AMX)	14 (PG) 71 (AMX) 67 (AMP)	6 out of 33: 18 (CFR) 3 out of 19: 29 (CFT) 0 out of 2: 0 (CFO) 2 out of 7: 29 (MRP) 1 out of 3: 33 (AZT)	3 out of 4: 75 (CVL) 8 out of 9: 89 (AMX-CVL)	0 out of 10: 0 (PG) 7 out of 11: 64 (AMX) 6 out of 10: 60 (AMP) 1 out of 7: 14 (CFT)



## **5. CONCLUSIONS**



## 5. CONCLUSIONS

The most relevant conclusions are summarized, according to the objectives initially proposed.

- ✓ A set of 13 diamine-derived haptens for AMX and PG has been synthesized for the first time with aliphatic diamines of different alkyl chain lengths in order to prepare HSA major and minor antigens. Their assessment in a multiplex assay revealed that even when they were recognized by specific IgG from immunized rabbits, they were not recognized after the analysis of a set of 35 allergic patients. On the other hand, the evaluation of five antigens prepared using both HSA and histone cationized carrier proteins has allowed the detection of specific IgE, even at low concentrations, only for H1-antigens (LOD = 0.07 IU/mL). In this respect, the importance of the selection of the carrier molecule in the production of antigens has been highlighted. The sensitivity of the developed assay resulted in 31 % for AMX and 60 % for PG, improving three-fold the sensitivity compared to the results obtained by using the existing *in vitro* diagnostic test for PG allergy (20 %). Therefore, the preparation of antigens by means of the employment of cationized proteins is a crucial strategy to improve the diagnostic sensitivity of *in vitro* tests.
- ✓ Penicillin metabolites PLA, 6-APA, PGoyl and PA permitted the formation of minor antigens which were specifically recognized by IgE from a cohort of 36 human samples. The clinical performances of the developed immunoassay were improved with respect to the current diagnostic tests, with a diagnostic specificity of 100 % (94 % to PLA). The diagnostic sensitivity ranged from 67 to 100 %, depending on the metabolite studied, with PLA, 6-APA and PGoyl antigens achieving the best sensitivities.

## Conclusions

- ✓ The evaluation of major antigens to the  $\beta$ -lactam antibiotics CFR, CFO, CFT, MRP and AZT showed that they are not recognized by specific IgE in a cohort from 37 allergic patients. However, when minor antigens were prepared, it has been demonstrated that they show immunogenic capacity, with high sensitivity ( $< 0.01$  IU/mL) and specificity (100 %). It has been concluded that the conjugation route followed when preparing antigens for  $\beta$ -lactam allergy diagnosis is a key step in order to specifically and selectively recognize IgE antibodies.
- ✓ The generation of reagents to determine specific IgE to clavulanic acid for the *in vitro* diagnostics of this target has been studied. To this end, the importance of the chemical structure for allergic diagnosis to clavulanic acid has been highlighted. The esterification of potassium clavulanate and the oxidation of the alcohol at the C-6 side chain permitted to use them for the production of specific IgG antibodies in a three-fold higher ratio than to other families of  $\beta$ -lactam antibiotics. However, only the antigen linked to HSA after esterification of potassium clavulanate, CVL-1, was able to detect specific IgE to clavulanic acid. Therefore, the diagnostic sensitivity was 50 %, permitting the *in vitro* quantification of specific IgE to clavulanic acid for the first time.
- ✓ A cohort of 20 skeletally-diverse compounds for the  $\beta$ -lactam antibiotics AMX, AMP, MRP and AZT, and the precursors 6-APA and 7-ACDA has been synthesized by DOS. These compounds have proved to be capable of recognizing different epitopes when tested with specific IgG from immunized rabbits, even when their chemical structures had been highly modified. Not only the diagnostic specificity of the *in vitro* immunoassay has been observed to be high, but also was the diagnostic selectivity, mostly with the AMX and AMP-based antigens. The use of a combinatorial technique in the production of selective antigens for  $\beta$ -lactam allergy testing has been assessed for the first time to the best of our knowledge. It has been demonstrated that the incorporation of skeletally diversity resulted in the recognition of additional epitopes, highlighting the potential of this approach for the preparation of antigens.

In conclusion, all the developed approaches contribute to the synthesis of novel haptens derived of  $\beta$ -lactam antibiotics. In consequence, the unprecedented haptens help to optimize an *in vitro* test with the diagnostic sensitivity and specificity needed to identify and discriminate allergic patients to  $\beta$ -lactam antibiotics, improving the performance of the tests currently in use.

Beyond the results presented herein, this dissertation opens up new possibilities for the diagnosis of allergies to  $\beta$ -lactam antibiotics that might help to improve the quality of life of allergic people to  $\beta$ -lactam antibiotics. The produced antigens could be integrated in commercial diagnostic tests in conjunction with the antigens currently in use what might improve the reliability of drug allergy diagnosis. This might enable clinical evidences that support the decision-making of the allergist, inform allergen-avoidance regimes, ameliorate the design of personalized therapeutic strategies, and advance the  $\beta$ -lactam delabeling initiatives in the clinical practice.



## **6. ANNEXES**





# 6.1. Annex 1

## Electronic supplementary information (ESI†)

### Boosting the sensitivity of *in vitro* $\beta$ -lactam allergy diagnostic tests

Eduarne Peña-Mendizabal, Sergi Morais and Ángel Maquieira

#### Experimental section

##### 1. General Experimental

NMR Spectra:  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra were recorded on Bruker AVIIIHD NanoBay 400MHz spectrometer using TOPSPIN software. Proton and carbon chemical shifts ( $\delta^1\text{H}$ ,  $\delta^{13}\text{C}$ ) are quoted in ppm and referenced to tetramethylsilane with residual protonated solvent as the internal standard. Resonances are described using the following abbreviations; s (singlet), d (doublet), t (triplet), q (quartet), quin. (quintet), m (multiplet), br (broad), app (apparent), dd (doublet of doublets), etc. Coupling constants (J) are given in Hz and are rounded to the nearest 0.1 Hz.

Mass Spectra: High resolution mass spectra (HRMS) were recorded using a AB SCIEX TripleTOF™ 5600 LC/MS/MS System. The LC system used was an Agilent 1290 HPLC system. The analyses were performed using an Agilent EC-C<sub>18</sub>. The data acquisition used are in positive mode, over a mass range of 100 - 950 m/z. The MS was using an IDA acquisition method with: the survey scan type (TOF-MS) and the dependent scan type (product ion) using 35 V of collision energy. Data was evaluated using the qualitatively evaluated using the Peak View™ software. m/z values are reported in Daltons; high resolution values are calculated to four decimal places from the molecular formula, all found values being within a tolerance of 5 ppm.

Chromatography techniques: TLC was performed on Merck Glass TLC silica gel 60 F<sub>254</sub> 0.2 mm precoated plates and visualised using ultraviolet light and potassium permanganate stain.

MS-MALDI-TOF: The samples were analyzed in a 5800 MALDI TOF-TOF (ABSciex) in positive linear mode (1,500 shots every position) in a mass range of 15,000-100,000 m/z.

## **2. Chemicals, immunoreagents and buffers**

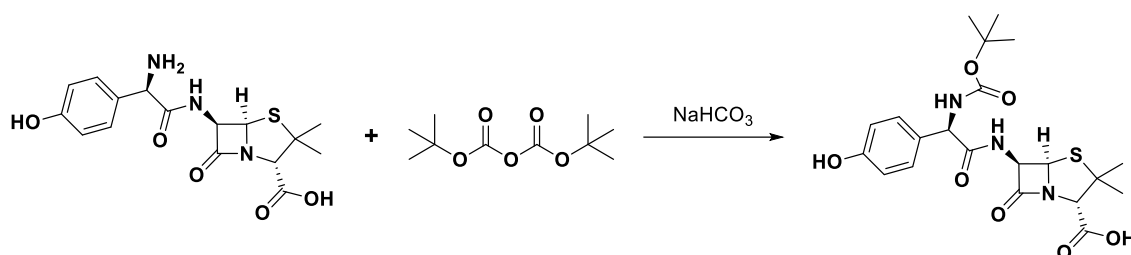
Benzylpenicillin sodium salt, amoxicillin trihydrate, aztreonam (AZT), 1,3-diaminopropane, 1,5-diaminopentane, 1,7-diaminoheptane, ethylene diamine dihydrochloride, 1,4-diaminobutane dihydrochloride, 1,4-phenylenediamine dihydrochloride, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), anhydrous di-tert-butyl dicarbonate ((Boc)<sub>2</sub>O), sodium bicarbonate, Tween 20, human serum albumin (HSA), histone from calf thymus (H1) and keyhole limpet hemocyanin (KLH) are from Sigma-Aldrich (Madrid, Spain). Dichloromethane (DCM), N,N-dimethylformamide (DMF), dioxane, ethyl acetate, hydrochloric acid 37 % (HCl) and buffer salts are from Scharlau (Sentmenat, Spain) and used without further purification. Deuterated dimethyl sulfoxide (DMSO-d<sub>6</sub>) is from ACROS Organics (New Jersey, USA). Specific anti-β-lactam IgE artificial human sera (ARTHUS) to PG is kindly provided by Dr. Fooke (Neuss, Germany) and anti-human IgE monoclonal antibody is from Ingenasa, S.A. (Madrid, Spain). Polyclonal secondary antibody goat α-rabbit IgG labelled with horseradish peroxidase (GAR-HRP) and goat anti-mouse antibody labelled with horseradish peroxidase (GAM-HRP) are purchased from Abcam (San Francisco, USA). IgE human serum (3rd WHO International Standard) is purchased from National Institute for Biological Standards and Control (NIBSC) (Hertfordshire, UK) and tetramethylbenzidine (TMB) substrate is from SDT GmbH (Baesweiler, Germany). Omalizumab antibody and Coomassie Brilliant Blue R-250 staining solution are from Bio-Rad (Madrid, Spain). Amicon Ultra 0.5 pre-concentrated 10 K filters are from Fisher Scientific (Madrid, Spain).

The employed buffers are: (I) sodium phosphate buffer 0.1 M, sodium chloride 0.15 M, pH 7.2; (II) MES 0.1 M, pH 4.7; (III) sodium carbonate 0.5 M, pH 11.0; (IV)

phosphate buffer saline (PBS 1X, 0.008 M sodium phosphate dibasic, 0.002 M sodium phosphate monobasic, 0.137 M sodium chloride, 0.003 M potassium chloride, pH 7.4); (V) PBS-T (PBS 1X containing 0.05 % Tween 20); and (VI) sodium carbonate/bicarbonate buffer 0.1 M, pH 9.6, as printing buffer. All buffers are filtered through a 0.45- $\mu\text{m}$  pore size nitrocellulose membrane from Thermo Fisher Scientific (Madrid, Spain) before being used.

### 3. Synthesis of BLC-derived haptens

#### *Boc-amoxicillin*



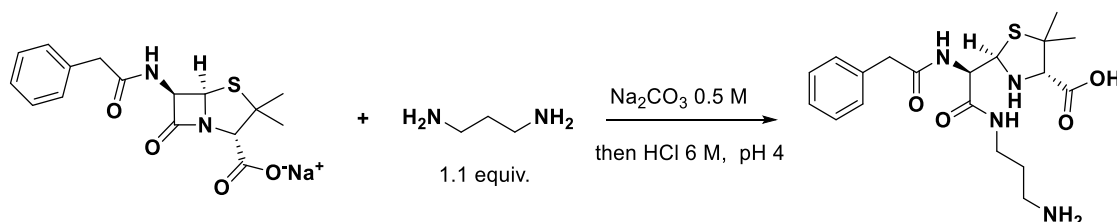
$\text{NaHCO}_3$  (200 mg, 2.38 mmol, 2 equiv.) is added to a solution of amoxicillin (440 mg, 1.19 mmol) in 150 mL  $\text{H}_2\text{O}$  and stirred at 5 °C. Anhydrous  $(\text{Boc})_2\text{O}$  (390 mg, 1.79 mmol, 1.5 equiv.) dissolved in 1.5 mL dioxane is added dropwise and the mixture is allowed to react at 0 °C for 1 h and then overnight at room temperature. The organic layer is extracted and washed twice with EtOAc and saturated  $\text{NaHCO}_3$ , respectively. Aqueous layers are mixed and acidified with HCl 6 M to pH 1 and extracted three times with EtOAc. Organic layers are collected and dried over  $\text{NaSO}_4$ . The solvent was evaporated and the desired compound dried under reduced pressure. Yield: 97 %.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-d}_6$ ):  $\delta$  9.36 (s, 1H), 8.73 (d,  $J = 7.9$  Hz, 1H), 7.18 (d,  $J = 9.7$  Hz, 2H), 6.66 (d,  $J = 8.3$  Hz, 2H), 5.45 (dd,  $J = 7.8, 4.2$  Hz, 1H), 5.37 (d,  $J = 4.2$  Hz, 1H), 5.25 (d,  $J = 8.5$  Hz, 1H), 4.15 (s, 1H), 1.91 (s, 1H), 1.55 (s, 3H), 1.41 (s, 3H), 1.38 (s, 9H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-d}_6$ ):  $\delta$  173.30, 170.46, 168.99, 156.80, 128.46, 114.86, 78.16, 70.47, 67.50, 66.36, 64.19, 59.80, 57.91, 30.52, 28.19, 26.71, 13.85. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{21}\text{H}_{27}\text{N}_3\text{O}_7\text{S}$  ( $[\text{M}+\text{H}^+]$ ): 466.1642, found 466.2539.

#### 3.1. BLC-oyl derivatives

## Annex 1

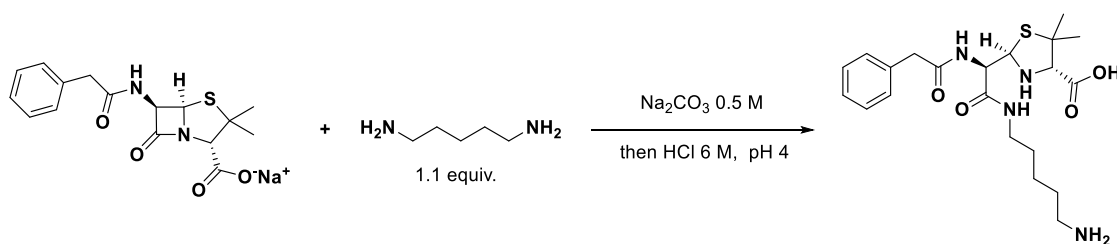
0.150 mmol of the corresponding BLC dissolved in deionized water (0.1 M) was basified by slow addition of sodium carbonate 0.5 M, pH 11.0. Then, 0.165 mmol (1.1 equiv) of the corresponding diamine was added dropwise at 0 °C under nitrogen atmosphere. The reaction was stirred at room temperature for 3 h followed by an acidification to pH 4.0 with HCl 6.0 M. The solution was filtrated and the desired precipitate washed twice with acidified water. The compound was allowed to dry under vacuum to give a white powder that was characterized by NMR and MS and used without further purification. Yield of the reaction was corrected by measuring the amount of salts ( $\text{Na}_2\text{CO}_3$  and  $\text{NH}_4\text{Cl}$ ) present. Haptens **1-6** (Scheme S1) were obtained through this route.

### Hapten 1



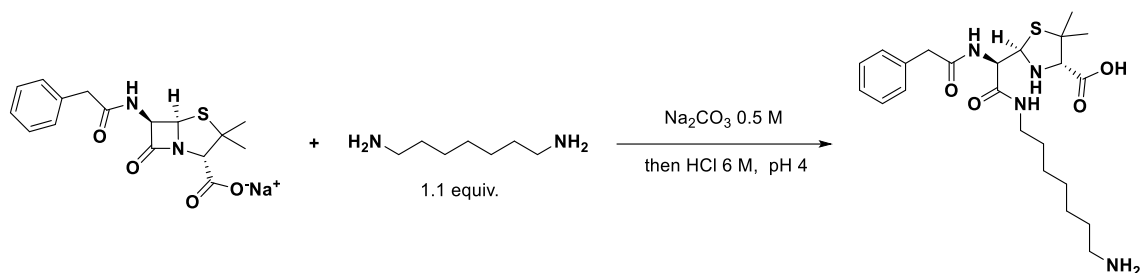
Yield: 97 %. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{19}\text{H}_{28}\text{N}_4\text{O}_4\text{S}$  ( $[\text{M}+\text{H}^+]$ ): 409.1904, found 409.1906.

### Hapten 2



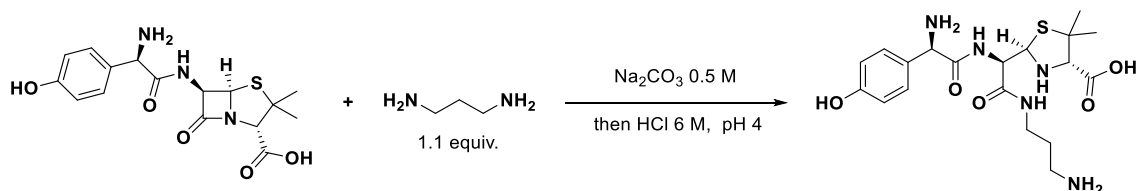
Yield: 61 %. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{21}\text{H}_{32}\text{N}_4\text{O}_4\text{S}$  ( $[\text{M}+\text{H}^+]$ ): 437.2217, found 437.2222.

### Hapten 3



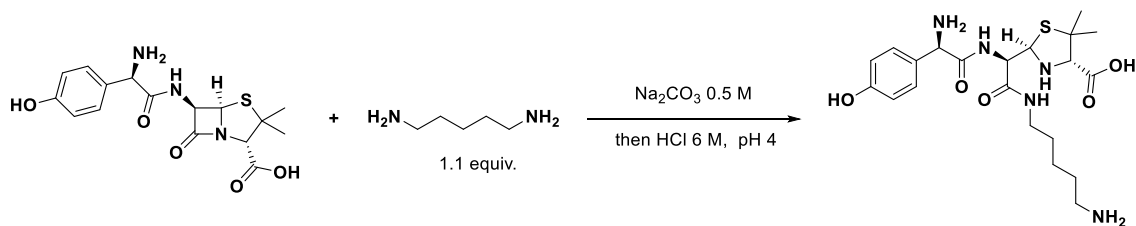
Yield: 77 %. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{23}\text{H}_{36}\text{N}_4\text{O}_4\text{S}$  ( $[\text{M}+\text{H}^+]$ ): 465.2530, found 465.2527.

#### Hapten 4



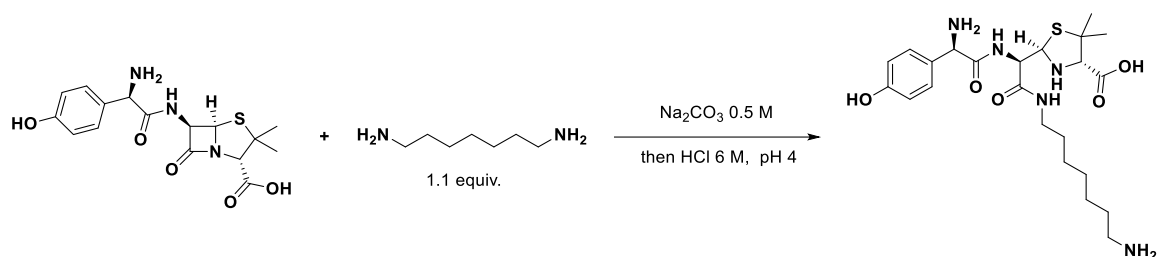
Yield: 100 %. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{19}\text{H}_{29}\text{N}_5\text{O}_5\text{S}$  ( $[\text{M}+\text{H}^+]$ ): 440.1962, found 440.1971.

#### Hapten 5



Yield: 80 %. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{21}\text{H}_{33}\text{N}_5\text{O}_5\text{S}$  ( $[\text{M}+\text{H}^+]$ ): 468.2275, found 468.3913.

#### Hapten 6

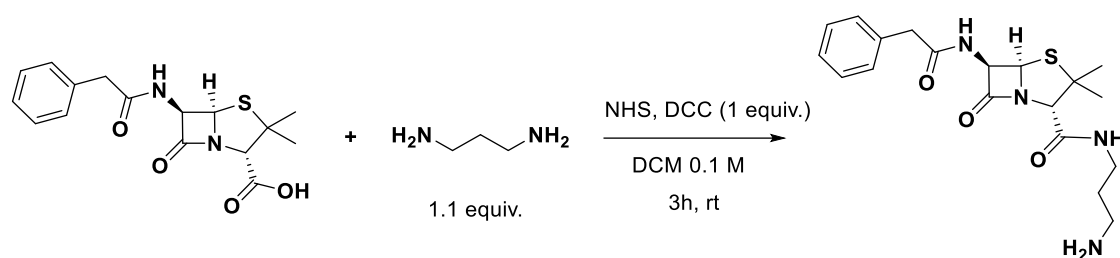


Yield: 100 %. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{23}\text{H}_{37}\text{N}_5\text{O}_5\text{S}([\text{M}+\text{H}^+])$ : 496.2588, found 496.2589.

### 3.2. BLC-anil derivatives

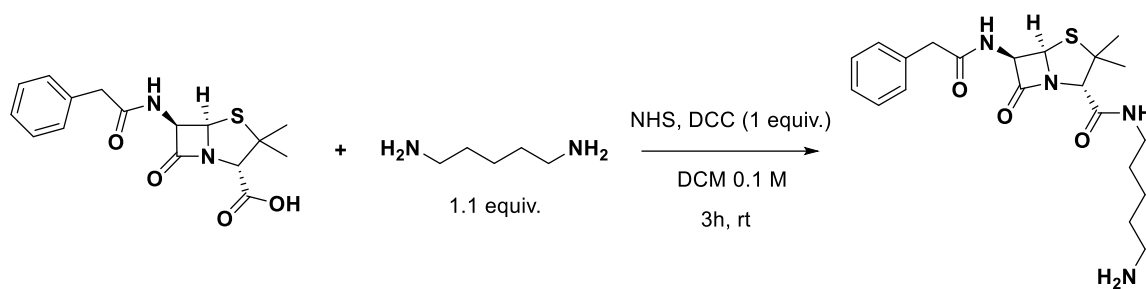
0.165 mmol (1.1 equiv) of the corresponding diamine was added dropwise at 0 °C under nitrogen atmosphere to 0.150 mmol of the corresponding BLC (the free acid) dissolved in DCM/DMF (0.1 M), treated with NHS and DCC (1 equiv.). The reaction was stirred for 3 h at room temperature. The solution mixture was filtered under vacuum, concentrated and dried under reduced pressure to give a white powder that was characterized by NMR and MS and used without further purification. Haptens **7-13** (Scheme S1) were obtained through this route.

### Hapten 7



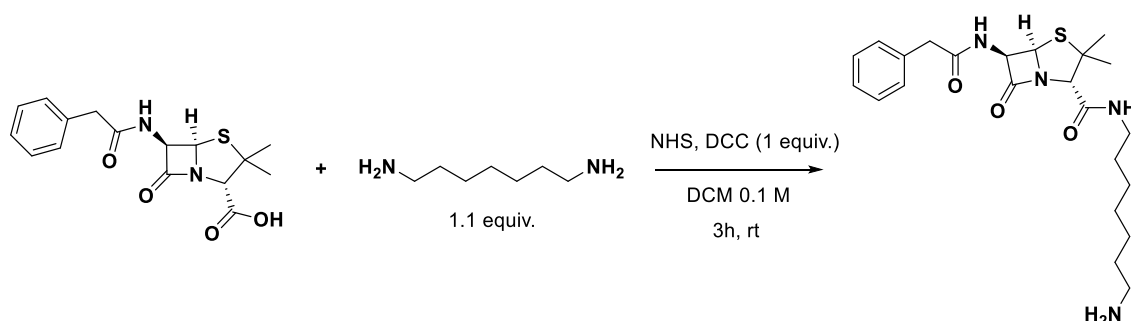
Yield: 100 %. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{19}\text{H}_{26}\text{N}_4\text{O}_3\text{S}([\text{M}+\text{H}^+])$ : 391.1798, found 391.1802.

### Hapten 8



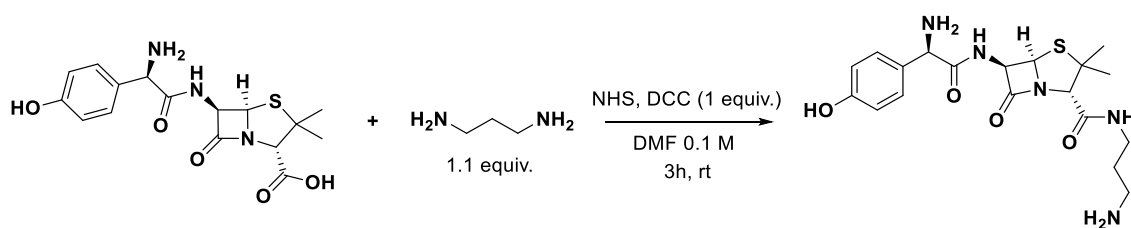
Yield: 66 %. HRMS (ESI-TOF)  $m/z$  calculated for  $C_{21}H_{30}N_4O_3S$  ( $[M+H]^+$ ): 419.2111, found 419.2119.

### Hapten 9



Yield: 77 %. HRMS (ESI-TOF)  $m/z$  calculated for  $C_{23}H_{34}N_4O_3S$  ( $[M+H]^+$ ): 447.2424, found 447.2430.

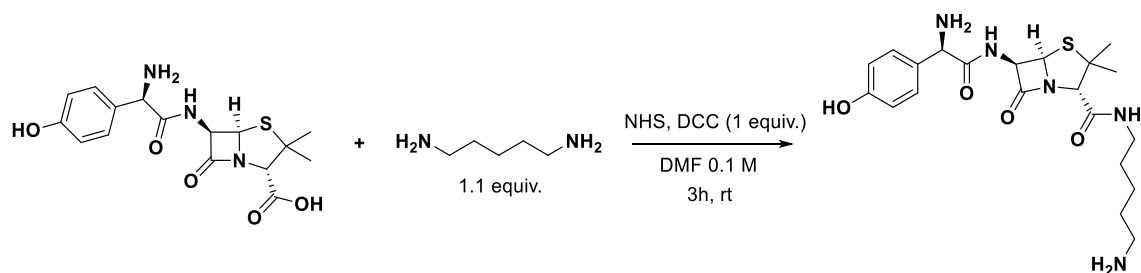
### Hapten 10



Yield: 51 %. HRMS (ESI-TOF)  $m/z$  calculated for  $C_{19}H_{27}N_5O_4S$  ( $[M+H]^+$ ): 422.1857, found 422.1862.

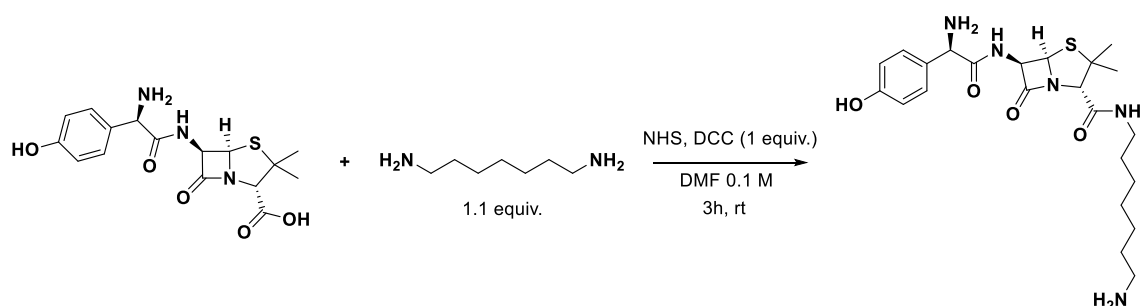
### Hapten 11

Annex 1



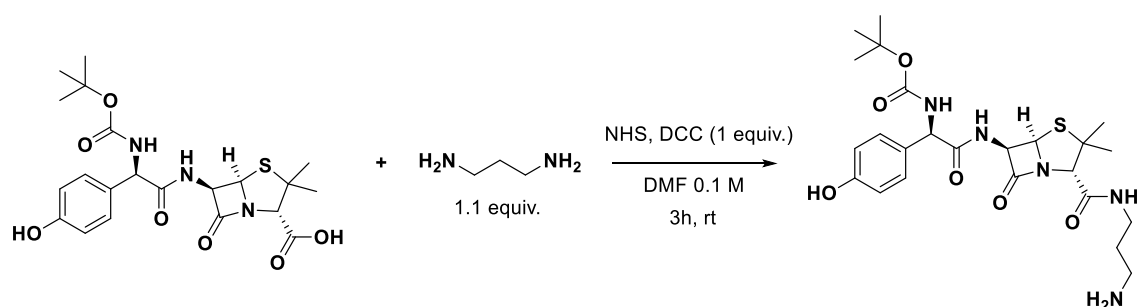
Yield: 14 %. HRMS (ESI-TOF)  $m/z$  calculated for  $C_{21}H_{31}N_5O_4S$  ( $[M+H]^+$ ): 450.2170, found 450.2911.

Hapten 12



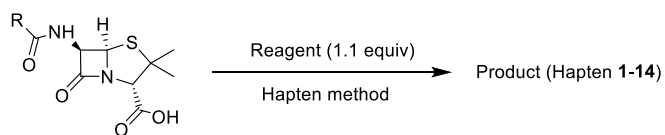
Yield: 81 %. HRMS (ESI-TOF)  $m/z$  calculated for  $C_{23}H_{35}N_5O_4S$  ( $[M+H]^+$ ): 478.2483, found 478.3226.

Hapten 13



Yield: 85 %. HRMS (ESI-TOF)  $m/z$  calculated for  $C_{24}H_{35}N_5O_6S$  ( $[M+H]^+$ ): 522.2381, found 522.2561.

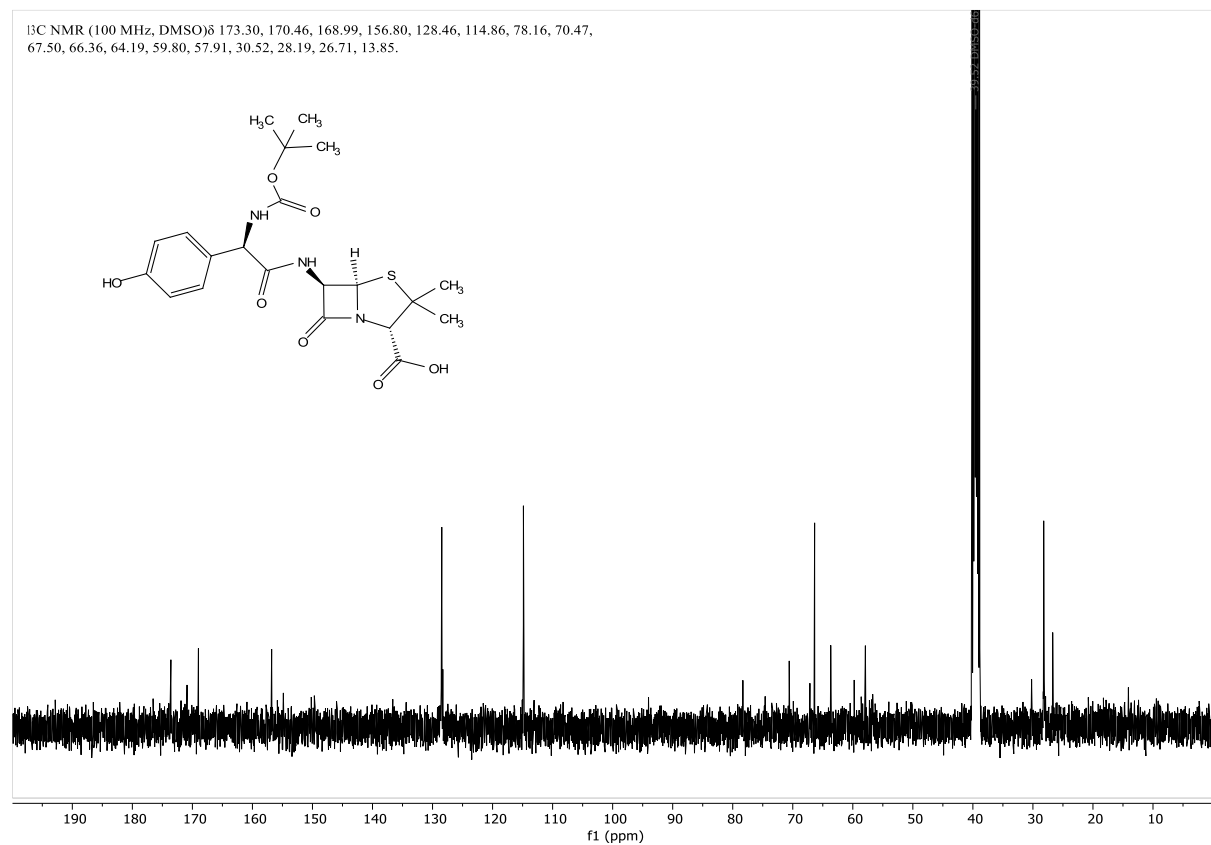
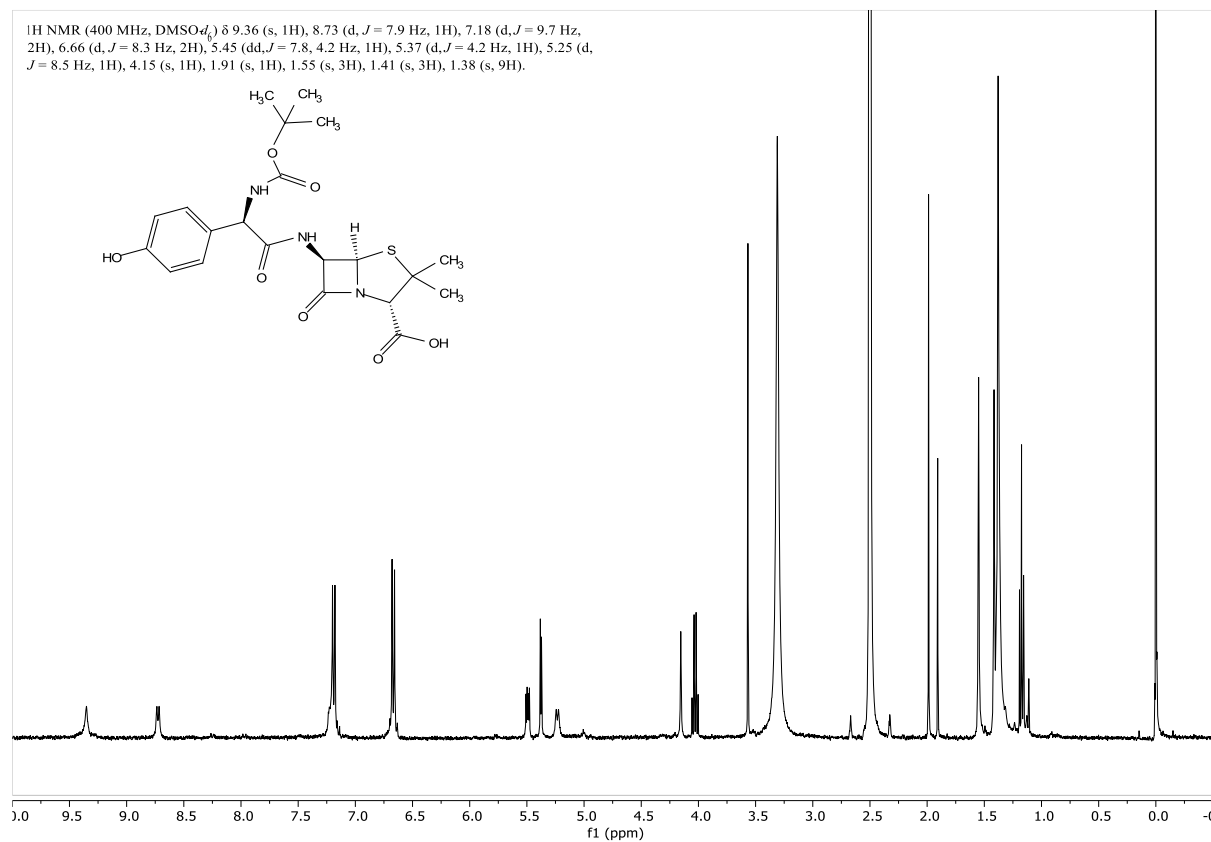




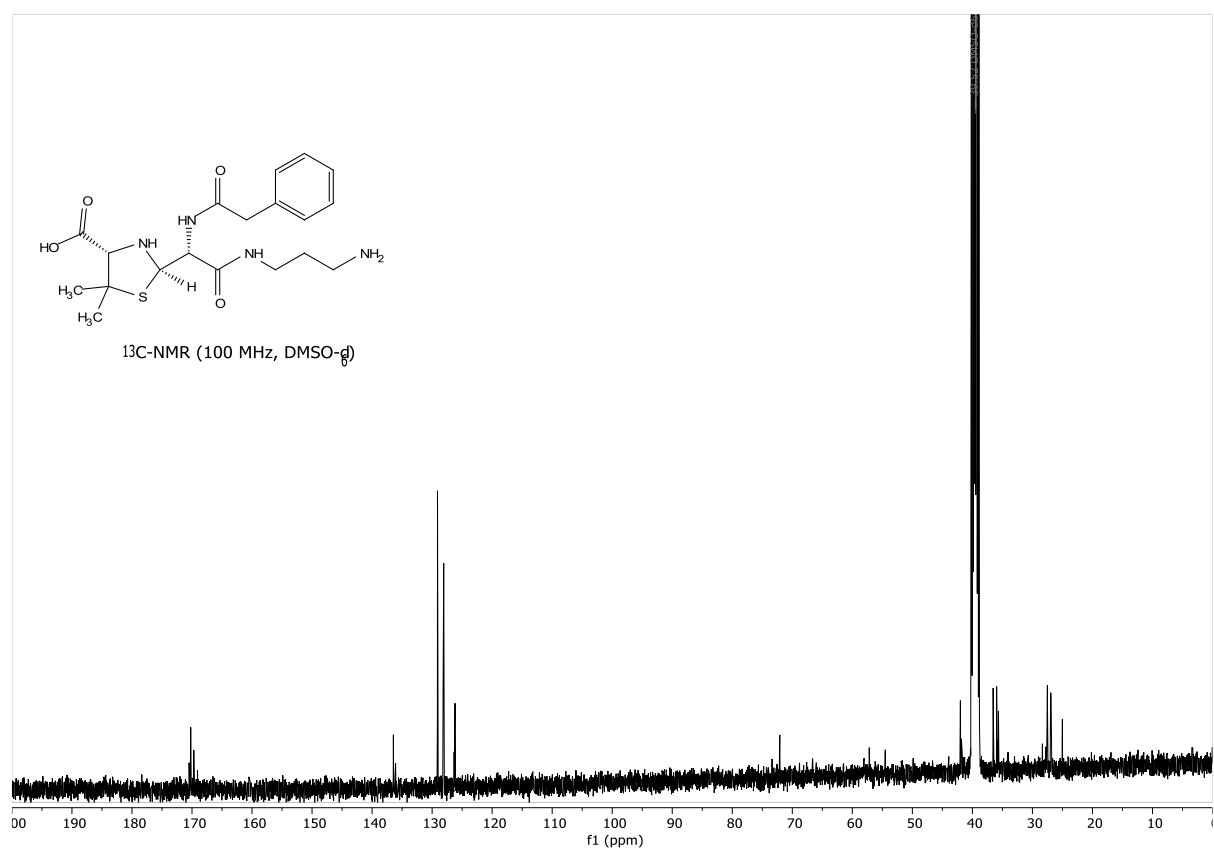
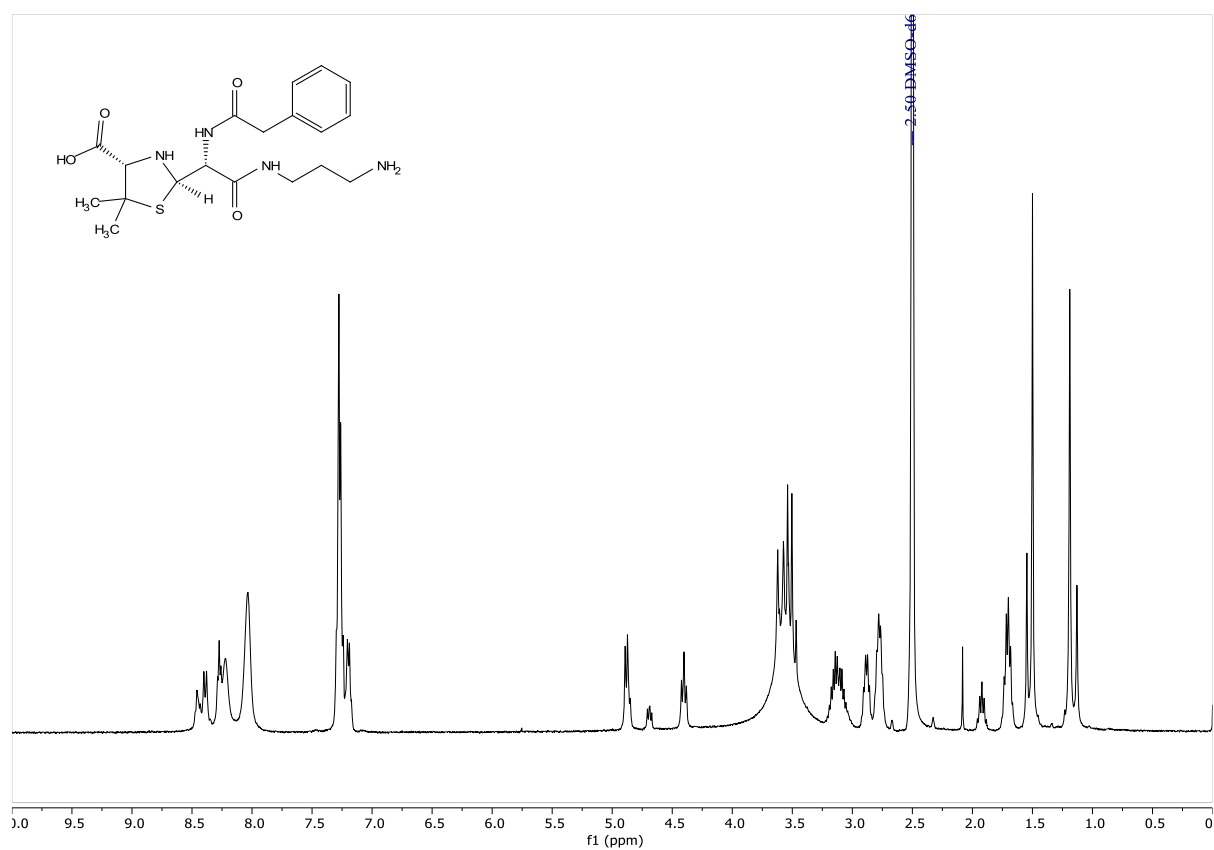
Entry	BLC	R	Reagent (1.1 equiv)	Yield <sup>[a]</sup> (%)	Hapten method <sup>[b]</sup>
1	PG		<chem>NCCCCN</chem>	97	-oyl
2	PG		<chem>NCCCCCCN</chem>	61	-oyl
3	PG		<chem>NCCCCCCCCN</chem>	77	-oyl
4	AMX		<chem>NCCCCN</chem>	100	-oyl
5	AMX		<chem>NCCCCCCN</chem>	80	-oyl
6	AMX		<chem>NCCCCCCCCN</chem>	100	-oyl
7	PG		<chem>NCCCCN</chem>	100	-anyl
8	PG		<chem>NCCCCCCN</chem>	66	-anyl
9	PG		<chem>NCCCCCCCCN</chem>	77	-anyl
10	AMX		<chem>NCCCCN</chem>	51	-anyl
11	AMX		<chem>NCCCCCCN</chem>	14 <sup>[c]</sup>	-anyl
12	AMX		<chem>NCCCCCCCCN</chem>	81	-anyl
13	AMX		<chem>NCCCCN</chem>	85	-anyl
14	PG		-		Reference

**Scheme 6.1.S1.** Chemical structures of the BLC synthesized haptens using alkyl diamines. [a] Isolated yields were reported. [b] Reaction conditions: (I) -oyl haptens:  $\text{Na}_2\text{CO}_3$  0.5 M, pH 11.0, then HCl 6.0 M, pH=4.0, 3h, rt; (II) -anyl haptens: DCM/DMF 0.1 M, 3h, rt. [c] Poor yield due to transfer losses.

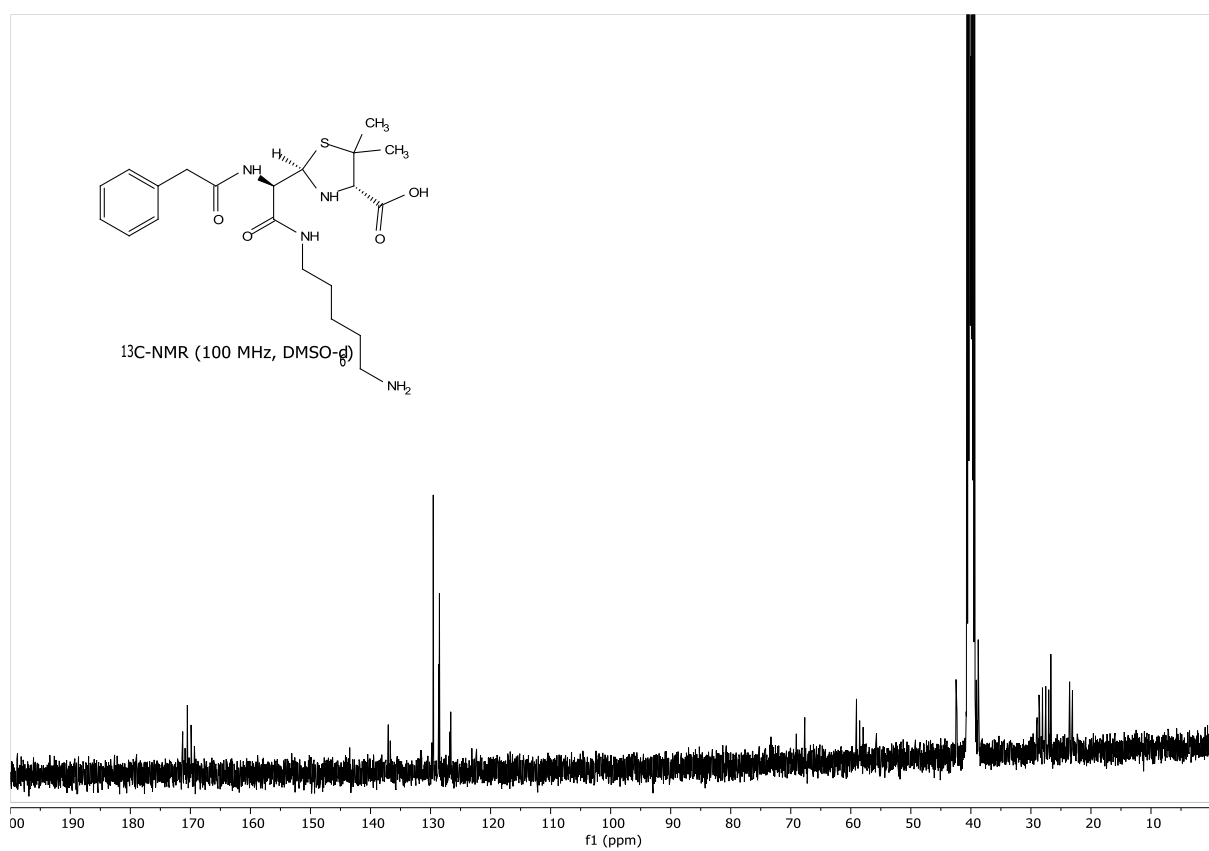
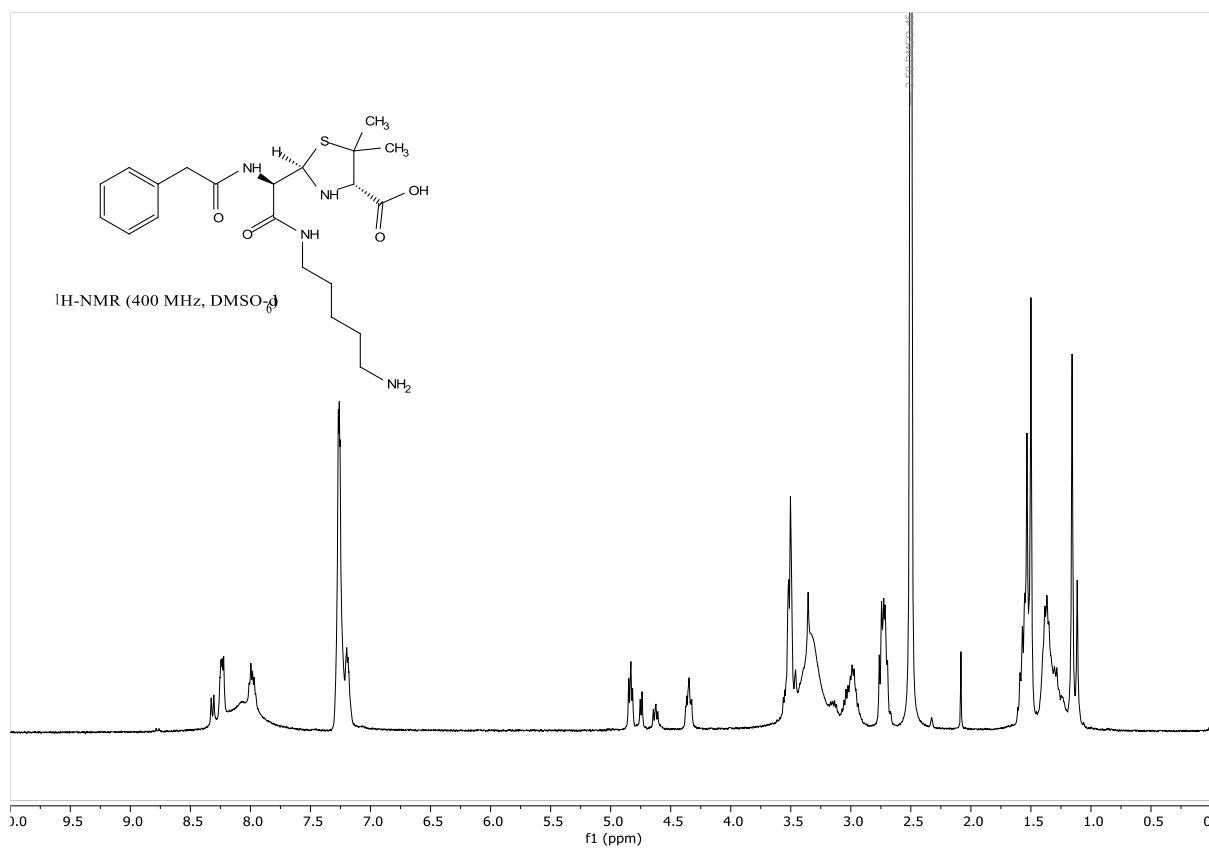
## 4. NMR spectra

*Boc-amoxicillin*

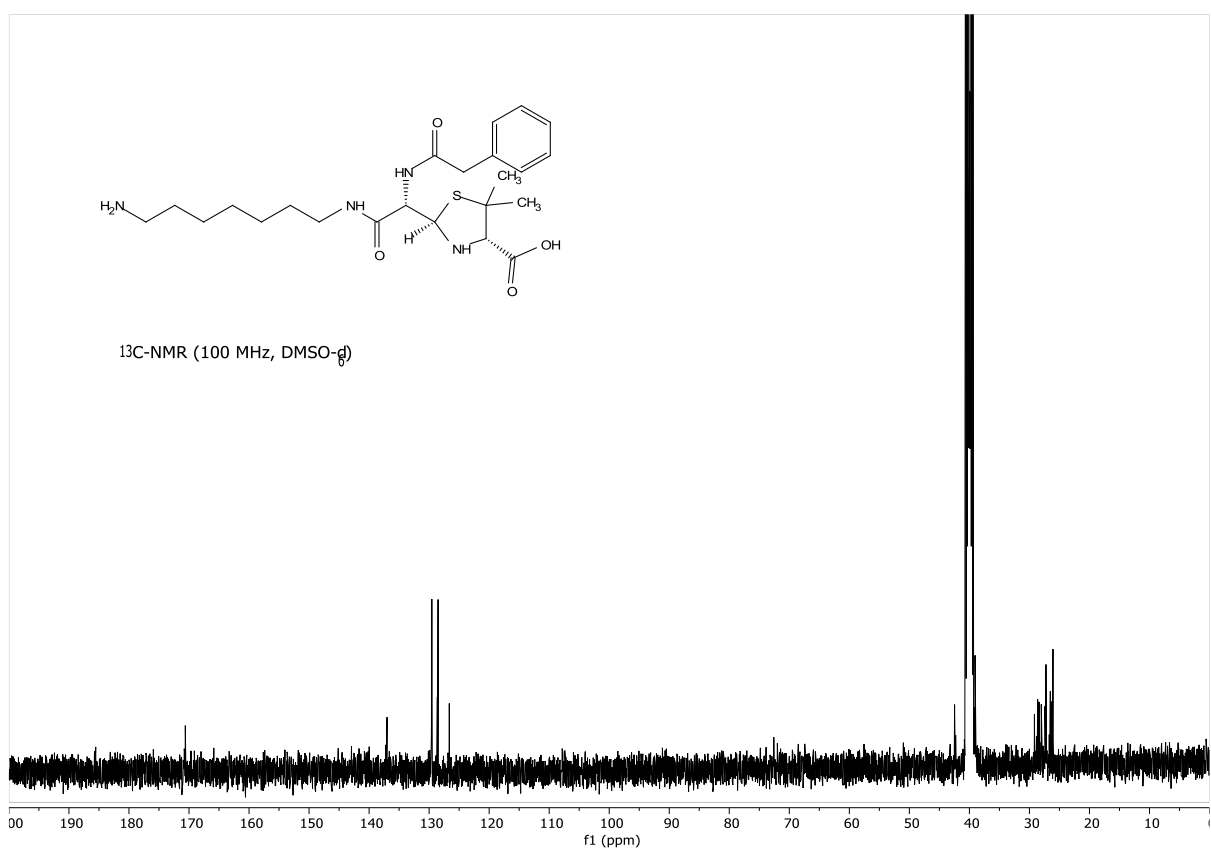
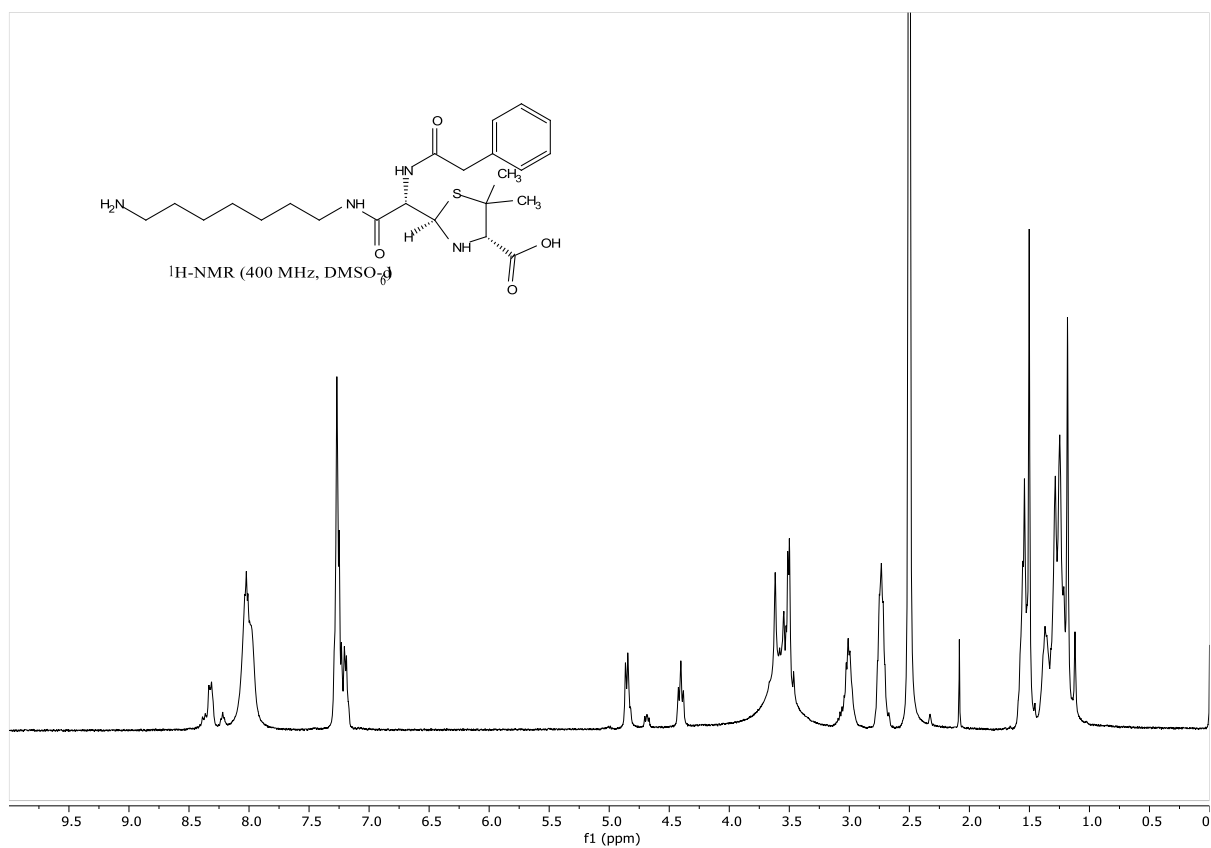
## Hapten 1



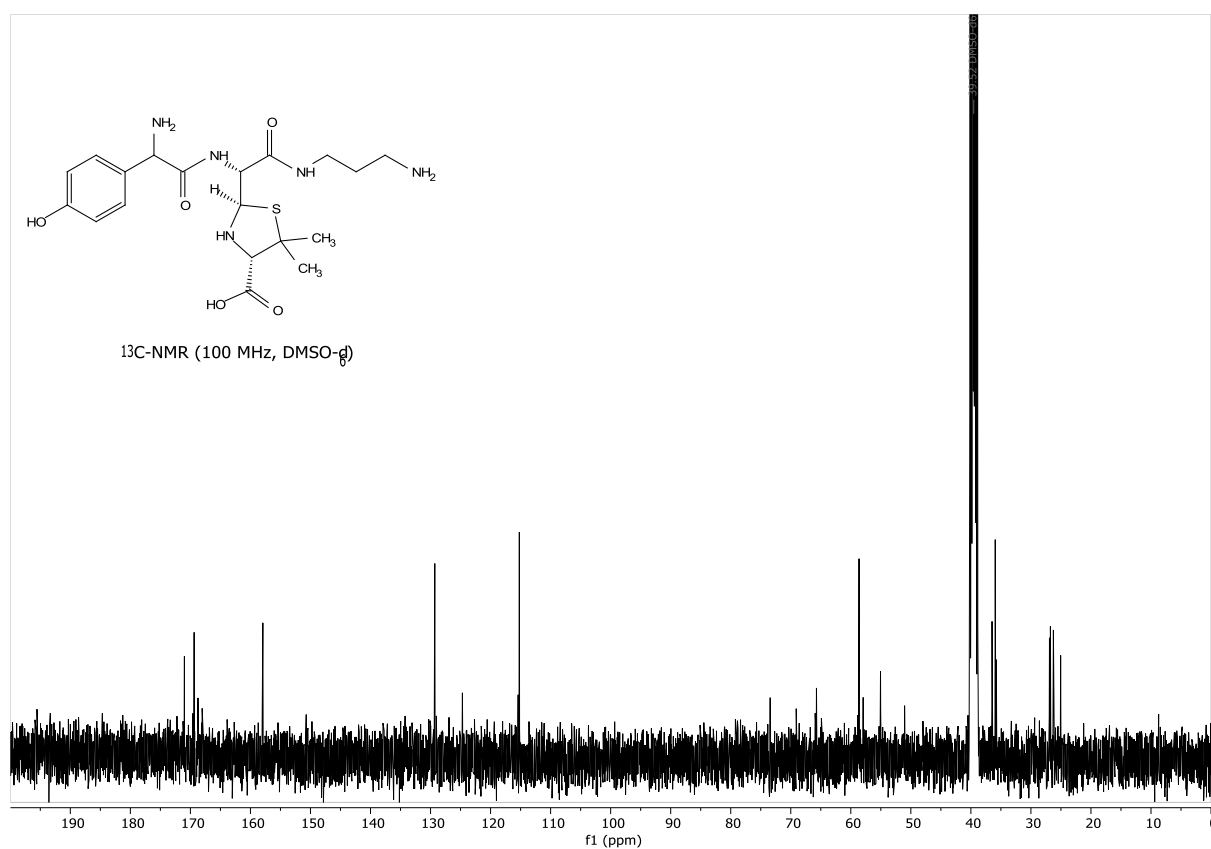
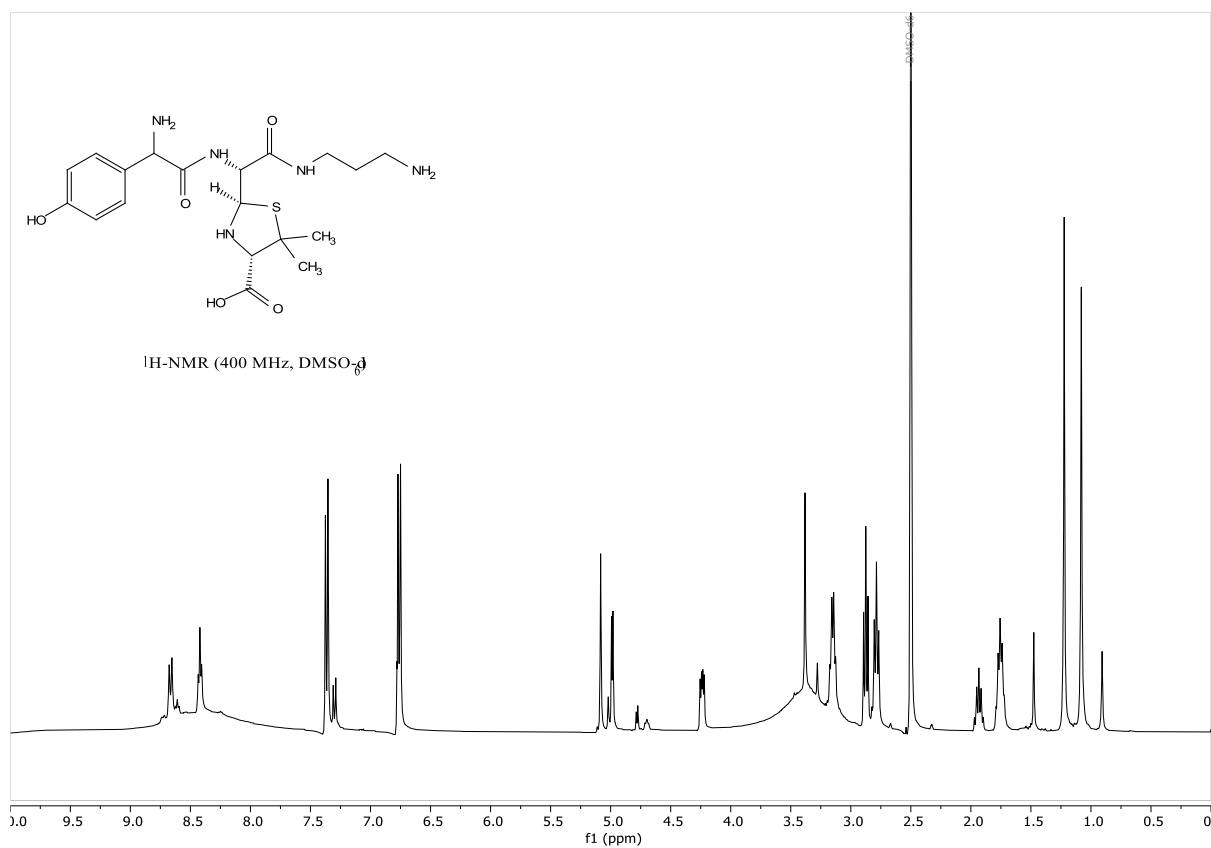
## Hapten 2



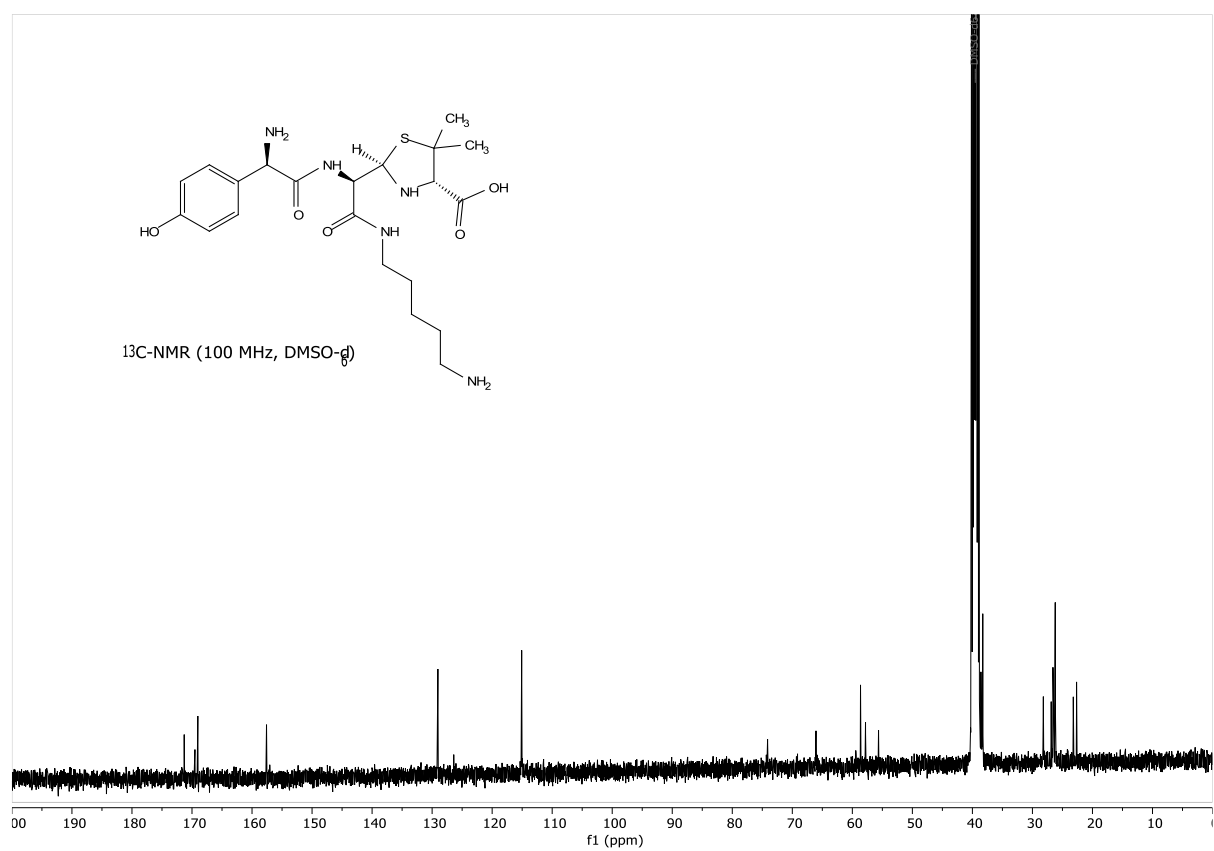
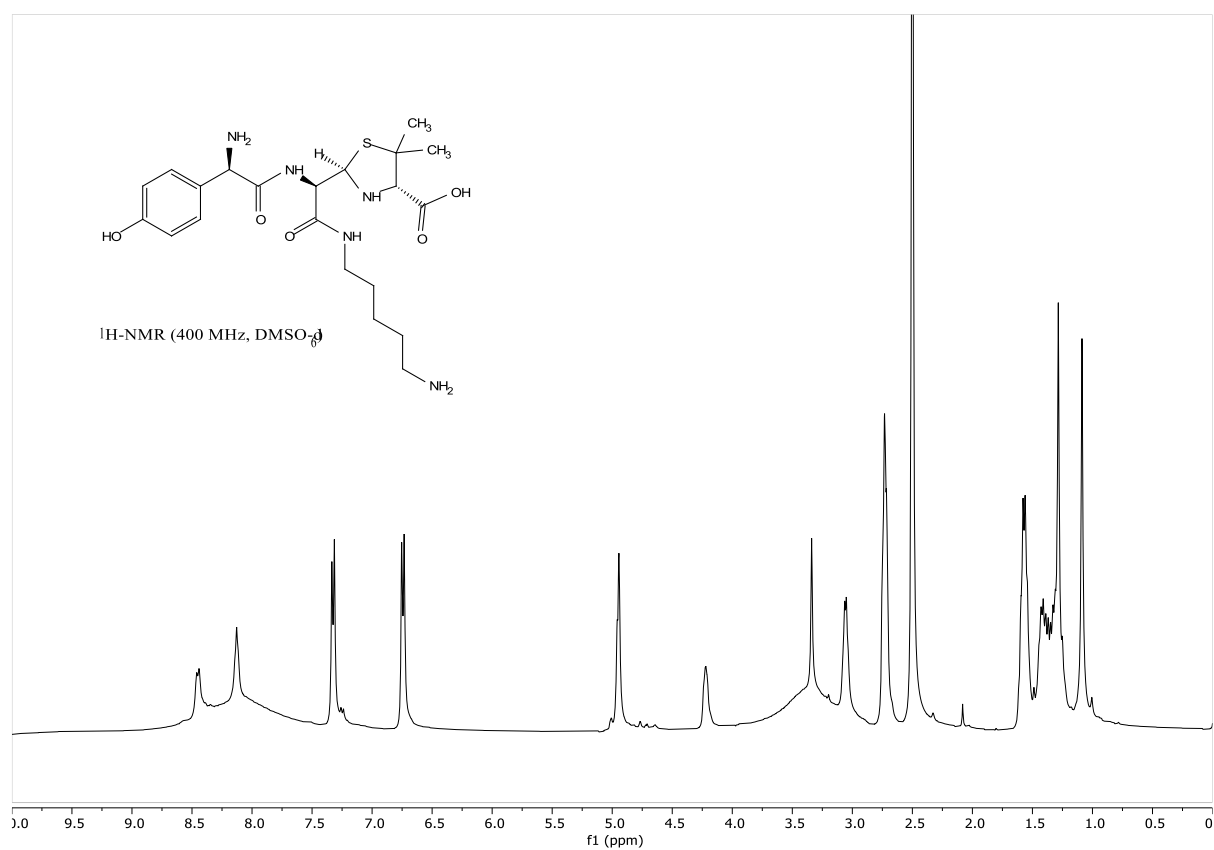
## Hapten 3



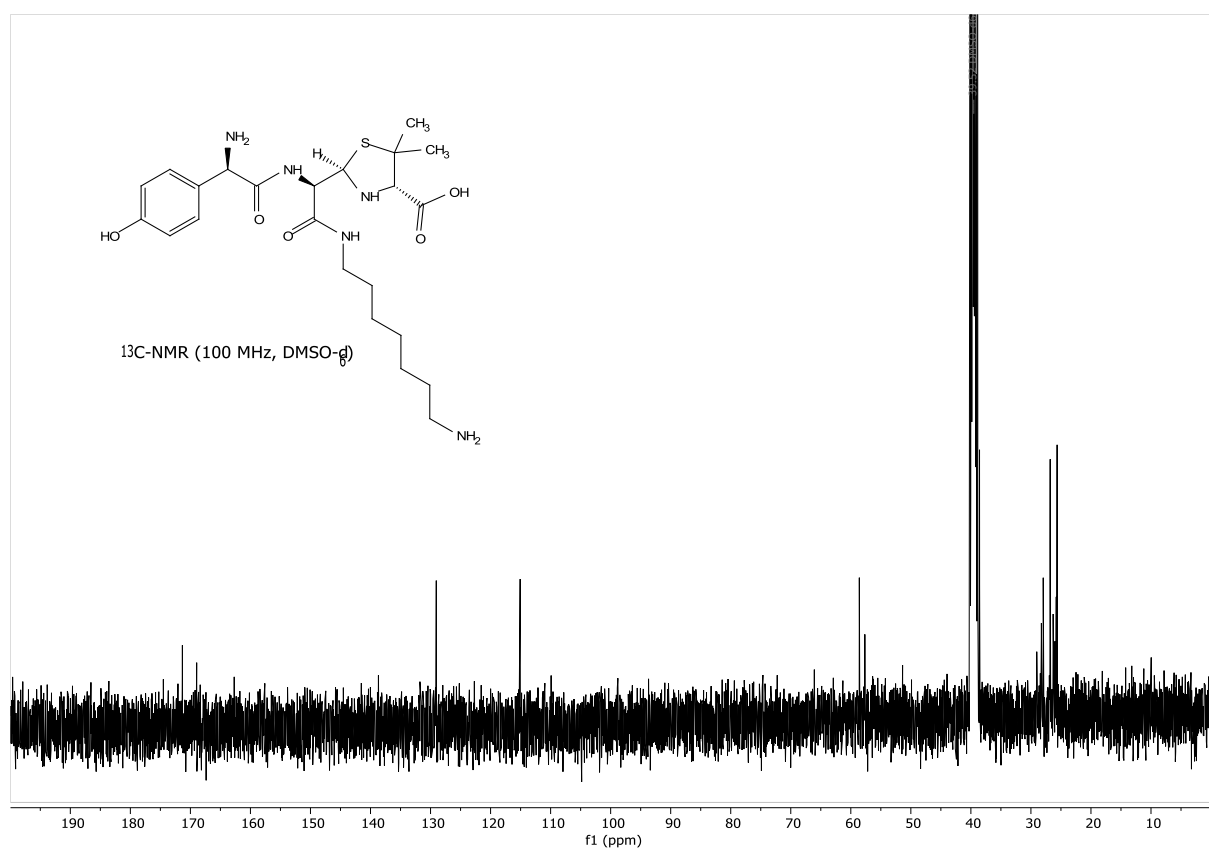
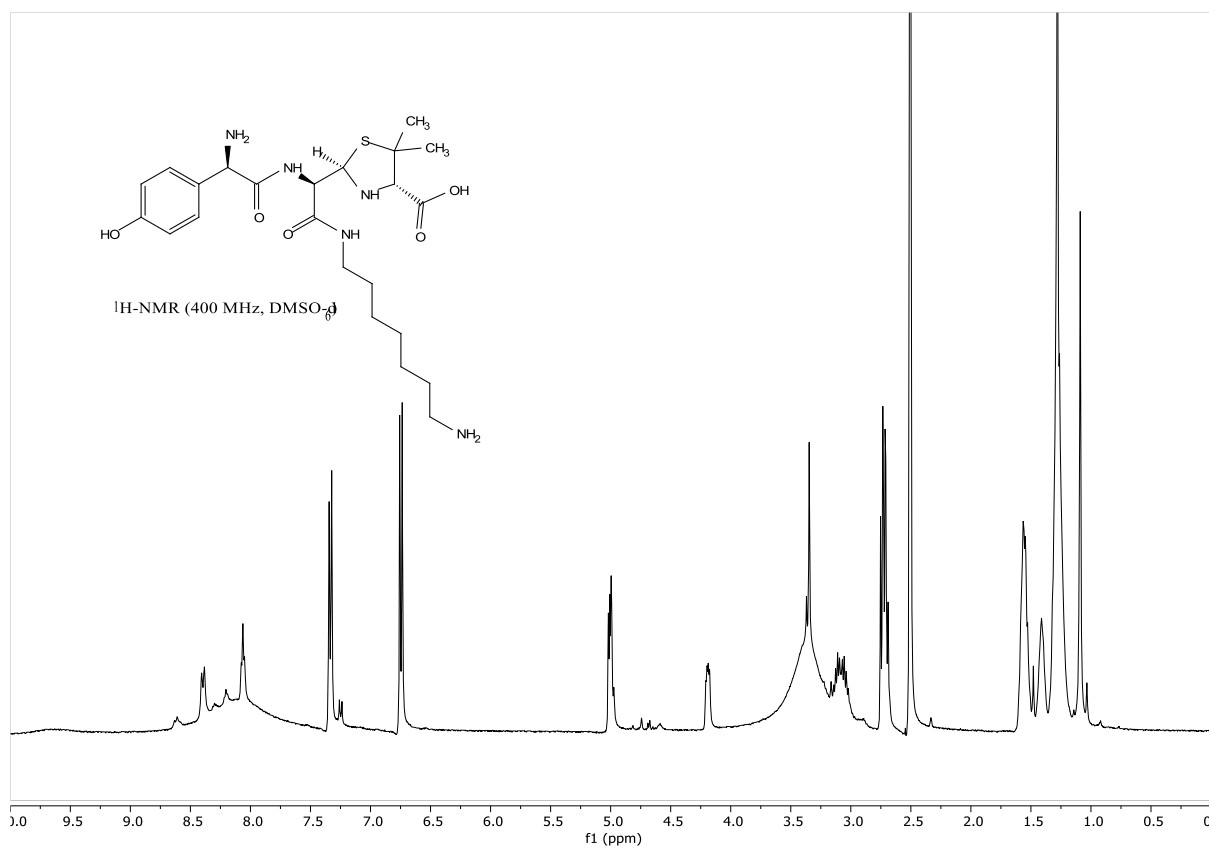
Hapten 4



## Hapten 5

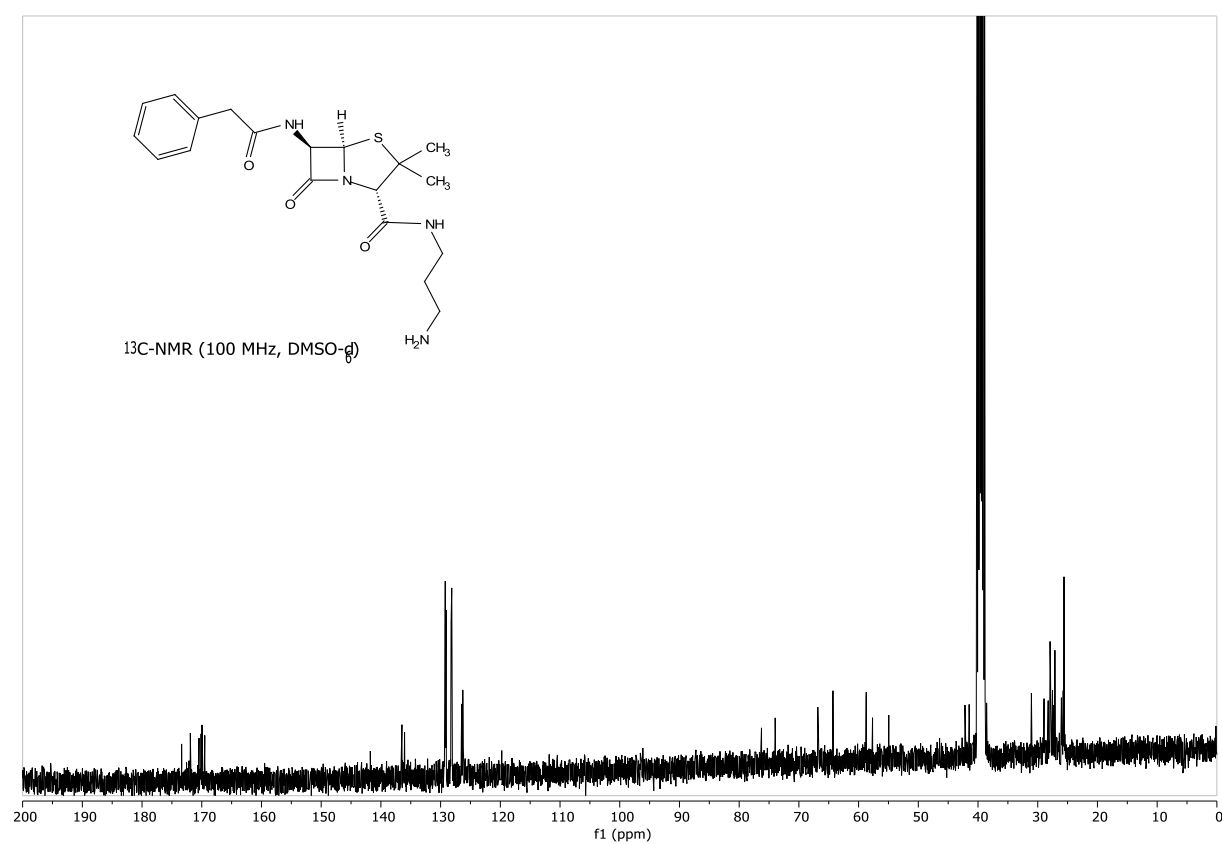
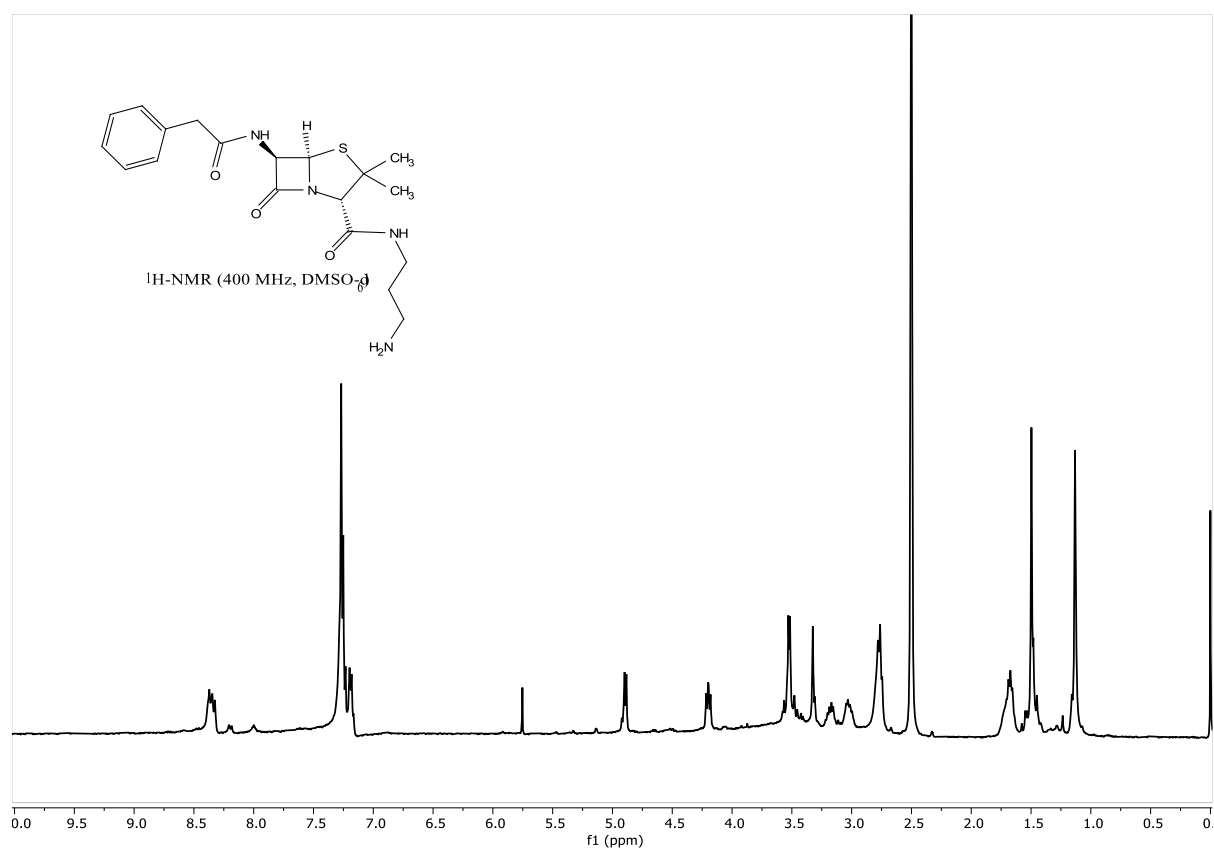


## Hapten 6

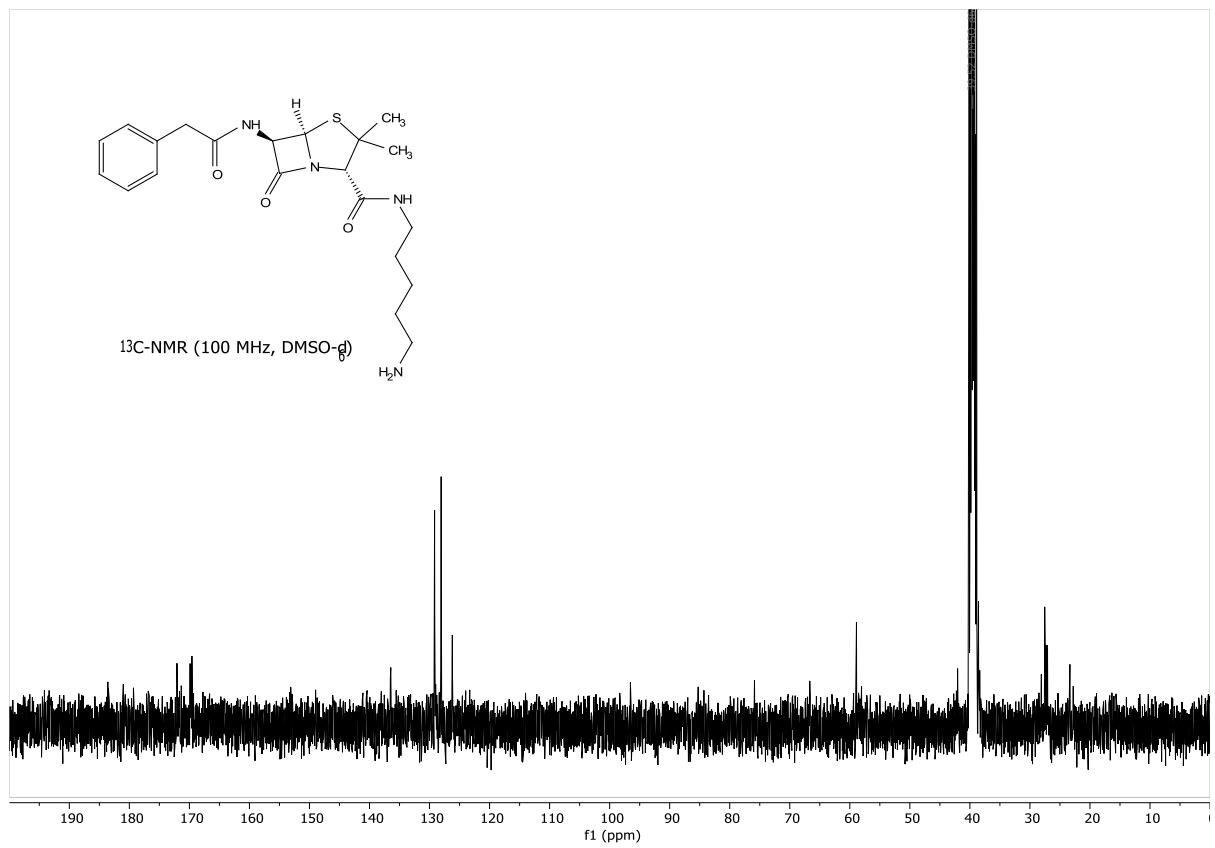
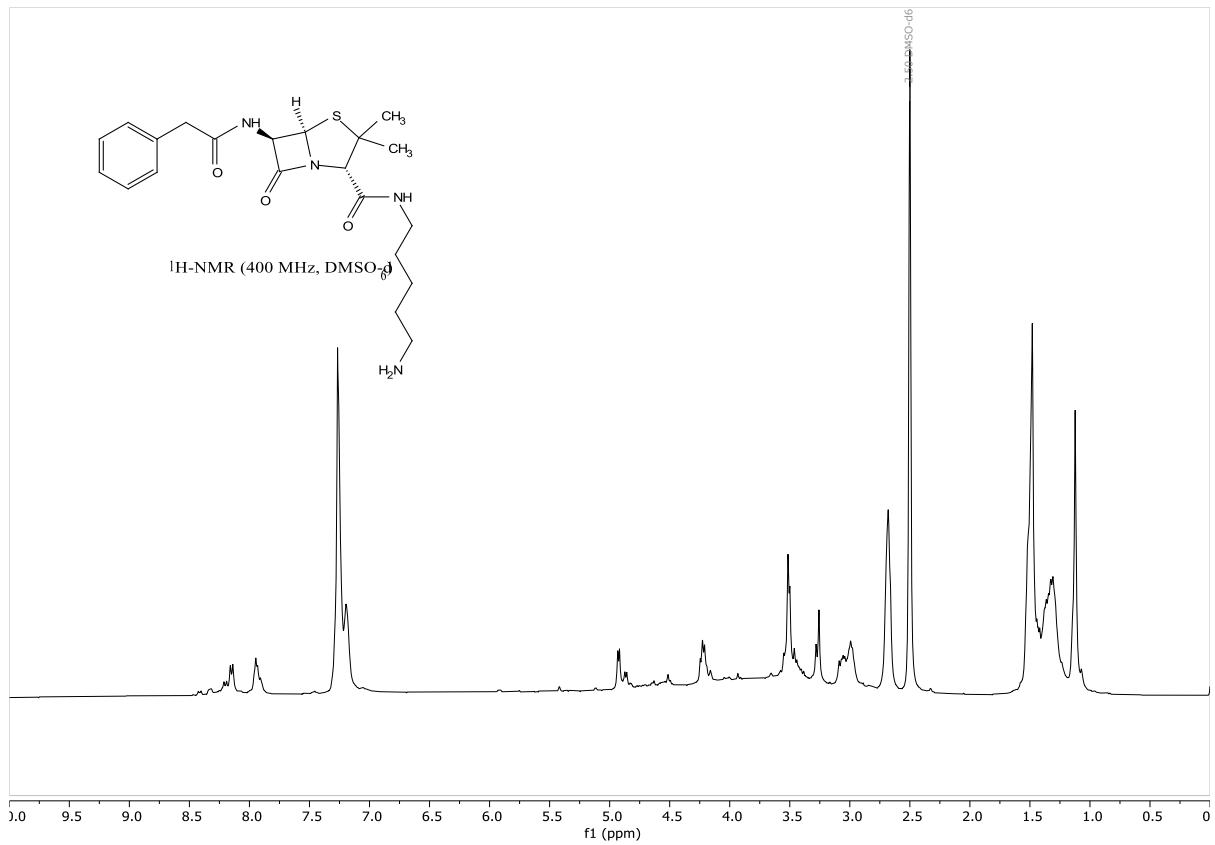




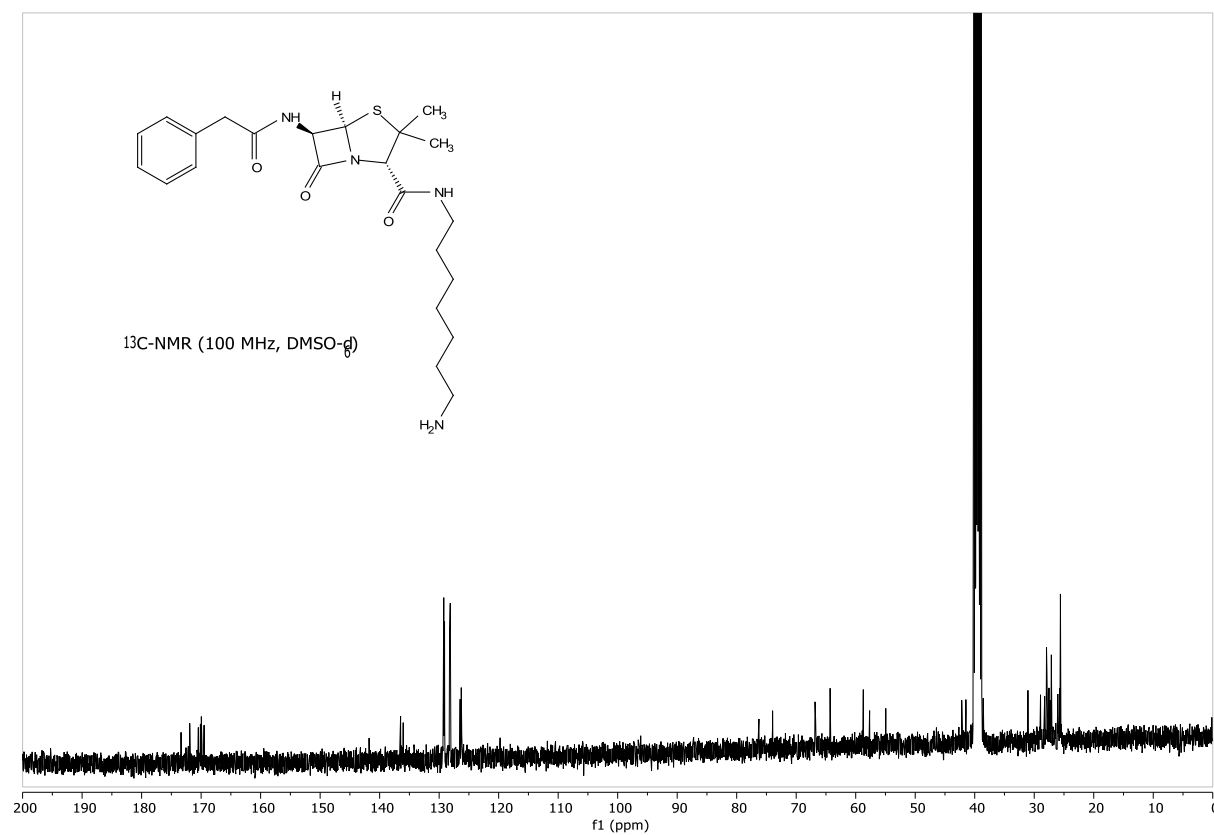
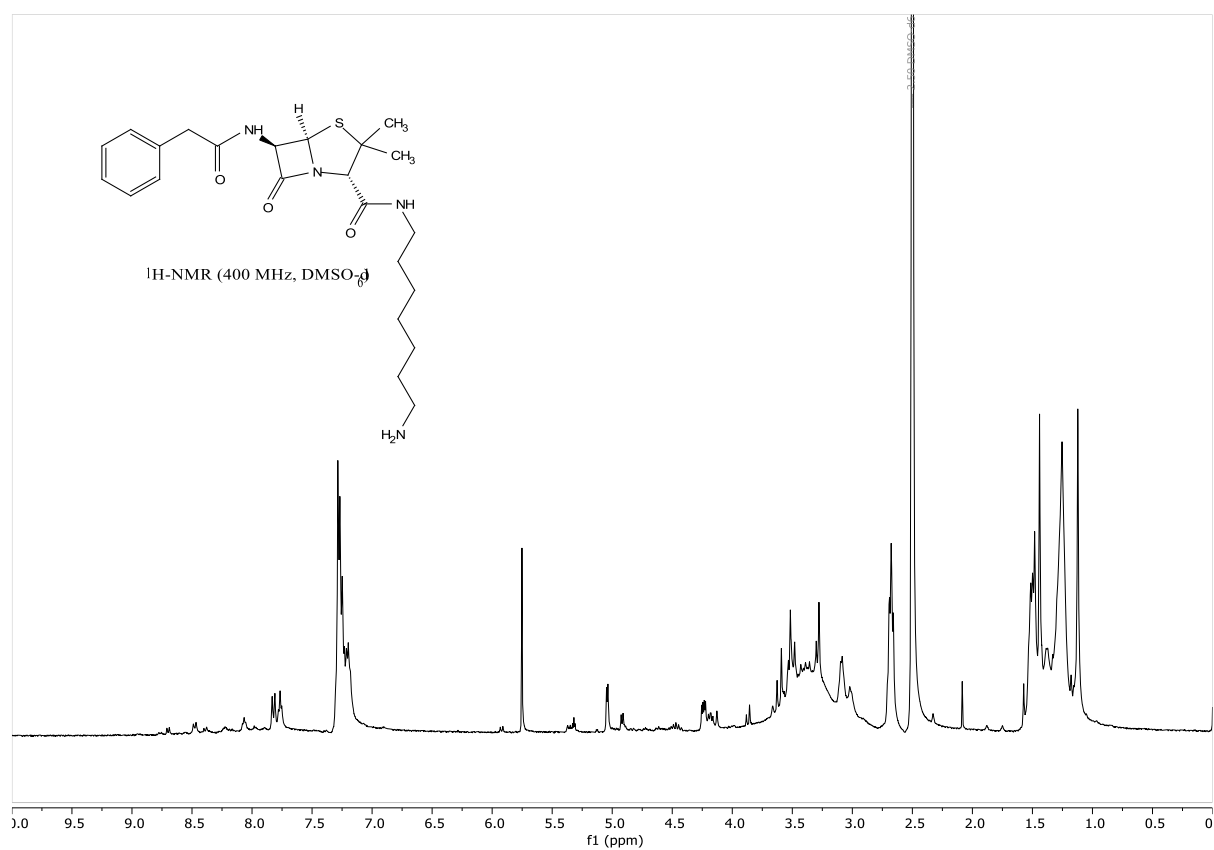
## Hapten 7



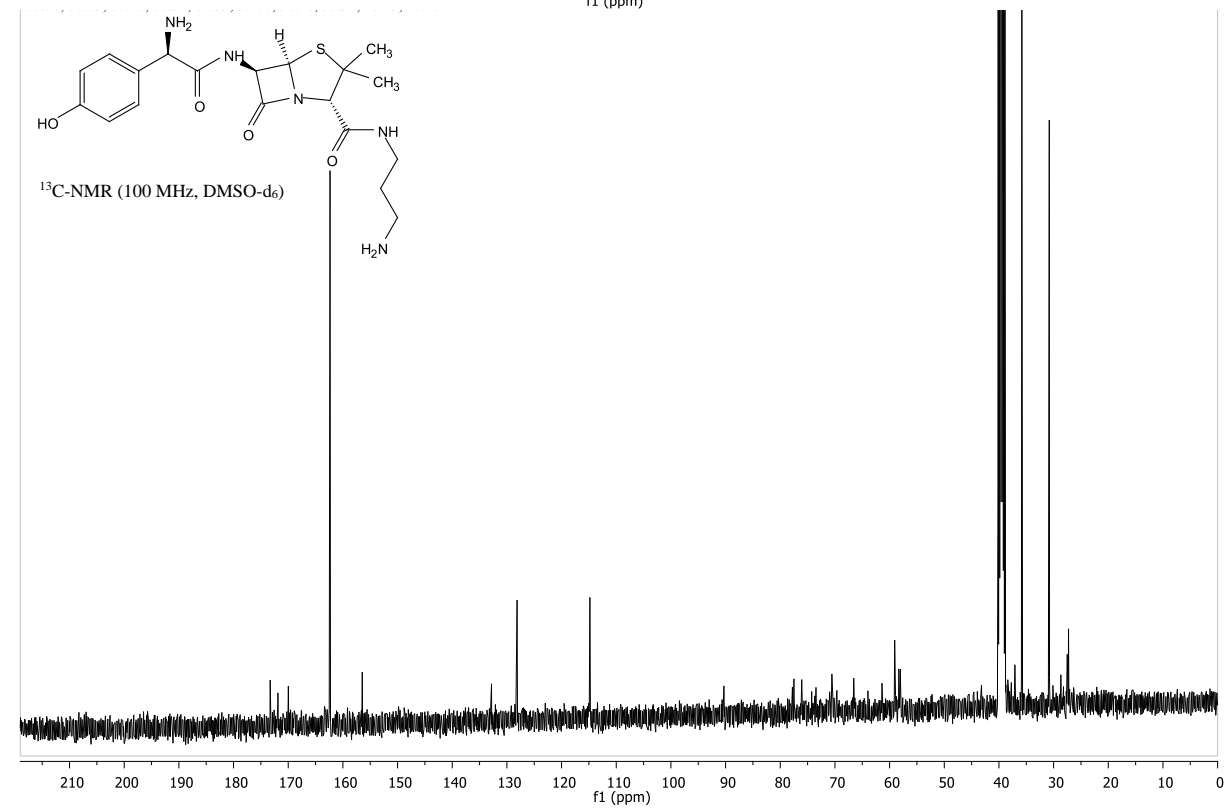
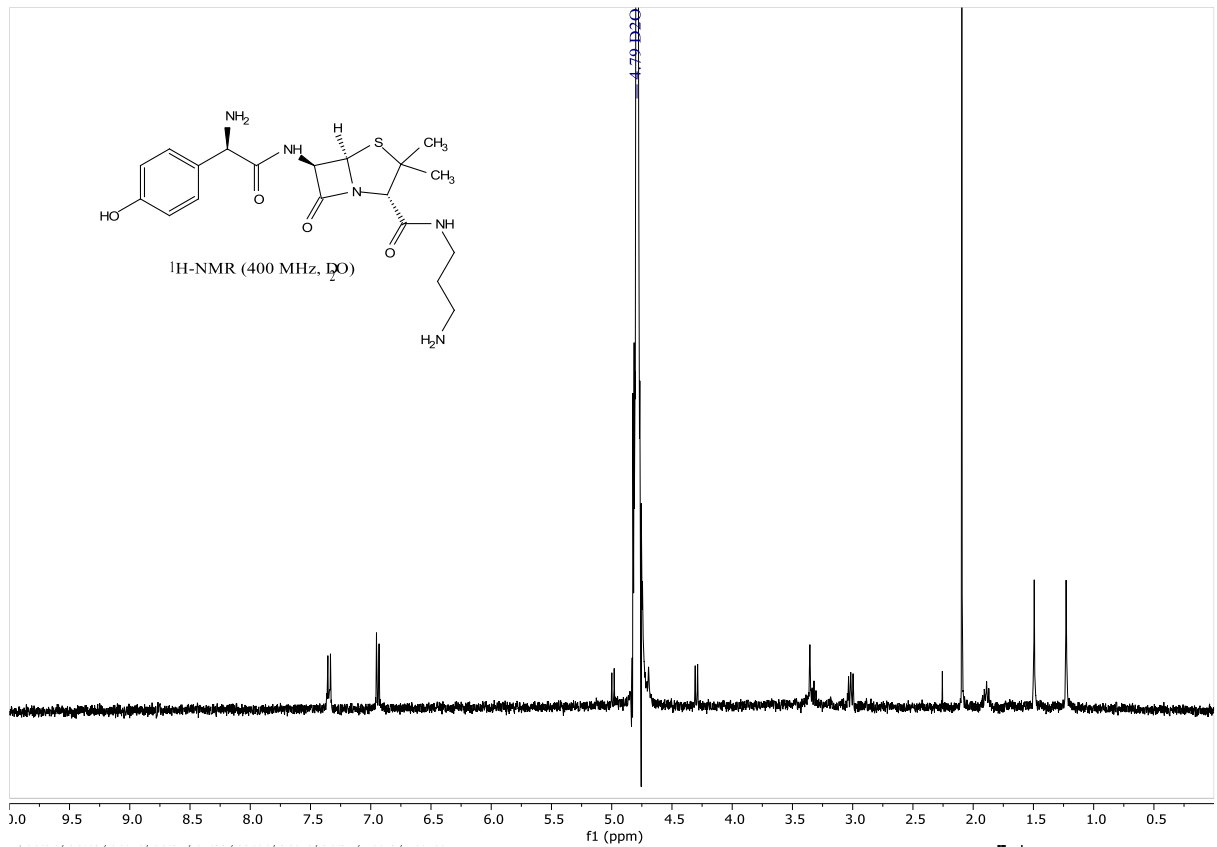
Hapten 8

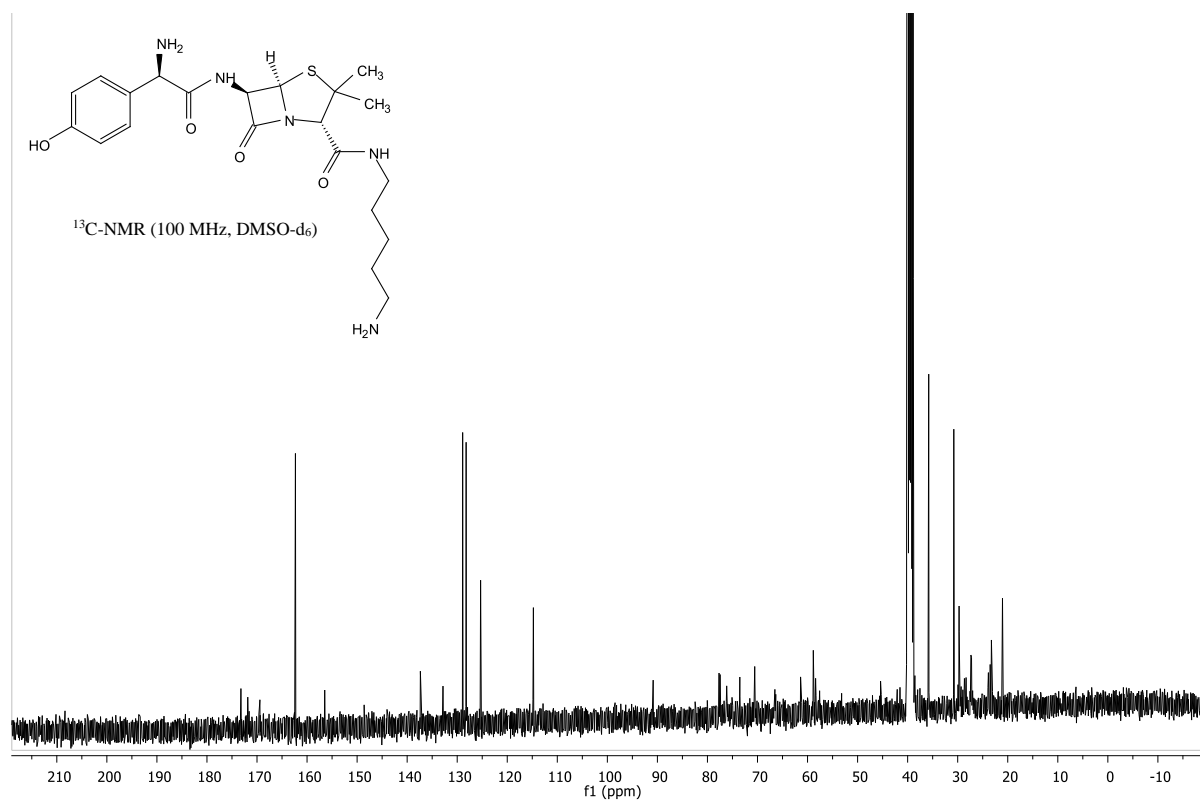
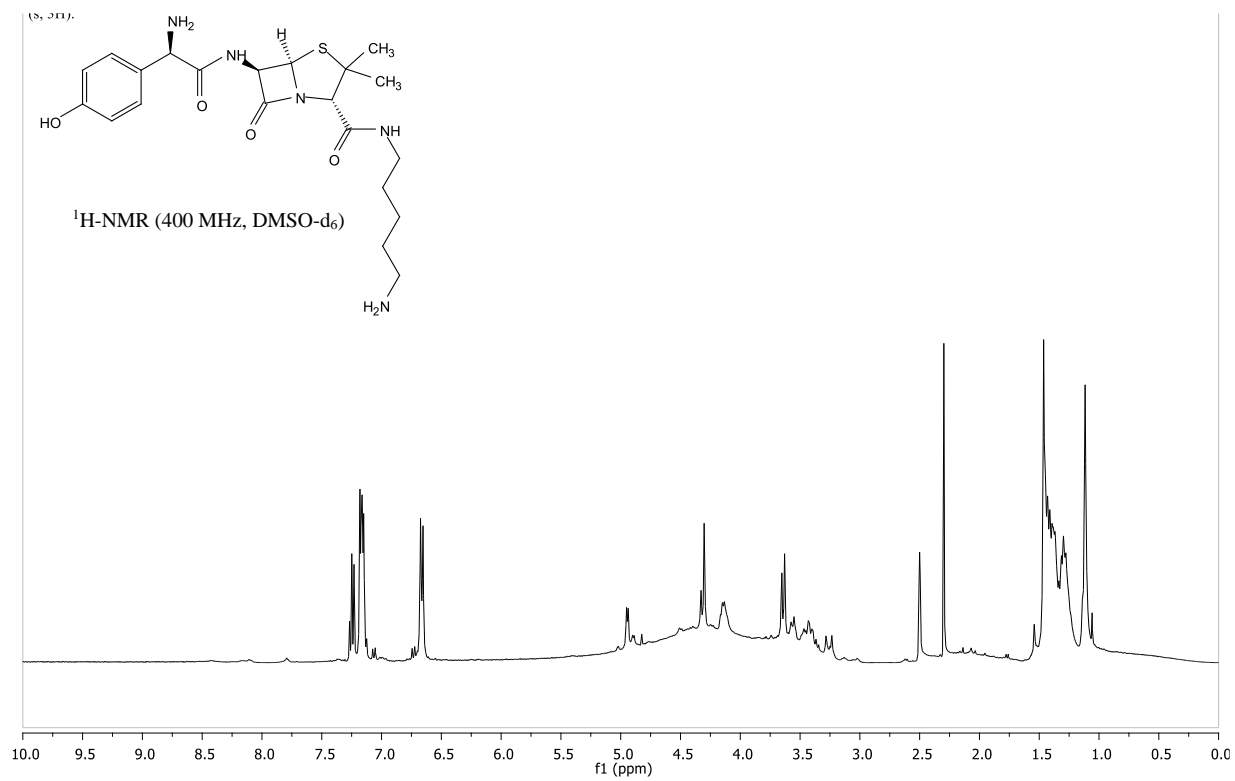


## Hapten 9

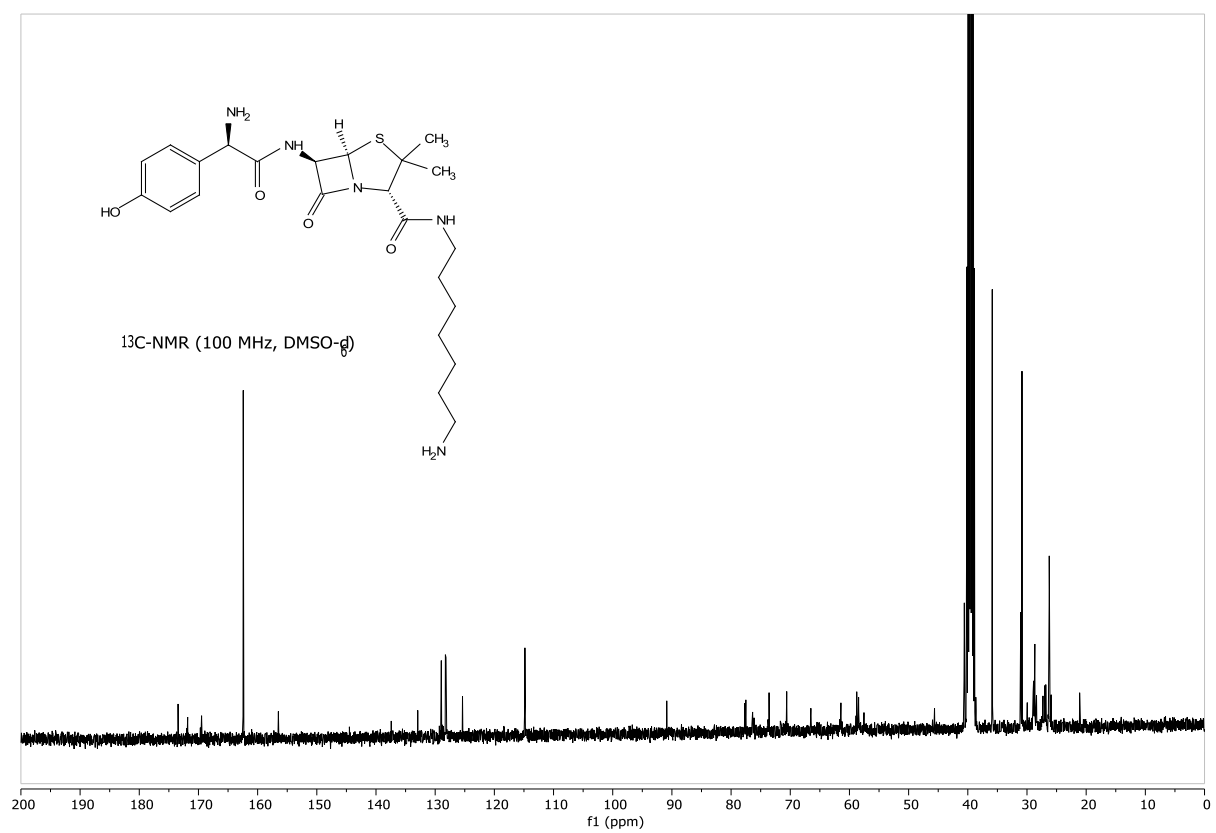
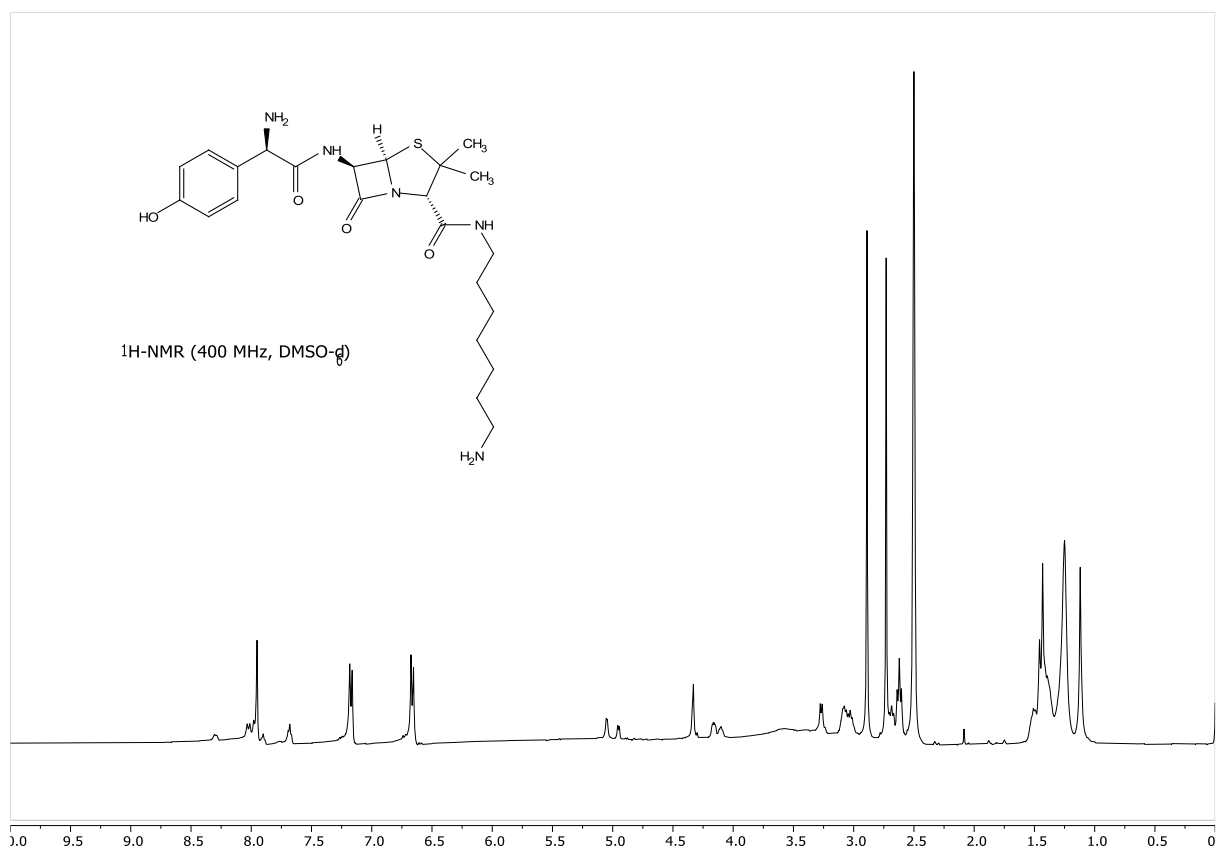


Hapten 10

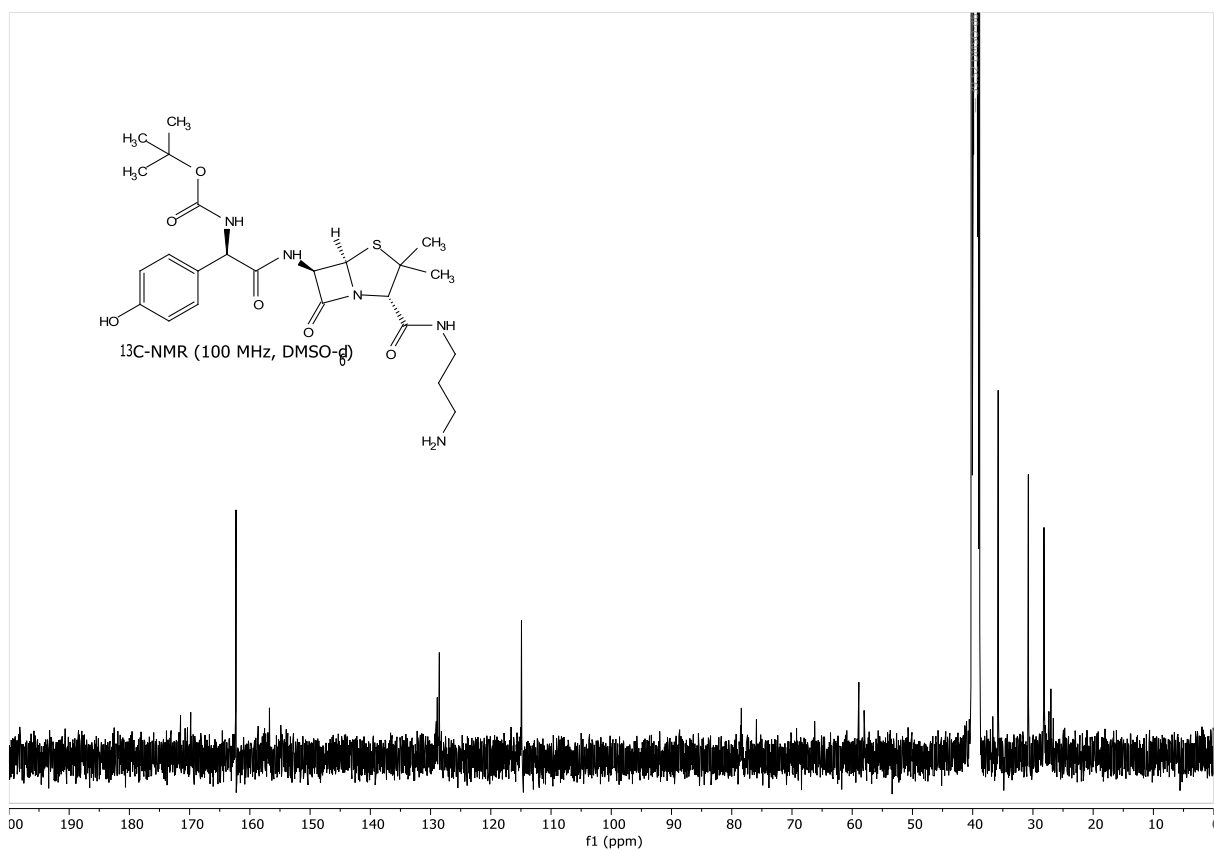
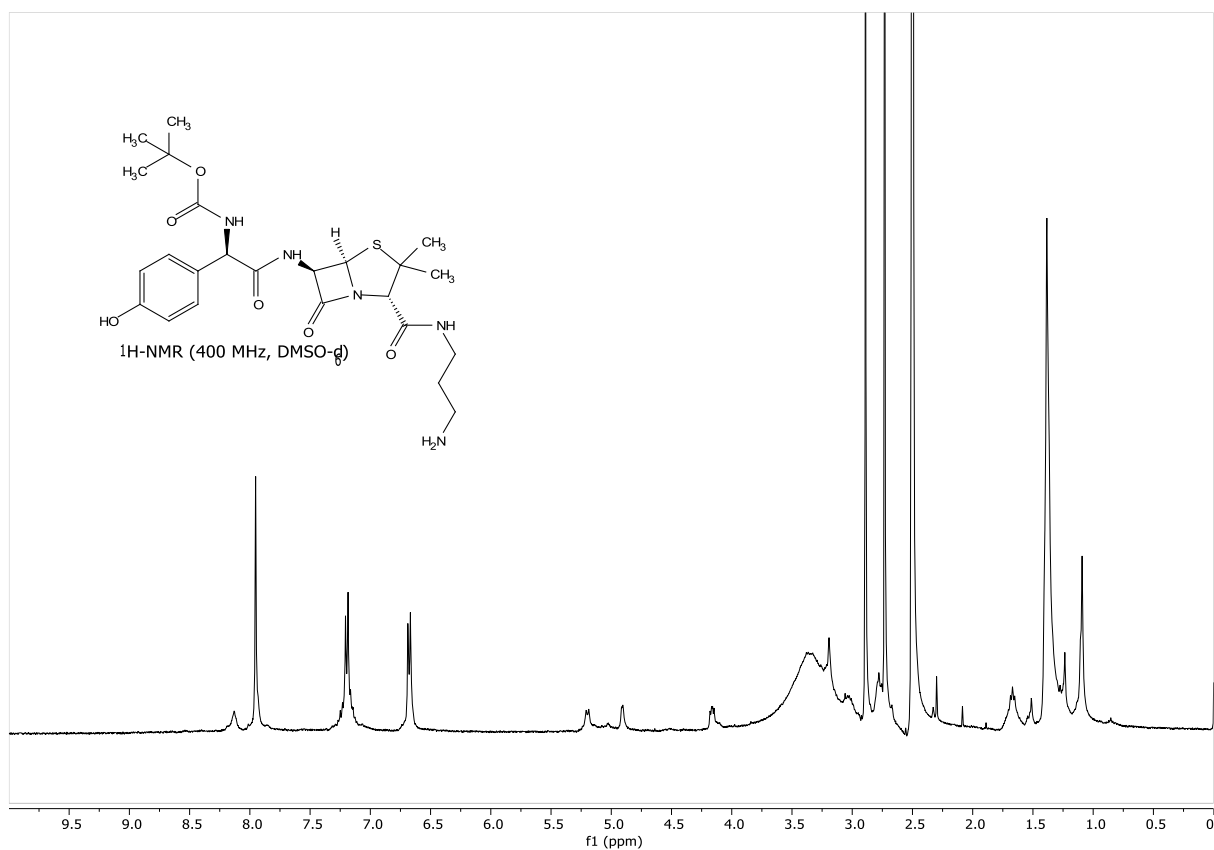


**Hapten 11**

## Hapten 12



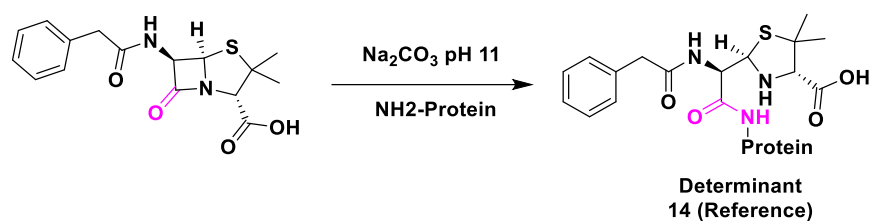
## Hapten 13



## 5. Preparation of the antigens

### Strategy I) Using synthesized haptens

In the case of the PG reference antigen (Scheme S1, entry **14**) and the negative control (AZT), the conjugation was performed through the lysine residues of the protein HSA by  $\beta$ -lactam ring opening, through amidation between the carbonyl carbon of the  $\beta$ -lactam ring and amino group of lysine residues (Scheme S2), as previously described,<sup>1</sup> with few modifications. Briefly, HSA (2.0 mg) dissolved in sodium carbonate 0.5 M, pH 11.0, reacted with PG or AZT (0.03 mmol, 1,000 equiv.) overnight at room temperature.



**Scheme 6.1.S2.** PG-oyl formation after  $\beta$ -lactam ring opening.

Modified -oyl and -anyl antigens were prepared following the carbodiimide chemistry. For that, HSA (2.0 mg) and the corresponding impure modified BLC hapten (4.0 mg) were dissolved in 200  $\mu\text{L}$  and 1 mL, respectively, of sodium phosphate buffer 0.1 M, NaCl 0.15 M, pH=7.2. Then, 500  $\mu\text{L}$  of the BLC hapten solution was mixed up in an Eppendorf with the HSA solution (200  $\mu\text{L}$ ) and EDC (10.0 mg) was added. The solution was allowed to react at room temperature for 2 hours.

All antigens were purified by gel filtration chromatography on Amicon Ultra 0.5 pre-concentrated 10 K filters using PBS 1X, pH 7.4, as elution buffer. Finally, they were diluted to 1.0 mg/mL and stored at  $-20\text{ }^\circ\text{C}$  until used. Protein quantification was performed by the Coomassie/Bradford colorimetric assay.<sup>2</sup> Molar ratios protein-hapten were determined by MS-MALDI-TOF.<sup>3</sup>



*Strategy II) Using cationized carrier molecules*

Diamine dihydrochloride salts used in this study were ethylene diamine, 1,4-diaminobutane and 1,4-phenylenediamine. Major antigens were produced with this strategy and the carrier proteins used were HSA and H1.

In order to cationize carrier proteins, 1.0 mmol of the corresponding diamine dihydrochloride salt (1 equiv) dissolved in 600  $\mu\text{L}$  of MES 0.1 M, pH 4.7 (coupling buffer) was mixed up with 2.0 mg of the carrier protein dissolved in 200  $\mu\text{L}$  of coupling buffer. Then, an EDC hydrochloride solution of 10 mg/mL in coupling buffer was prepared and 200  $\mu\text{L}$  of this solution was added to the reaction mixture. The solution was allowed to react overnight at room temperature. Cationized proteins were purified by gel filtration chromatography on 10 K precentred filters using sodium carbonate 0.5 M, pH 11.0, as the elution buffer for the following conjugation step.

Then, a solution of the corresponding BLC (1,000 equiv.) in sodium carbonate 0.5 M, pH 11.0, was added to the solutions. The reactions were allowed to stir at room temperature overnight. Purification and quantification of the protein-modified antigens were performed as explained in Strategy I.

## 6. MS-MALDI-TOF spectra

The molecular weight (MW) of each antigenic determinant was calculated from the peak centroid of the peaks according to the following equation:

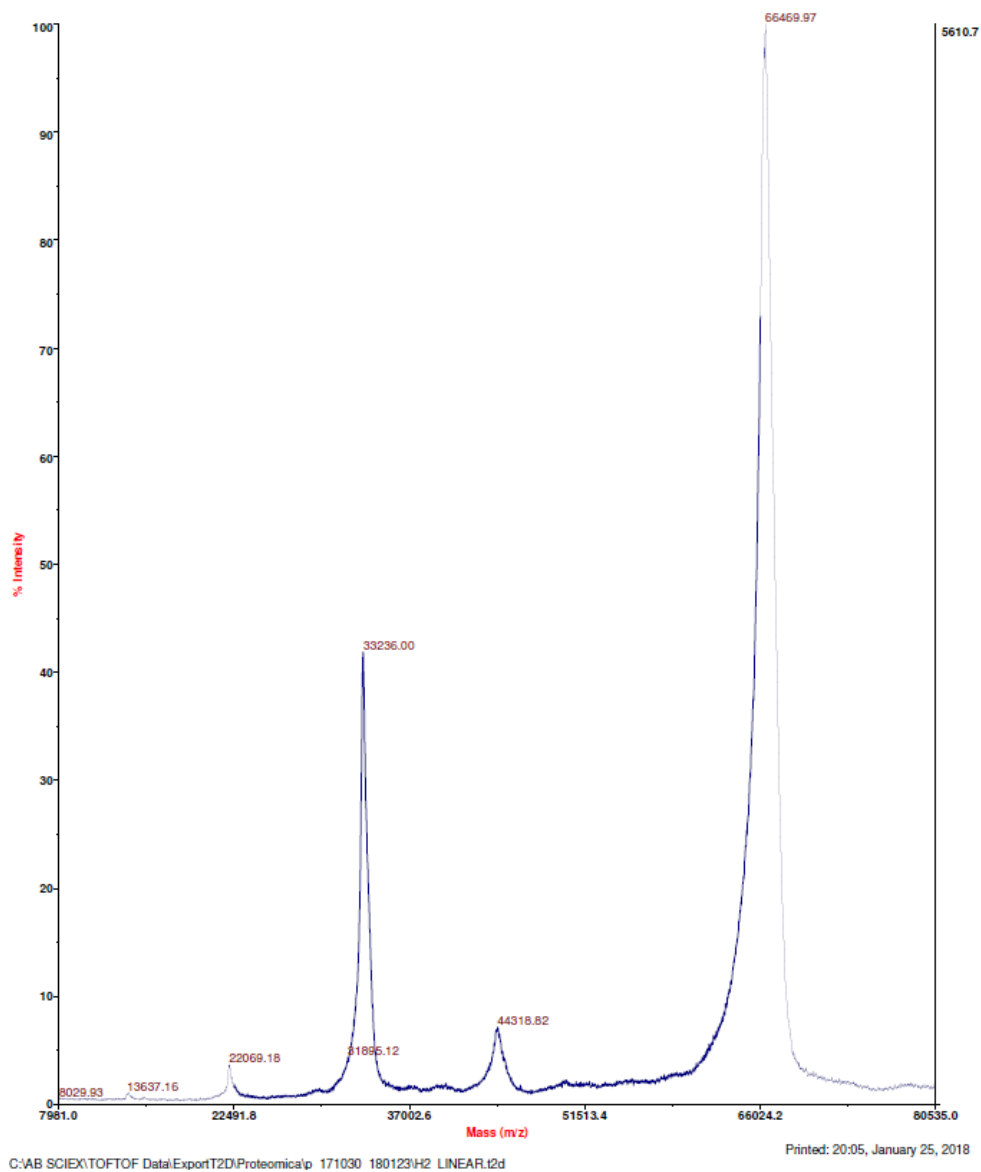
$$[\text{MW}(\text{determinant}) - \text{MW}(\text{protein})] / \text{MW}(\text{hapten}).$$

The incremental change in molecular weight due to incorporation of hapten molecules to protein corresponds to the number of hapten molecules per protein molecule.

*Control HSA*

AB Sciex TOF/TOF™ Series Explorer™ 72094

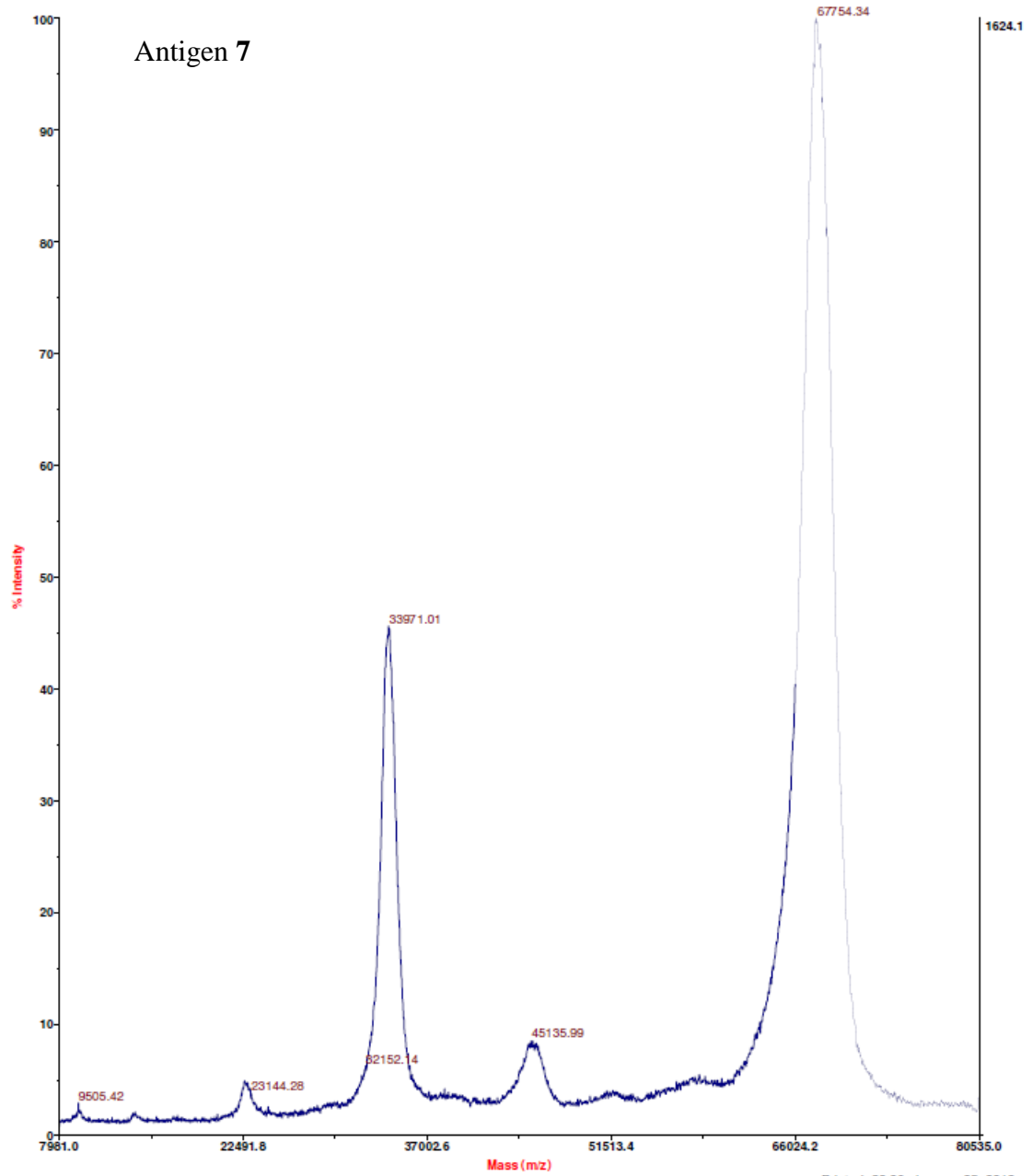
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PG antigens

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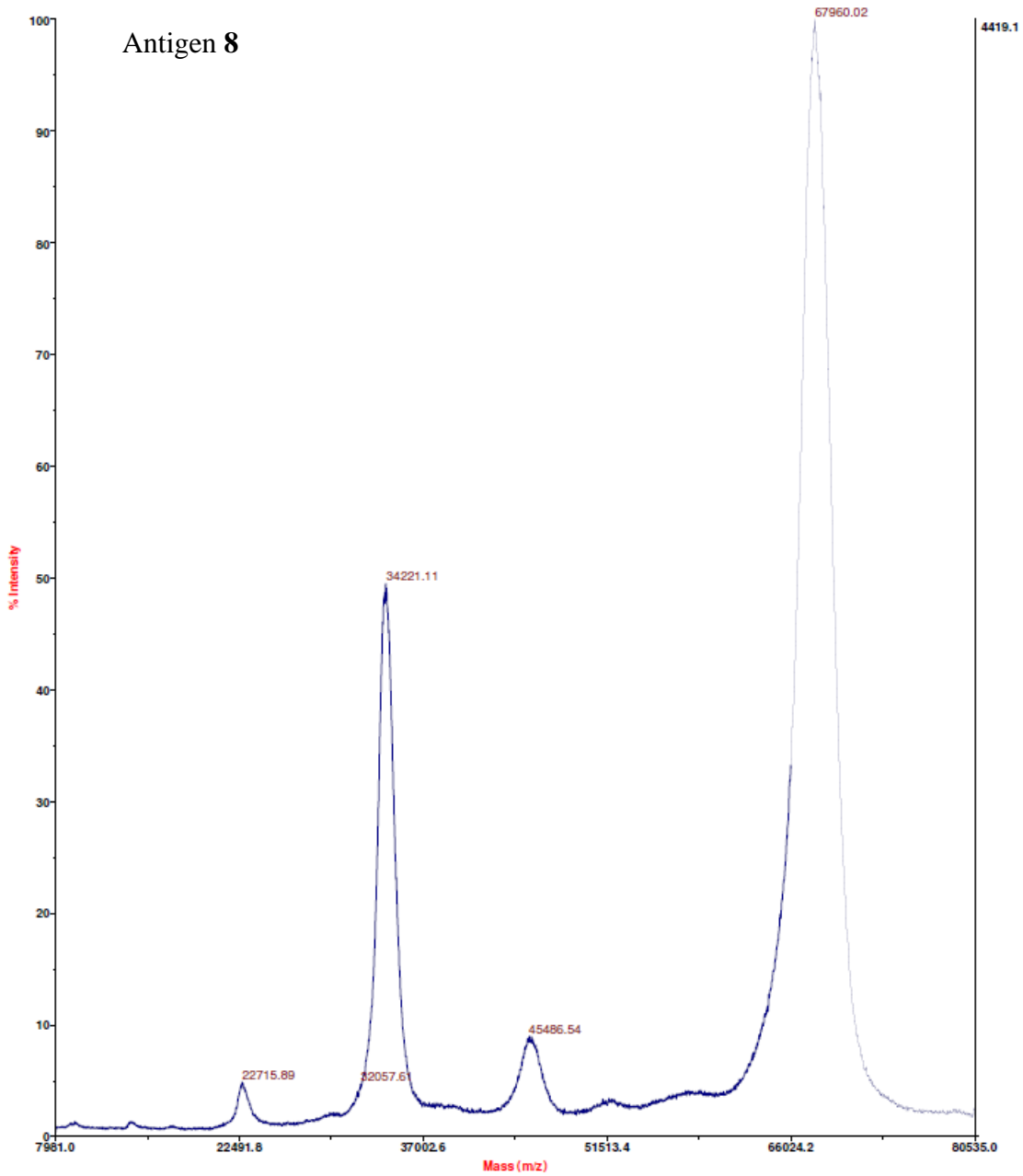
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AB Sciex TOF/TOF™ Series Explorer™ 72094

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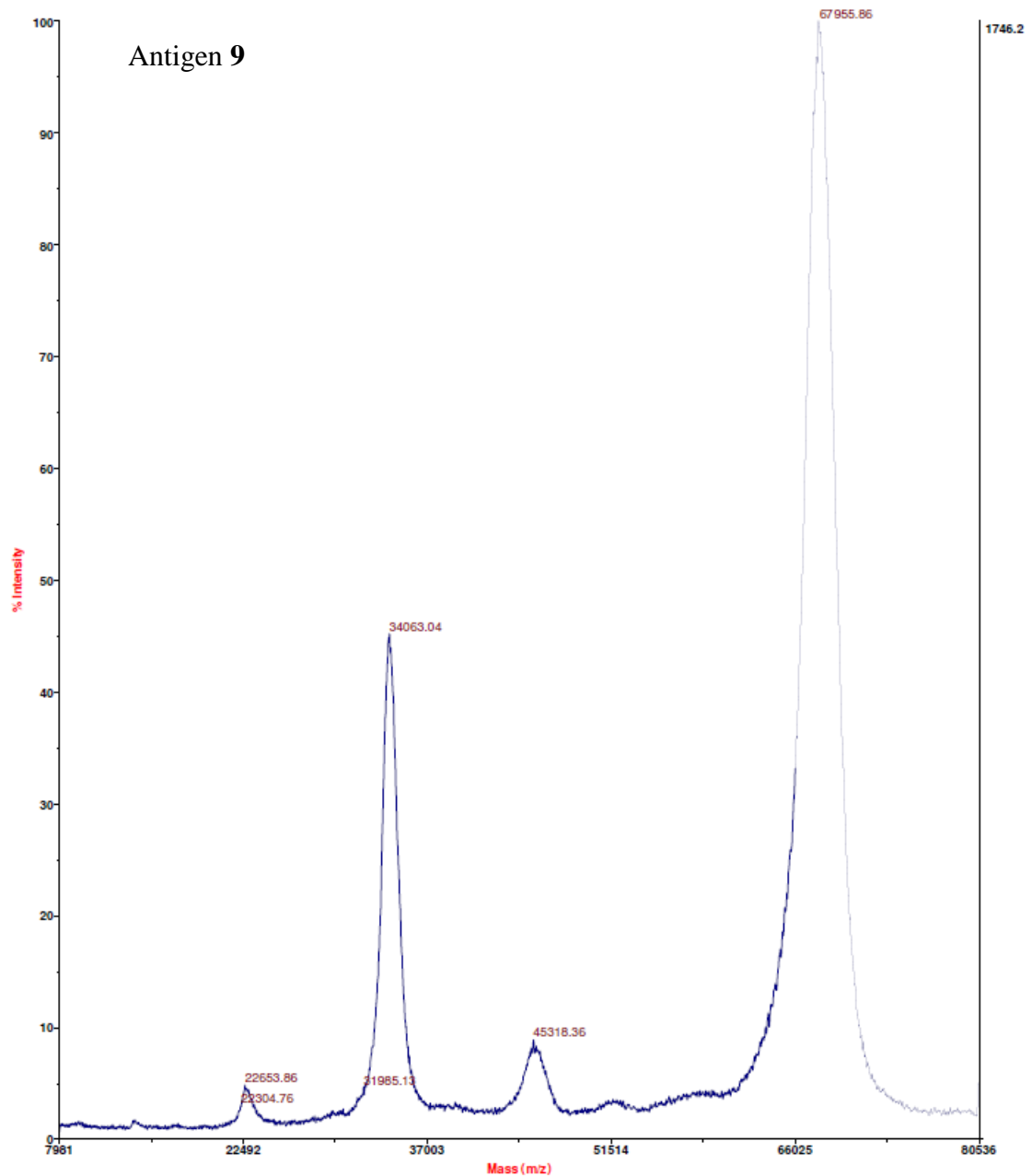


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AB Sciex TOF/TOF™ Series Explorer™ 72094

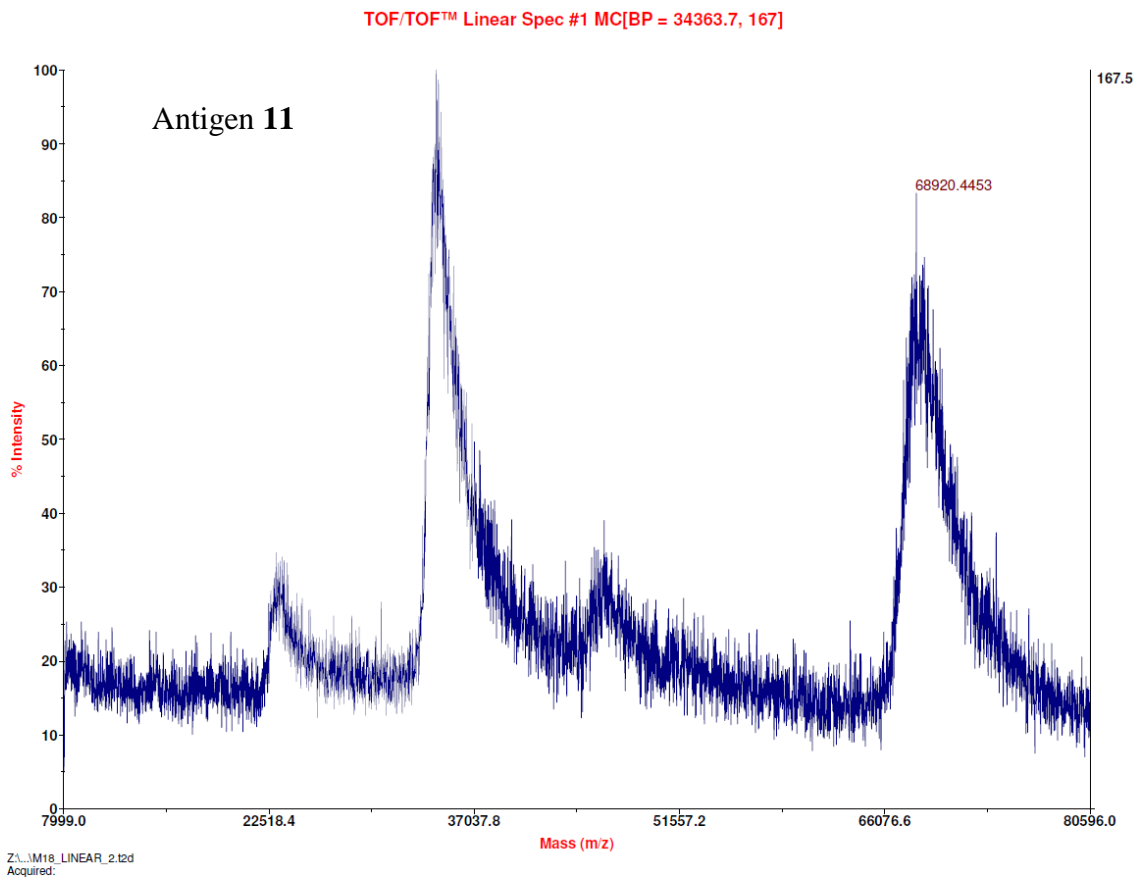
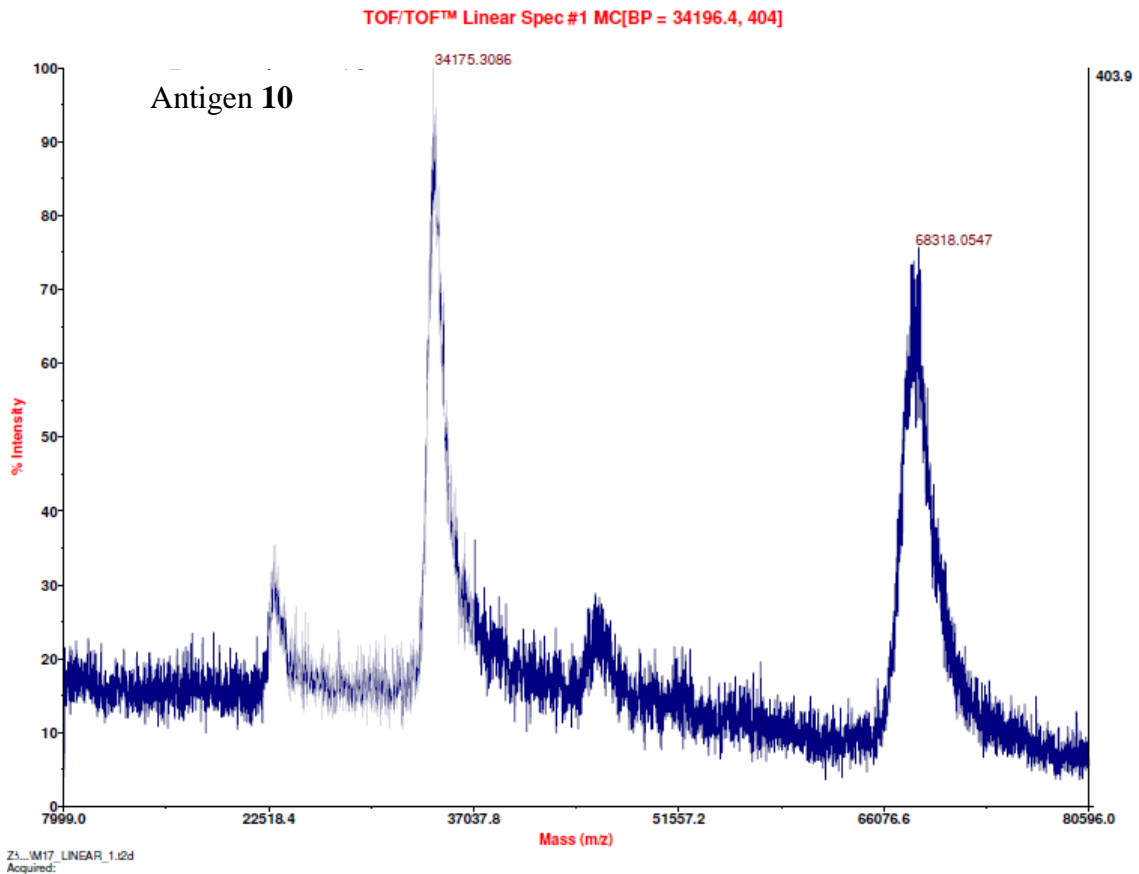
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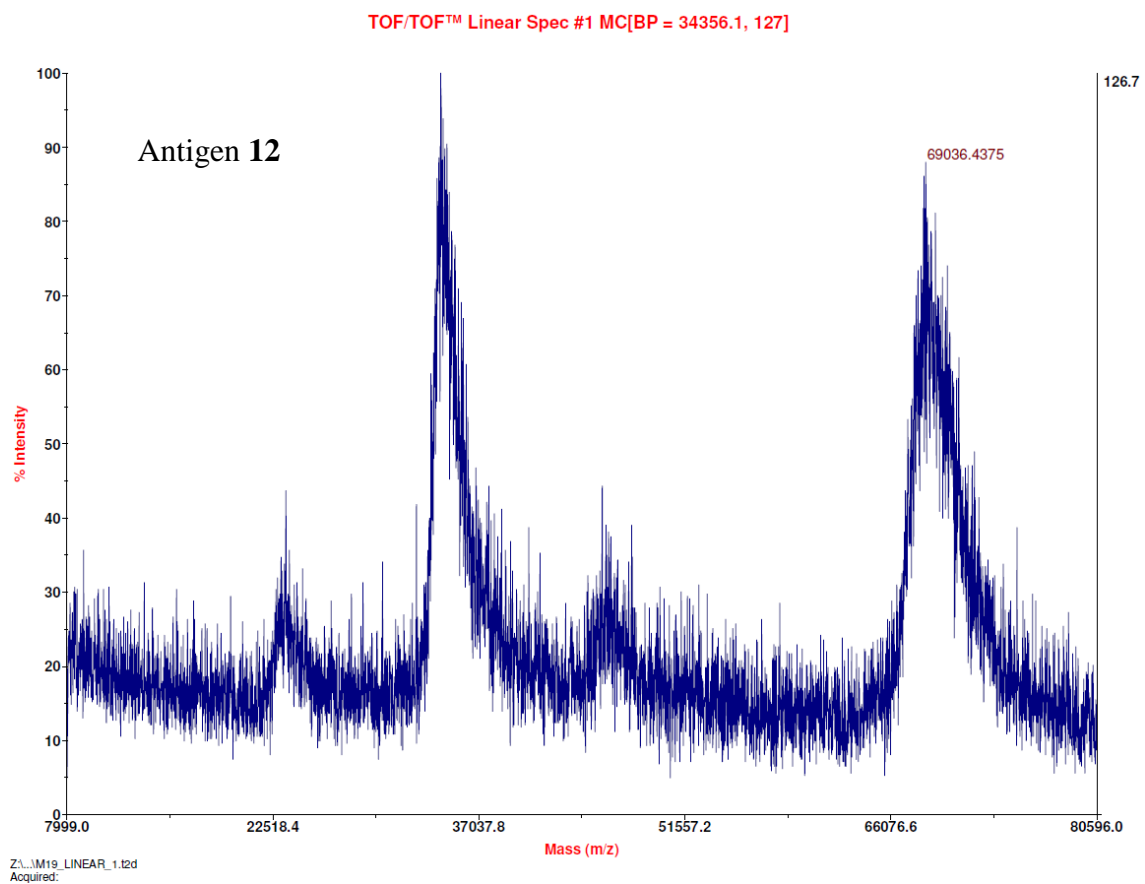


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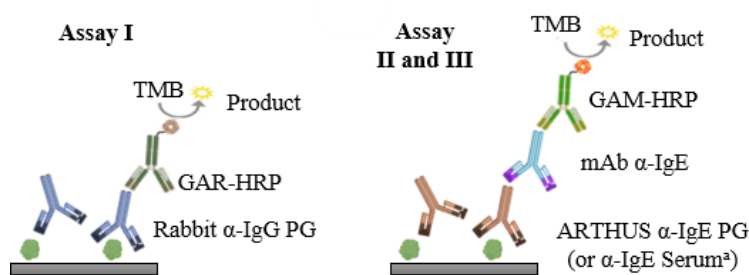
AMX antigens





## 7. Assay protocol for the evaluation of the structural antigens

Assays consist on the detection of specific IgG (Figure S1, assay I) and IgE (Figure S1, assays II and III) on standard DVDs (CD Rohling-up GmbH, Saarbrücken, Germany). For that, antigens (40  $\mu\text{g/mL}$ ) and controls (negative and positive), prepared in printing buffer, were spotted in microarray format (20 arrays per disk of  $4 \times 4$  spots, Figure S2a) by dispensing 25 nL of each one, using a noncontact printing device (AD 1500 BioDot, Inc., Irvine, CA). The spots were 500  $\mu\text{m}$  in diameter with a center-to-center distance of 1.0 mm. Within each microarray (Figure S2b-c), spots for each antigen (two replicates, position 3-8) and negative (HSA, position 1) and positive (rabbit IgG or human IgE, position 2) controls are included. After printing, the DVD was incubated for 16 h at 37  $^{\circ}\text{C}$ .



**Figure 6.1.S1.** Scheme of the microimmunoassays I, II and III based on a direct format with colorimetric detection. <sup>a</sup> $\alpha$ -IgE serum from allergic patients and controls was used in assay III.



**Figure 6.1.S2.** A) Image of the array on the DVD surface; B) Lay-out of the antigens and controls printed on the DVD (Position 1: HSA, negative control, C(-); 2: human IgE, positive control, C(+); 3: Aztreonam-oyl antigen, negative control C(-); 4-7: antigen 1-4; 8: Reference antigen); C) Representative image of the array with specific antigens and controls printed on the DVD. Artificial human serum specific to PG was used.

For the detection of specific IgG to penicillins, different dilutions (1/1,000-1/32,000) of rabbit sera and control (PBS-T) (25  $\mu\text{L}$  per sample) were added to each array and incubated for 15 min. Then, the DVD was washed with PBS-T and water and 25  $\mu\text{L}$



of the polyclonal secondary antibody GAR-HRP in PBS-T buffer (dilution 1/400) was added for 15 min followed by the washing step. For the detection of specific IgE, 25  $\mu$ L of sample was added to each array. Samples were incubated for 15 min when artificial human serum was used,<sup>4</sup> while serum samples from allergic patients and controls were incubated for 30 min. After washing, 25  $\mu$ L of mAb-IgE in PBS-T buffer (1.0  $\mu$ g/mL) was added and incubated for 15 min. After washing as before, 25  $\mu$ L of a 1/100 dilution of GAM-HRP was added for 15 min. Finally, immunoreaction was developed in all immunoassays by homogeneously dispensing 1.0 mL of TMB along the whole disc surface. The reaction was stopped by washing the disk with water after 15 min. Signals were read by a modified DVD drive and the data was analyzed as previously described.<sup>5,6</sup> All experiments were repeated 3 times.

BLC-specific IgE levels expressed as units of specific IgE (IU/mL) were determined, using the 3<sup>rd</sup> WHO standard for total serum IgE content involving heterologous interpolation. The calibration curve was built performing a sandwich immunoassay where 3<sup>rd</sup> WHO International Standard was used as calibrator, and Omalizumab as the capture antibody.<sup>7</sup> All the other immunoreagents were the same as used for the determination of specific IgE (assays II and III). The standard data points, signal versus semi-log concentration, were the mean of 10 curves performed on different disks during several days. A four-parameter logistic (4PL) curve was fitted through the points, using SigmaPlot 11 software.

Concentrations of specific IgE were calculated from the calibration curve for total IgE. Statistical significances between the obtained IgE concentrations for the diamine-derived antigens and the reference were determined by the Holm-Sidak method using the SigmaPlot 11 software and P values of < 0.001 were considered significant.

## 8. Patients

The study of the reactivity of the prepared antigens towards the sera from allergic patients included patients (I) whose culprit drug was PG, AMX or augmentin and (II) whose culprit drug was another BLC. A cohort of 35 subjects with negative skin test to BLCs and with good tolerance to them were used as controls. Clinical characteristics from the 35 patients included in the study are shown in Supplementary Table S1. All samples from patients and controls were kindly provided by the Hospital Universitari i Politènic La Fe, Valencia, Spain, and informed consent for the diagnostic procedures was obtained from all patients. Patients were diagnosed following the procedure described in the European Network of Drug Allergy (ENDA) protocol based on skin testing, *in vitro* tests or drug provocation test, when necessary. This study was approved by the Hospital Universitari i Politènic La Fe ethical review committee. All experiments were performed in accordance with the relevant guidelines and regulations.

**Table 6.1.S1.** Clinical characteristics of the allergic patients.

<b>Patient</b>	<b>Sex<sup>[a]</sup></b>	<b>Year birth</b>	<b>Clinical manifestation</b>	<b>Clinical Entity</b>	<b>Time</b>
01	M	1969	Augmentin	Anaphylaxis	Immediate
02	F	1978	Augmentin	Anaphylaxis	Immediate
03	M	1936	Augmentin	Anaphylaxis	Immediate
04	F	1939	Cefazolin	Anaphylaxis	Immediate
05	M	1949	Augmentin	Cutaneous	Delayed
06	F	1948	Augmentin	Anaphylaxis	Immediate
07	F	1967	Amoxicillin	Anaphylaxis	Immediate
08	F	1960	Augmentin	Anaphylaxis	Immediate
09	M	1956	Augmentin	Cutaneous	Delayed
10	F	1982	Cefuroxime	Anaphylaxis	Immediate
11	M	1972	Augmentin	Cutaneous	Delayed
12	F	1964	Augmentin	Cutaneous	Immediate
13	M	1978	Amoxicillin	Cutaneous	NR <sup>[b]</sup>
14	F	1961	Augmentin	Cutaneous	Immediate
15	F	1948	Amoxicillin	Inespecific	Immediate
16	F	1946	Penicillin G	Inespecific	Delayed
17	M	1977	Penicillin G	Cutaneous	Delayed
18	M	1953	Augmentin	Cutaneous	Immediate
19	M	1951	Amoxicillin	Cutaneous	Immediate
20	M	1964	Augmentin	Anaphylaxis	Immediate
21	M	1976	Amoxicillin	Cutaneous	Immediate
22	M	1961	Amoxicillin	Anaphylaxis	Immediate
23	M	1968	Amoxicillin	Cutaneous	NR
24	M	1965	Augmentin	Anaphylaxis	Delayed
25	M	1982	Amoxicillin	Cutaneous	Delayed
26	M	1982	Amoxicillin	Anaphylaxis	Immediate
27	F	1970	Piperacillin	Cutaneous	Immediate
28	F	1951	Amoxicillin	Cutaneous	Immediate
29	F	1982	Augmentin	Cutaneous	Immediate
30	F	1984	Augmentin	Cutaneous	Delayed
31	F	1963	Augmentin	Cutaneous	NR
32	F	1972	Augmentin	Anaphylaxis	Immediate
33	F	1962	Cefuroxime	Cutaneous	Delayed
34	M	1963	Amoxicillin	Cutaneous	Immediate
35	M	1975	Augmentin	Anaphylaxis	NR

[a]Sex: F=Female, M=Male; [b]NR=No reported in the clinical history

**Table 6.1.S2.** Results of the analysis of human serum samples

Patient No	Prick-test <sup>[a]</sup>		ImmunoCAP (in IU/mL <sup>[b]</sup> )		Multiplex Immunoassay <sup>[c]</sup> (in IU/mL)			
					Antigen No			
	PG	AMX	PG	AMX	PG	AMX		
					1b	3b	4b	5b
01	N	N	13.60	10.20	0.23	0.48	<LOD <sup>[e]</sup>	<LOD
02	N	P	0.03	0.16	0.34	0.23	0.10	0.09
03	N	P	2.16	1.64	3.65	0.20	0.07	<LOD
04	N	P	0.00	0.82	<LOD	0.44	<LOD	<LOD
05	NR	NR	2.14	1.01	0.66	0.18	0.07	<LOD
06	N	P	1.83	0.91	3.47	<LOD	<LOD	<LOD
07	NR	NR	1.02	0.79	1.11	0.21	0.10	0.08
08	N	P	0.08	0.13	0.15	0.16	<LOD	<LOD
09	N	N	0.00	0.95	<LOD	2.75	<LOD	<LOD
10	N	P	0.09	0.74	<LOD	<LOD	<LOD	<LOD
11	N	P	0.01	0.03	<LOD	<LOD		
12	N	P	0.02	0.04	<LOD	0.20		
13	N	P	0.00	0.15	0.11	<LOD		
14	N	P	0.12	0.29	<LOD	<LOD	<LOD	0.28
15	N	N	0.03	0.07	0.18	<LOD		
16	N	N	0.01	0.03	0.16	<LOD		
17	N	N	0.04	0.06	<LOD	<LOD		
18	N	P	0.30	0.24	<LOD	<LOD	<LOD	<LOD
19	NR	N	0.02	0.12	0.16	<LOD		
20	P	NR	0.03	0.08	0.14	0.21		
21	N	P	0.17	0.46	0.08	<LOD		
22	P	P	0.00	0.03	<LOD	<LOD		
23	P	P	0.00	0.09	<LOD	<LOD		
24	N	P	0.17	0.33	0.21	<LOD		
25	N	P	0.03	0.24	0.20	<LOD	<LOD	<LOD
26	N	N	0.43	0.99	0.62	<LOD	<LOD	<LOD
27	N	N	0.00	0.00	0.46	<LOD		
28	N	P	0.00	0.00	0.25	<LOD		
29	NR	NR	3.01	7.02	2.62	1.39	0.23	0.40
30	N	N	0.05	0.41	<LOD	<LOD	<LOD	<LOD
31	N	P	0.00	0.01	0.15	<LOD		
32	P	P	0.03	0.24	<LOD	<LOD		
33	N	N	0.00	0.01	0.49	<LOD		
34	N	P	0.01	0.69	<LOD	<LOD	<LOD	<LOD
35	N	P	0.08	0.09	<LOD	<LOD	<LOD	0.07

Immunoassay values are the mean of at least three replicates and relative standard deviation (RSD) ranged from 4 to 13 %. [a] P= Positive, N= Negative, NR=Not Realized; [b] IU/mL= 2.4 ng/mL; [c]: Multiplex-DVD assay, using H1-oyl antigens; [d] Augmentin is a combination of AMX and potassium clavulanate; [e] <LOD= Value below the limit of detection (LOD).

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## 6.2. Annex 2

### Supplementary Information

#### Penicillin antigens based on metabolites for *in vitro* drug allergy testing

#### Experimental section

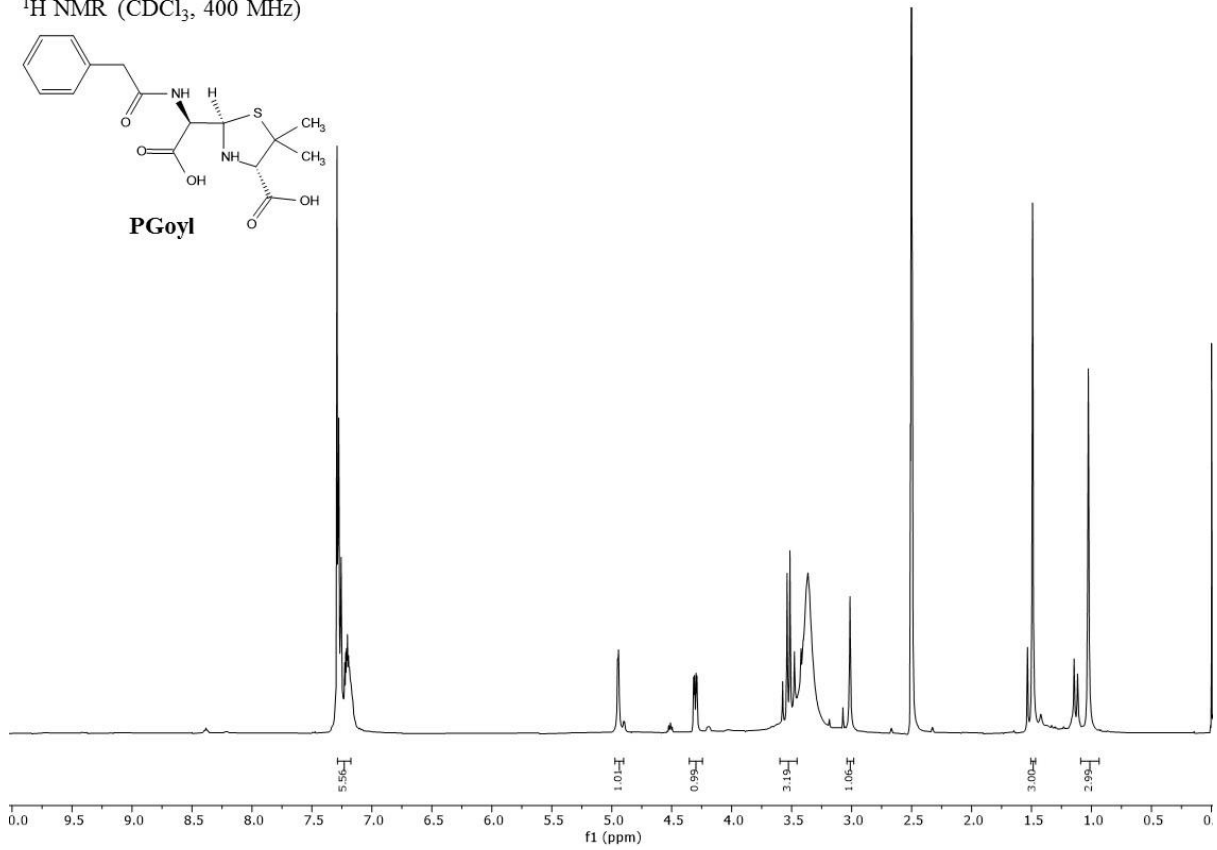
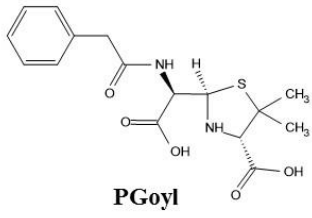
**Table 6.2.S1.** Clinical characteristics of the allergic patients

Patient Number	Sex <sup>[a]</sup>	Age (Years)	Culprit Drug	Clinical Manifestation	Route	Time
1	M	68	Augmentin <sup>[b]</sup>	Anaphylaxis	Parenteral	Immediate
2	F	72	Cefuroxime	Anaphylaxis	Oral	Immediate
3	F	37	Augmentin	Anaphylaxis	Oral	Immediate
4	F	77	Cefazolin	Anaphylaxis	Parenteral	Immediate
5	M	57	Augmentin	Anaphylaxis	Oral	Immediate
6	M	67	Augmentin	Cutaneous	Oral	Delayed
7	F	68	Augmentin	Anaphylaxis	Parenteral	Immediate
8	F	49	Amoxicillin	Anaphylaxis	Oral	Immediate
9	F	52	Augmentin	Anaphylaxis	Oral	Immediate
10	M	61	Augmentin	Cutaneous	Oral	Delayed
11	F	56	Augmentin	Cutaneous	Oral	Immediate
12	M	64	Augmentin	Cutaneous	Parenteral	Immediate
13	M	53	Augmentin	Anaphylaxis	Oral	Immediate
14	M	41	Amoxicillin	Cutaneous	Oral	Immediate
15	M	35	Amoxicillin	Anaphylaxis	Oral	Immediate
16	F	34	Augmentin	Cutaneous	Oral	Delayed
17	F	24	Augmentin	Anaphylaxis	Oral	Immediate
18	M	39	Augmentin	Cutaneous	Oral	Immediate

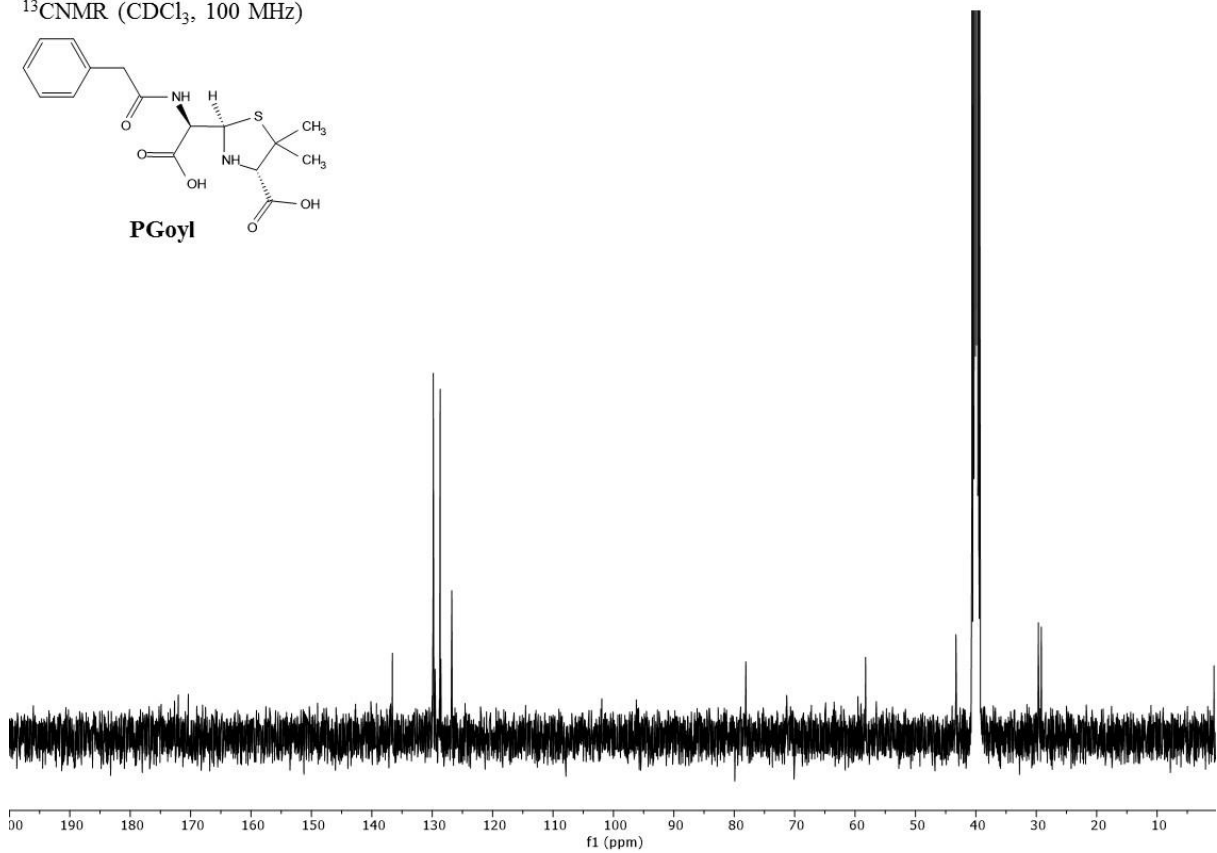
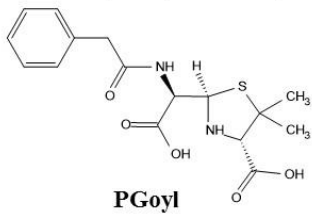
[a] F: female, M: male; [b] Augmentin is a combination of amoxicillin and potassium clavulanate

Annex 2

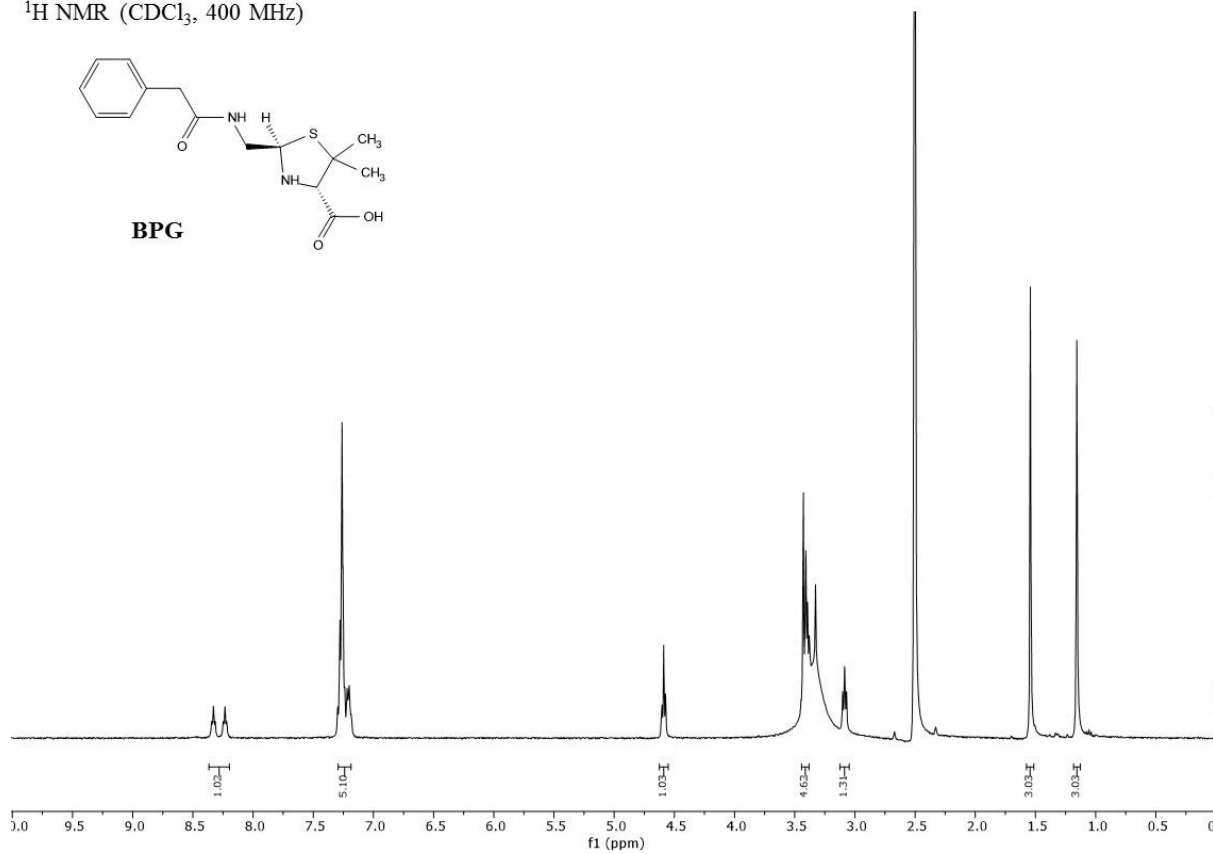
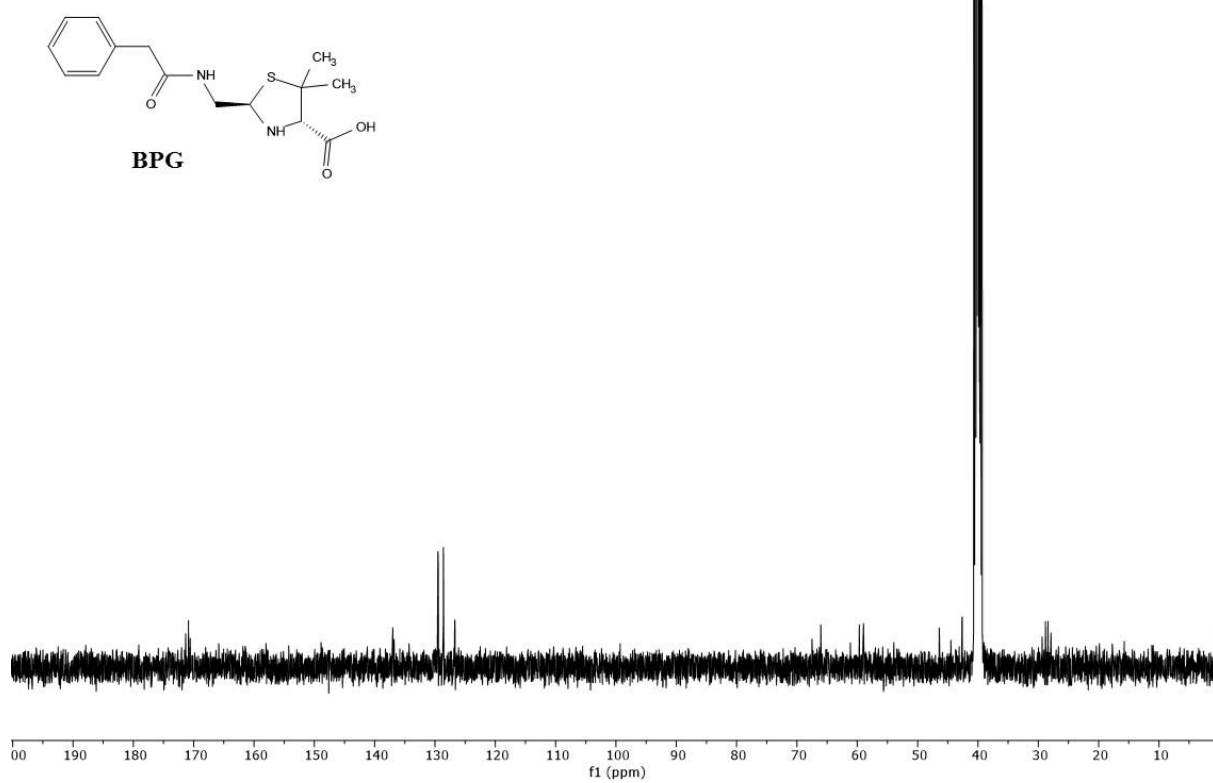
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)





$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)



## 6.3. Annex 3

### Supplementary information

#### Neo-antigens for the serological diagnosis of IgE-mediated drug allergic reactions to antibiotics cephalosporin, carbapenem and monobactam

Edurne Peña-Mendizabal, Sergi Morais and Ángel Maquieira

#### Experimental section

##### 1. Instrumental methods

NMR Spectra:  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra were recorded on Bruker AVIIIHD NanoBay 400 MHz spectrometer using TOPSPIN software. Proton and carbon chemical shifts ( $\delta\text{H}$ ,  $\delta\text{C}$ ) are quoted in ppm and referenced to tetramethylsilane with residual protonated solvent as the internal standard. Resonances are described using the following abbreviations; s (singlet), d (doublet), t (triplet), q (quartet), quin. (quintet), m (multiplet), br (broad), app (apparent), dd (doublet of doublets), etc. Coupling constants (J) are given in Hz and are rounded to the nearest 0.1 Hz.

Mass Spectra: High resolution mass spectra were recorded using an AB SCIEX Triple TOF<sup>TM</sup> 5600 LC/MS/MS System. The LC system used was an Agilent 1290 HPLC system. The analyses were performed using an Agilent EC-C<sub>18</sub>. The data acquisition used are in positive mode, over a mass range of 100 – 950 m/z. The MS was using an IDA acquisition method with the survey scan type (TOF-MS) and the dependent scan type (product ion) using 35 V of collision energy. Data was evaluated using the qualitatively evaluated using the PeakView<sup>TM</sup> software. m/z values are reported in Daltons; high resolution values are calculated to four decimal places from the molecular formula, all found values being within a tolerance of 5 ppm.

Chromatography techniques: TLC was performed on Merck Glass TLC silica gel 60 F<sub>254</sub> 0.2 mm precoated plates and visualised using ultraviolet light and potassium permanganate stain.

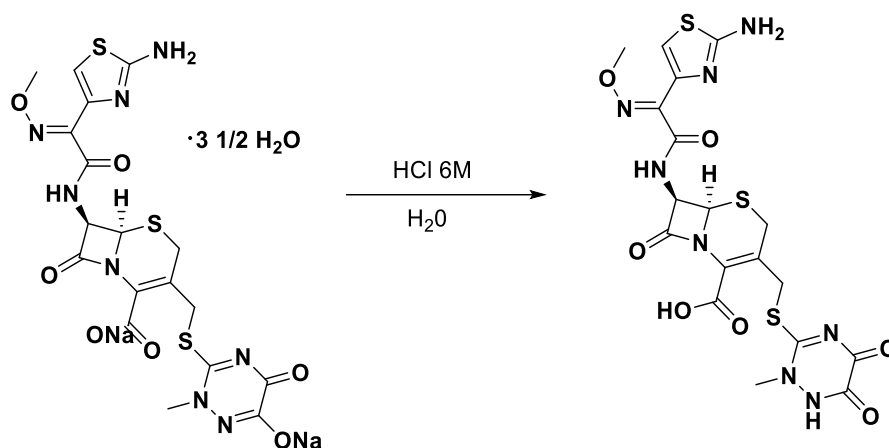
MS-MALDI-TOF: The samples were analyzed in a 5800 MALDI TOF-TOF (ABSciex) in positive linear mode (1500 shots every position) in a mass range of 15,000-100,000 m/z. For that, 1,0  $\mu$ L of every sample solution was spotted onto the MALDI plate. After the droplets were air-dried at room temperature, 1,0  $\mu$ L of matrix (10 mg/mL sinapinic acid in 0.1 % TFA-ACN/H<sub>2</sub>O (7:3, v/v)) was added and allowed to air-dry at room temperature.

## 2. Chemical and characterization procedures

### *Acidification of cephalosporin salts*

#### *Acidification of ceftriaxone sodium salt hemi(heptahydrate)*

4-((E)-2-(((6R,7R)-2-carboxy-3-(((2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl)thio)methyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-7-yl)amino)-1-(methoxyimino)-2-oxoethyl)thiazol-2-aminium

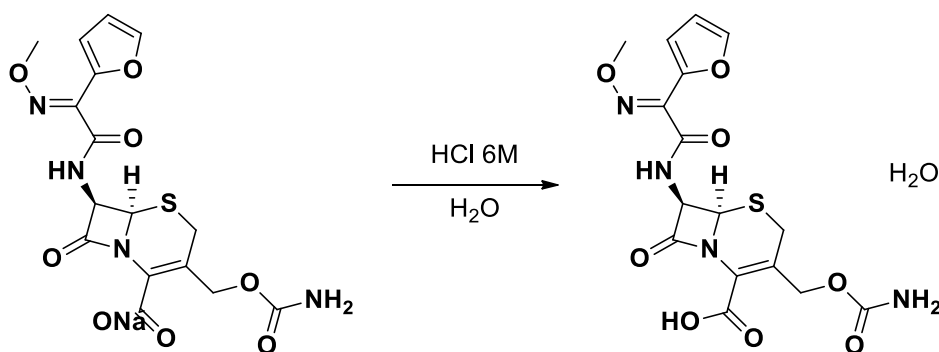


A dried round bottom flask (RBF) is filled with a solution of ceftriaxone sodium salt hemi(heptahydrate) (508.4 mg, 0.77 mmol, 1 eq) in H<sub>2</sub>O (7 mL). Then, the solution is acidified with 280  $\mu$ L of HCl 6 M to pH=1 and vacuum filtration is performed. Finally, the product is dried over high vacuum to give a yellow solid in 99 % yield. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta$  13.73 (s, 1H), 11.87 (s, 1H), 9.58 (d,  $J$  = 8.1 Hz, 1H), 7.23 (s, 2H), 6.73 (s, 1H), 5.76 (dd,  $J$  = 7.9, 4.8 Hz, 1H), 5.13 (d,  $J$  = 4.8 Hz, 1H), 4.38 (d,  $J$  = 13.3

Hz, 1H), 4.09 (d,  $J = 13.2$  Hz, 1H), 3.83 (s, 3H), 3.73 (d,  $J = 18.4$  Hz, 1H), 3.59 (s, 3H), 3.55 (d,  $J = 18.7$  Hz, 1H).  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ ):  $\delta$  167.63, 164.39, 162.98, 160.34, 156.19, 153.23, 148.52, 125.92, 108.93, 61.89, 58.64, 56.98, 42.79, 32.04, 26.21. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{18}\text{H}_{18}\text{N}_8\text{O}_7\text{S}_3$  ( $[\text{M}+\text{H}^+]$ ): 555.0533, found: 555.0542.

#### Acidification of cefuroxime sodium salt

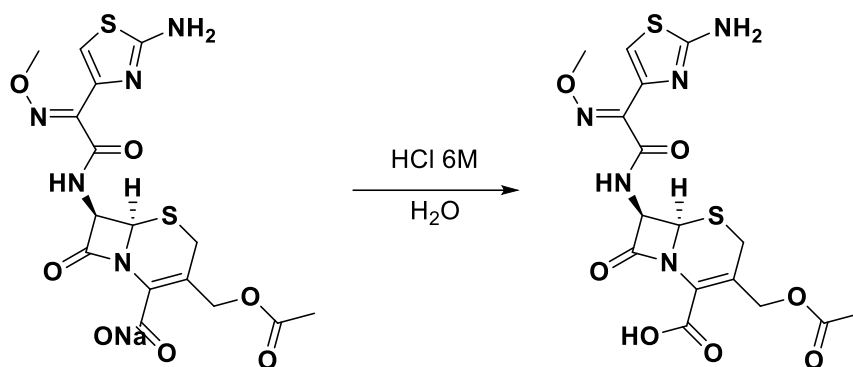
(6R,7R)-3-((carbamoyloxy)methyl)-7-((E)-2-(furan-2-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate



A dried RBF is filled with a solution of cefuroxime sodium salt (301.8 mg, 0.68 mmol, 1 eq) in  $\text{H}_2\text{O}$  (3 mL). Then, the solution is acidified with 200  $\mu\text{L}$  of HCl 6 M to pH=1 and vacuum filtration is performed. Finally, the product is dried over high vacuum to give a yellow solid in 79 % yield.  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  13.62 (s, 1H), 9.77 (d,  $J = 7.9$  Hz, 1H), 7.84 (d,  $J = 1.2$  Hz, 1H), 6.69 (d,  $J = 3.4$  Hz, 1H), 6.65 – 6.61 (m, 1H), 5.79 (dd,  $J = 7.6, 4.8$  Hz, 1H), 5.19 (d,  $J = 4.8$  Hz, 1H), 4.89 (d,  $J = 12.9$  Hz, 1H), 4.61 (d,  $J = 12.8$  Hz, 1H), 3.89 (s, 3H), 3.63 (d,  $J = 17.6$  Hz, 2H), 3.45 (d,  $J = 17.6$  Hz, 1H).  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ ):  $\delta$  163.40, 162.84, 161.61, 156.43, 145.39, 144.91, 112.87, 112.02, 62.32, 62.27, 58.72, 57.37, 25.72. HRMS (ESI-TOF)  $m/z$  calculated for  $\text{C}_{16}\text{H}_{16}\text{N}_4\text{O}_8\text{S}$  ( $[\text{M}+\text{H}^+]$ ): 443.0867, found: 443.1064.

#### Acidification of cefotaxime sodium salt

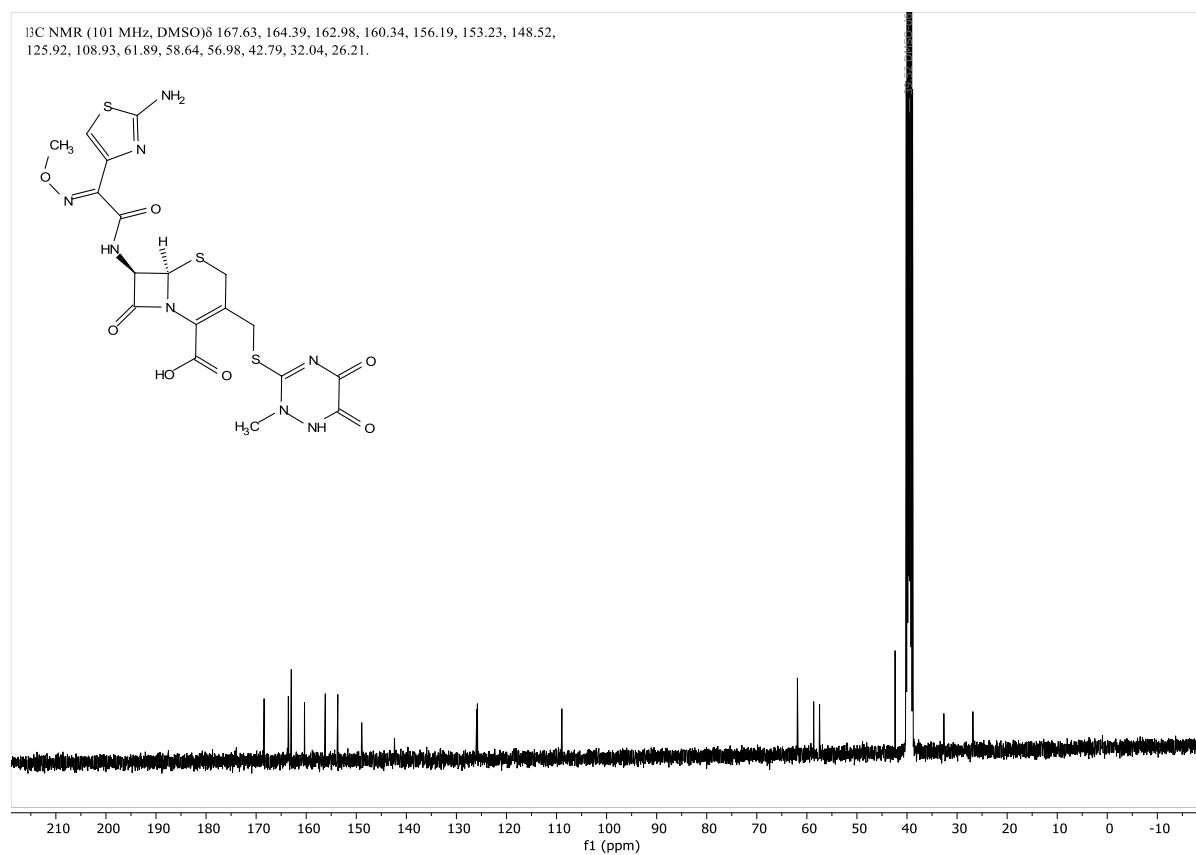
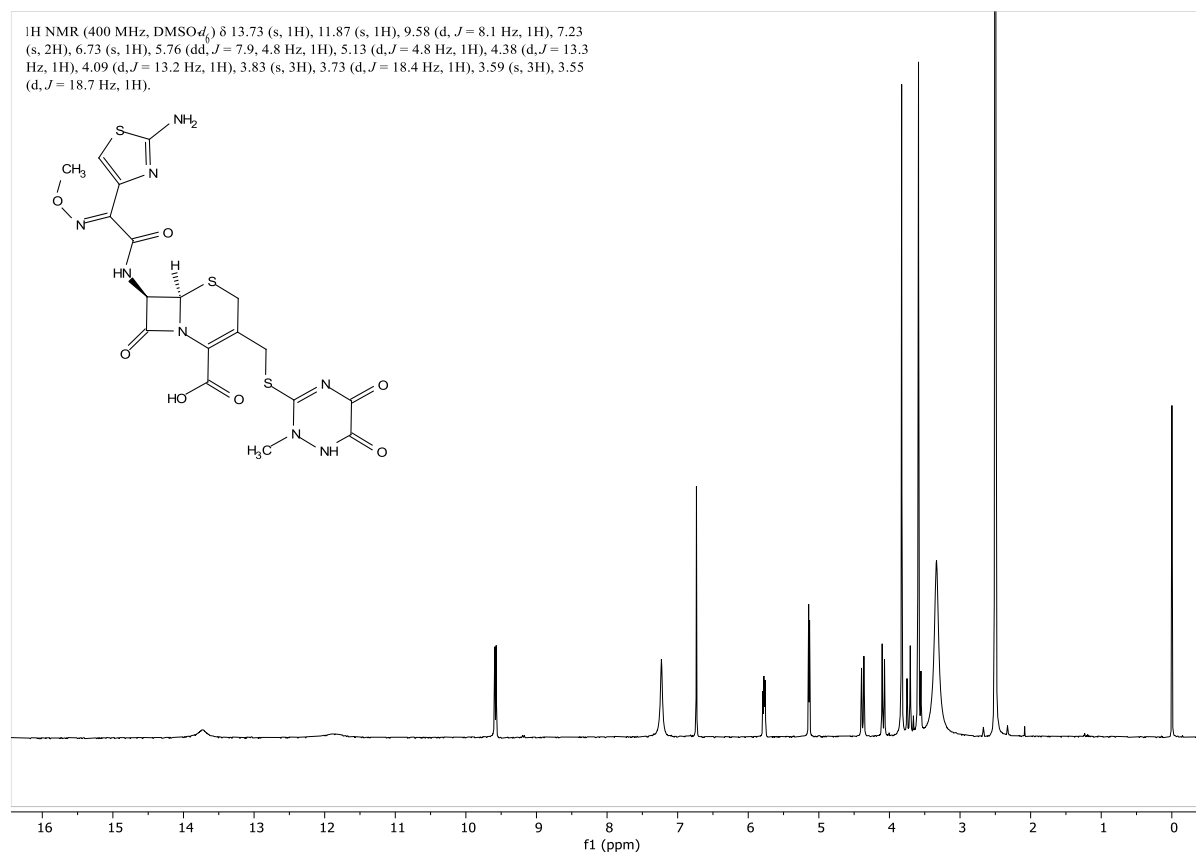
(6R,7R)-3-(acetoxymethyl)-7-((E)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid



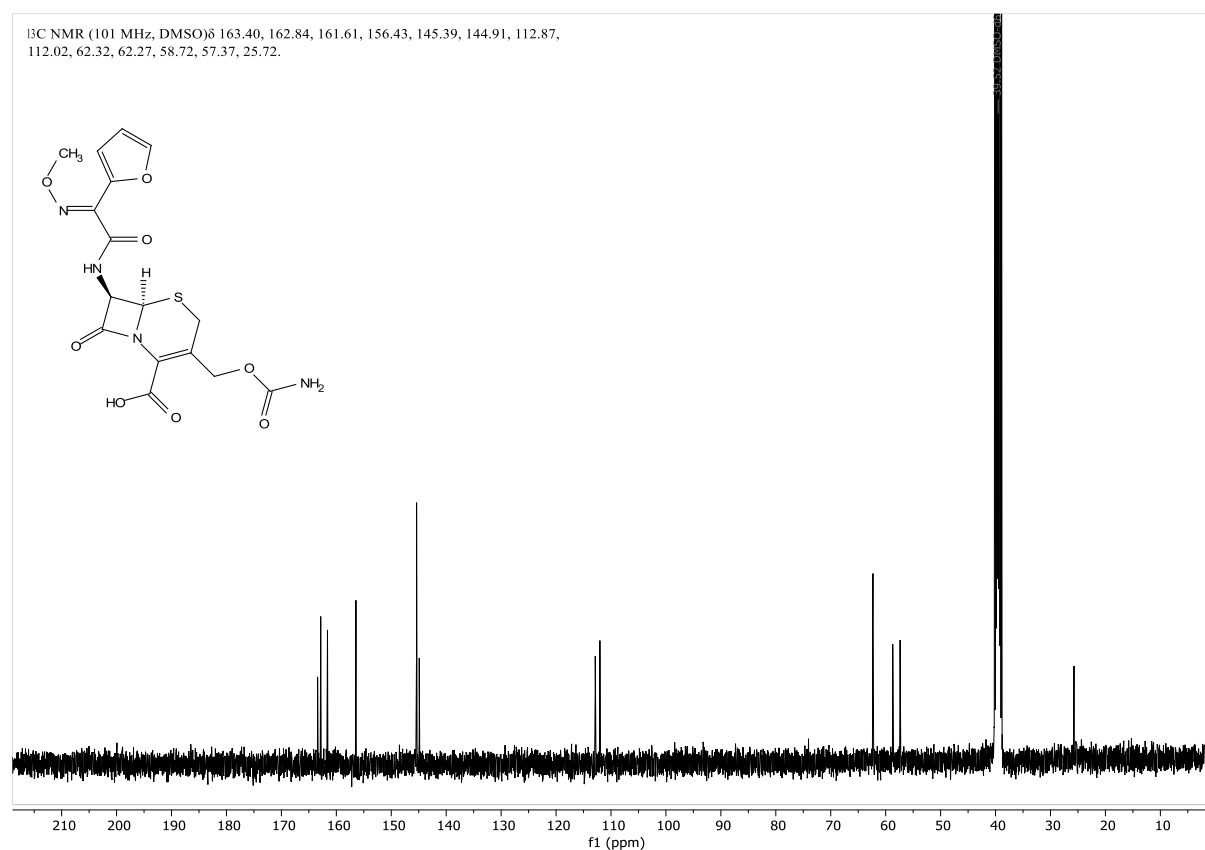
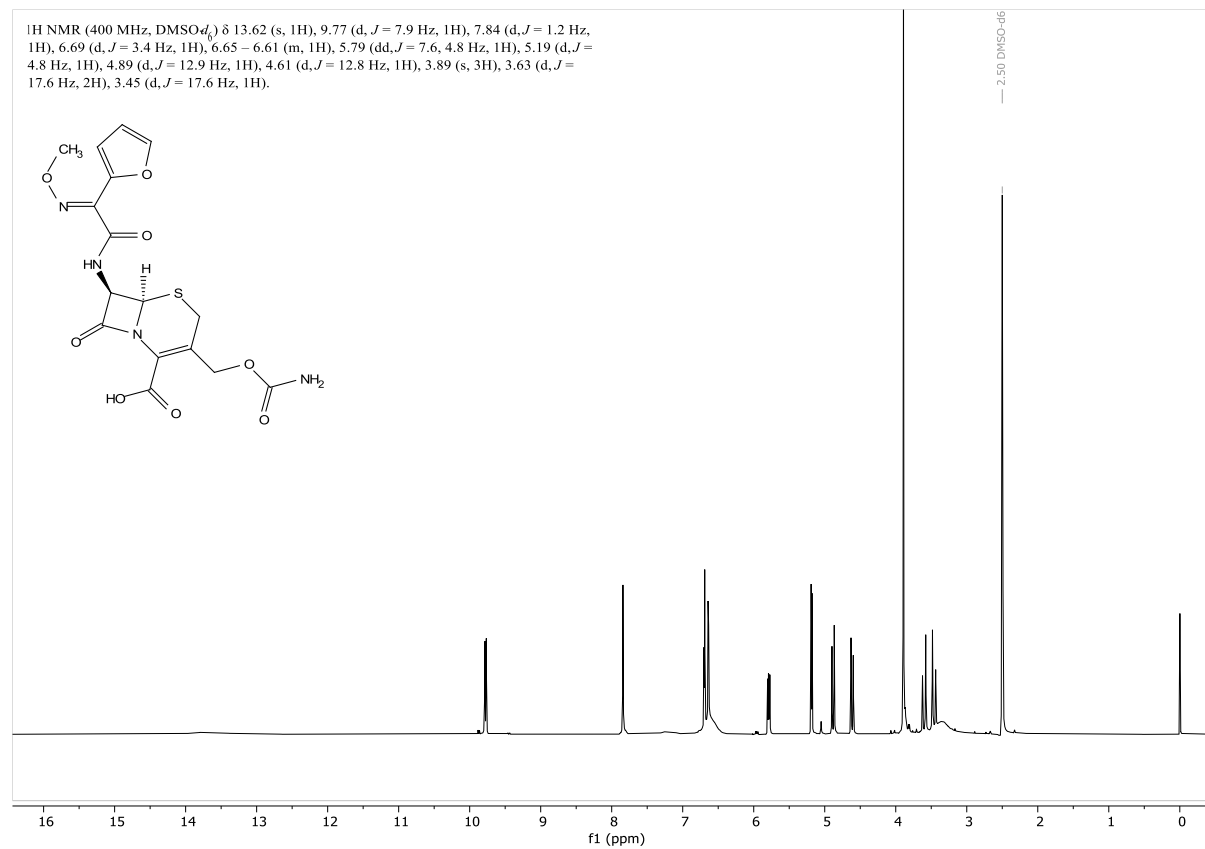
A dried RBF is filled with a solution of cefotaxime sodium salt (300.6 mg, 0.63 mmol, 1 eq) in H<sub>2</sub>O (4 mL). Then, the solution is acidified with 160 μL of HCl 6 M to pH=1 and vacuum filtration is performed. Finally, the product is dried over high vacuum giving a yellow solid in 69 % yield. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 13.68 (s, 1H), 9.59 (d, *J* = 8.2 Hz, 1H), 7.21 (s, 2H), 6.74 (s, 1H), 5.79 (dd, *J* = 7.9, 4.9 Hz, 1H), 5.15 (d, *J* = 4.8 Hz, 1H), 4.99 (d, *J* = 12.8 Hz, 1H), 4.69 (d, *J* = 12.8 Hz, 1H), 3.84 (s, 3H), 3.62 (d, *J* = 18.1 Hz, 1H), 3.48 (d, *J* = 18.1 Hz, 1H), 2.03 (s, 3H). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>): δ 170.22, 168.46, 164.15, 162.89, 148.69, 126.40, 123.00, 108.95, 62.70, 61.93, 58.67, 57.48, 54.92, 25.77, 20.58. HRMS (ESI-TOF) *m/z* calculated for C<sub>16</sub>H<sub>17</sub>N<sub>5</sub>O<sub>7</sub>S<sub>2</sub> ([M+H<sup>+</sup>]): 456.0642, found: 456.0646.

### 3. NMR spectra

*4-((E)-2-(((6R,7R)-2-carboxy-3-((2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl)thio)methyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-7-yl)amino)-1-(methoxyimino)-2-oxoethyl)thiazol-2-aminium*

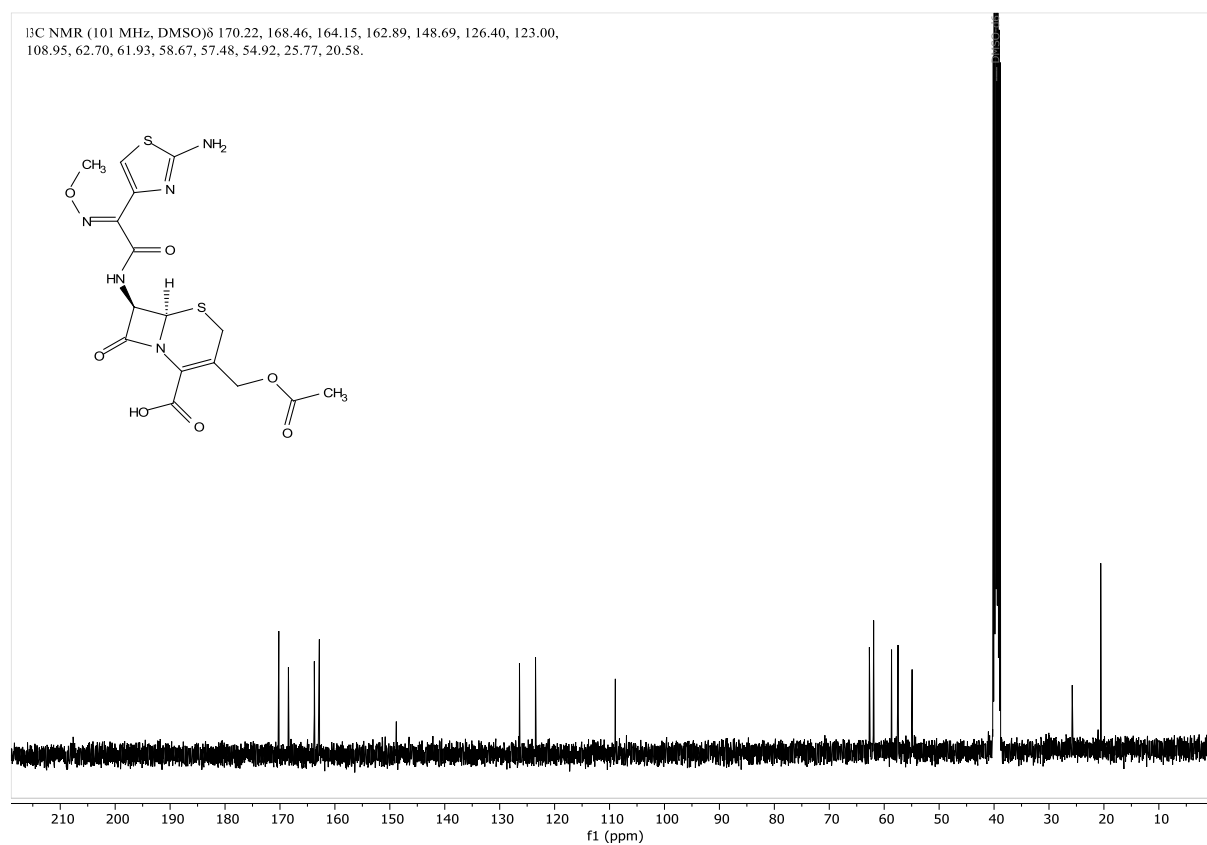
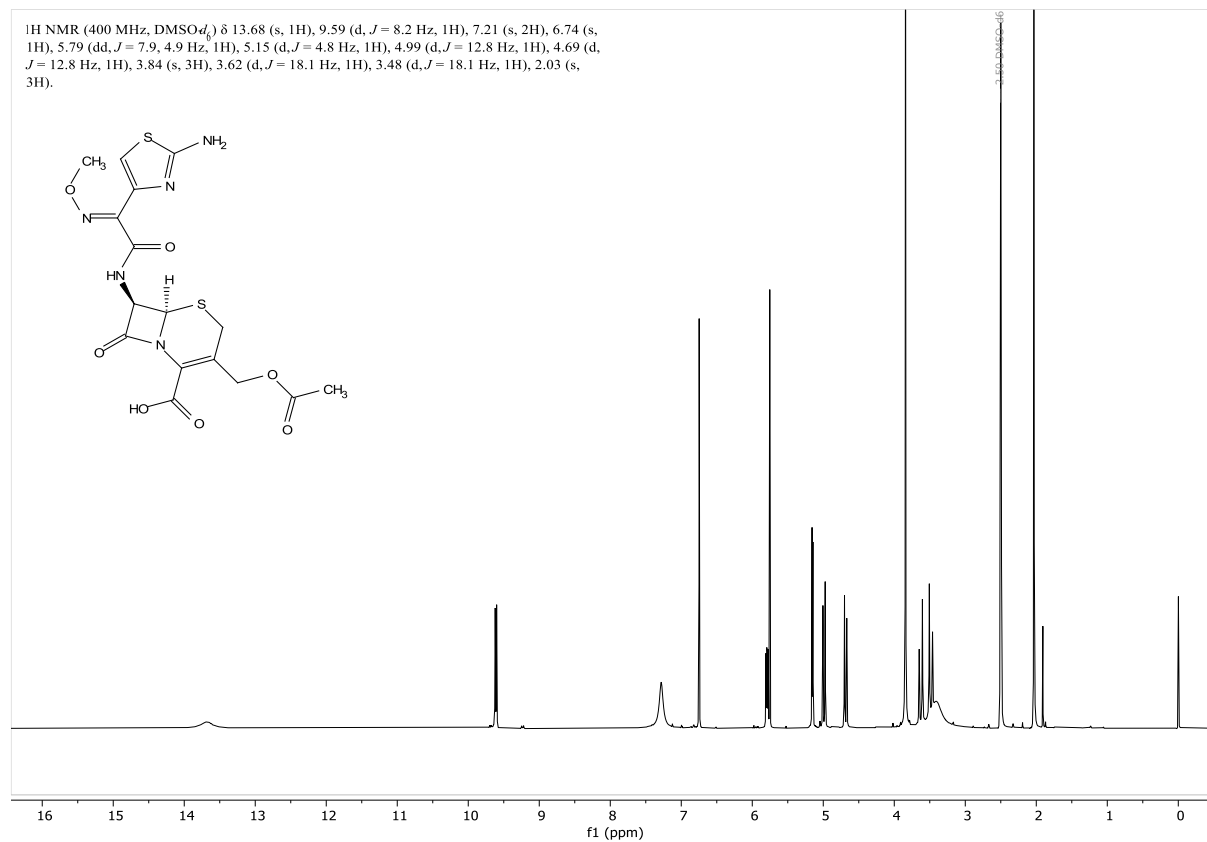


*(6R,7R)*-3-((*carbamoyloxy*)methyl)-7-((*E*)-2-(*furan-2-yl*)-2-(*methoxyimino*)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate





(6*R*,7*R*)-3-(acetoxymethyl)-7-((*E*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

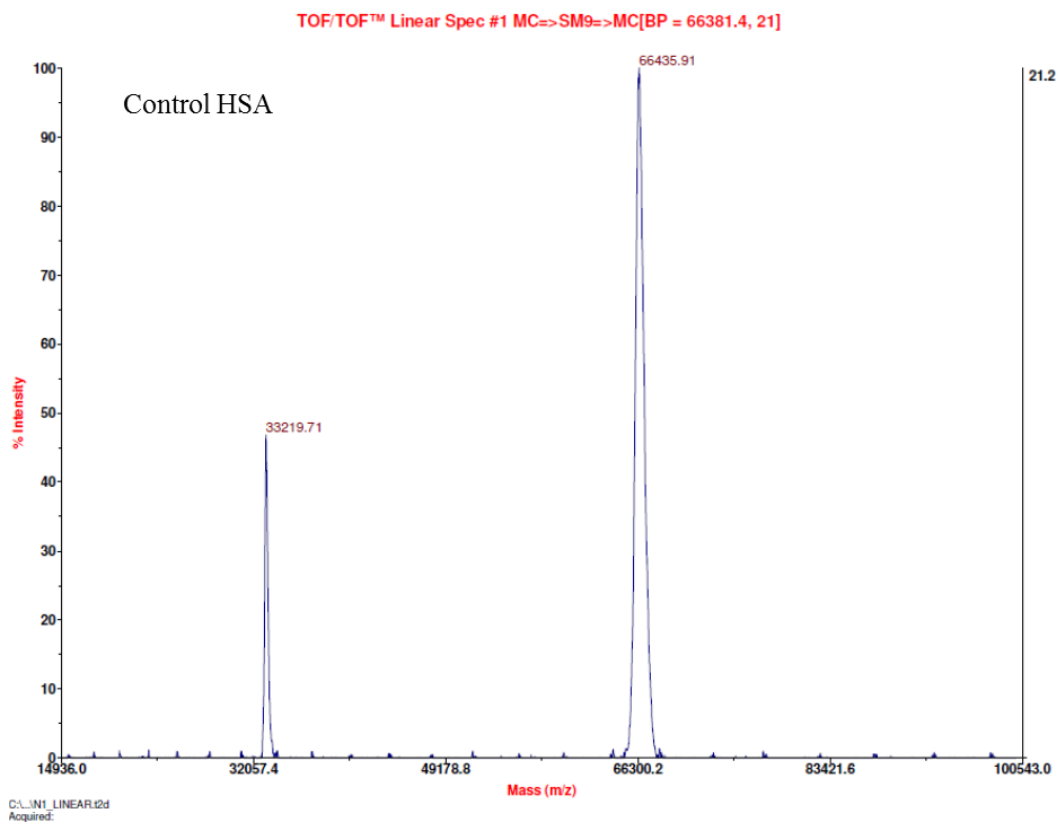


#### **4. MS-MALDI-TOF spectra**

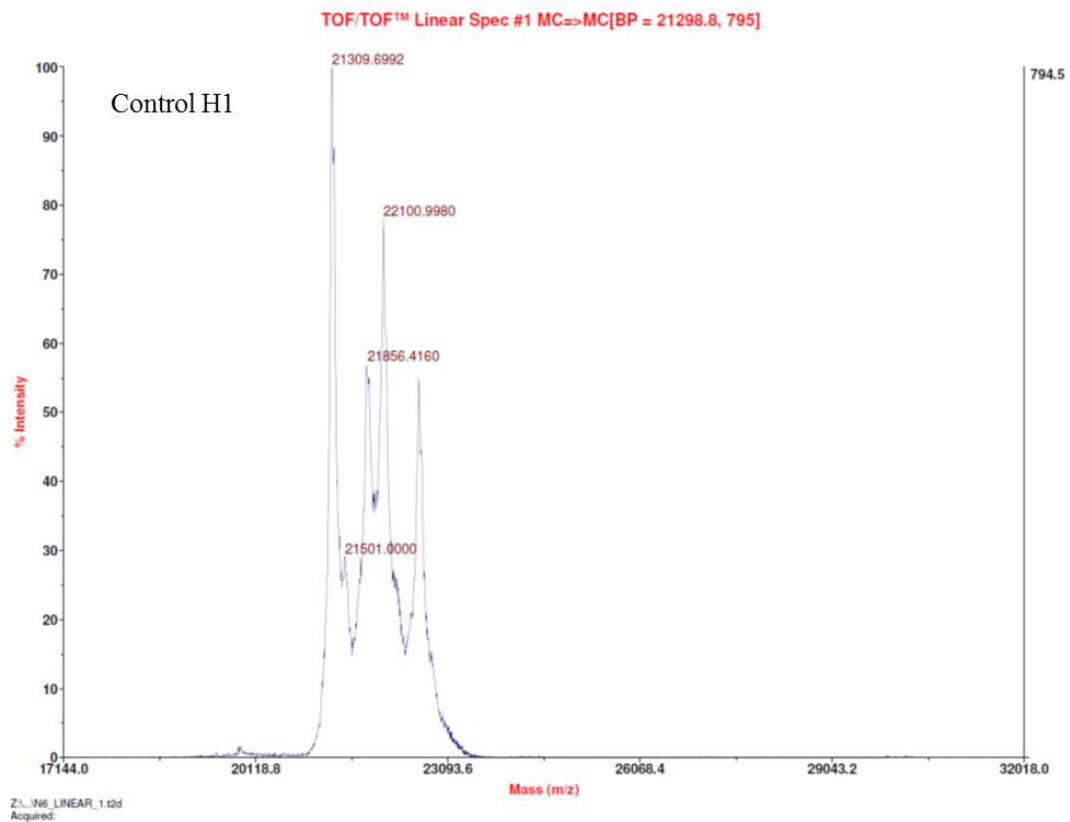
The histone H1 used in this study is an isolated lysine rich fraction of the mainly subfraction f1, which still have presence of the other subfractions that appear as multiple peaks in the spectra. Although H1 is not able to be analyze by MS-MALDI-TOF, HSA and H1 antigens were prepared following the same experimental procedure and both proteins present approximately 60 free lysine residues. These are the reasons why molar ratios ( $\beta$ -lactam/carrier protein) of H1 antigens were estimated to be the same as those obtained for the respective HSA antigens. Spectra showed corresponded to the HSA antigens. Both control HSA and H1 spectra have been included.

The molecular weight (MW) of each antigenic determinant was calculated from the peak centroid of the peaks according to the following equation:  $[MW(\text{determinant}) - MW(\text{protein})] / MW(\text{hapten})$ . The incremental change in molecular weight due to incorporation of hapten molecules to protein corresponds to the number of hapten molecules per protein molecule.

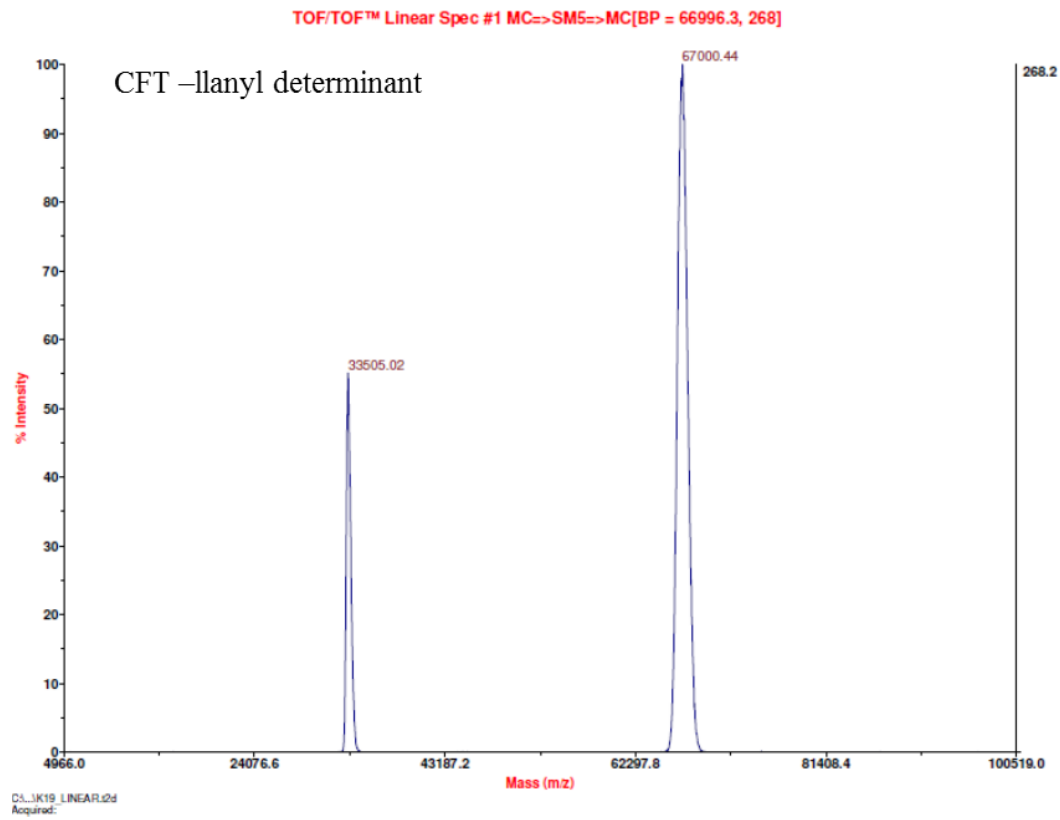
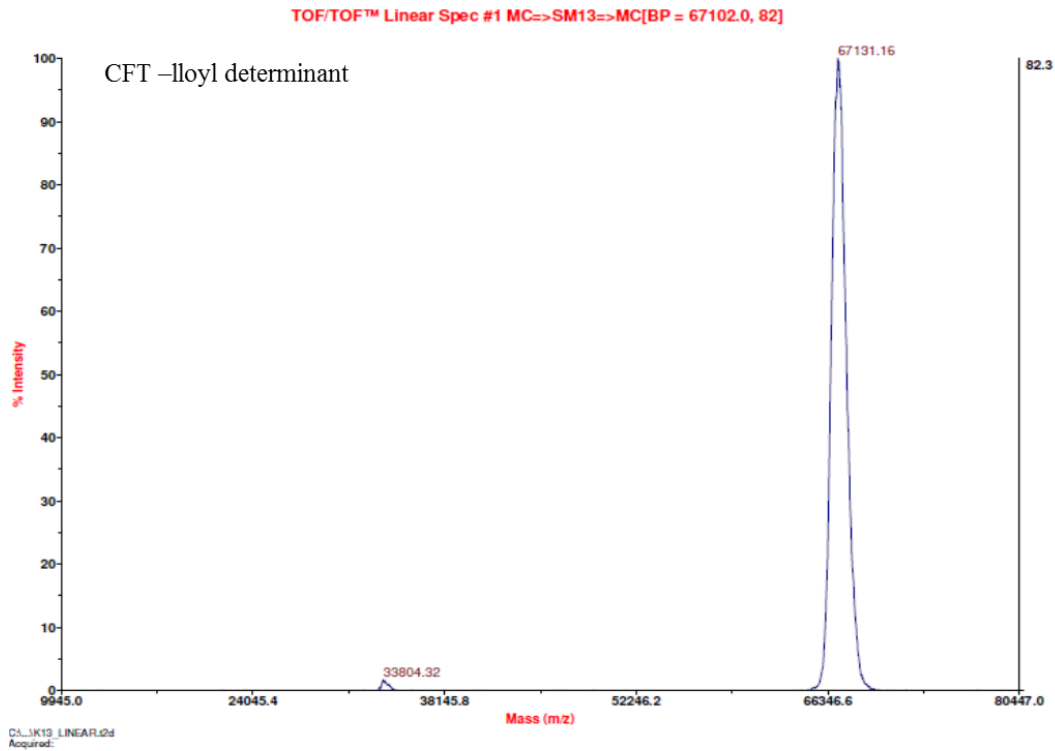
## Control HSA



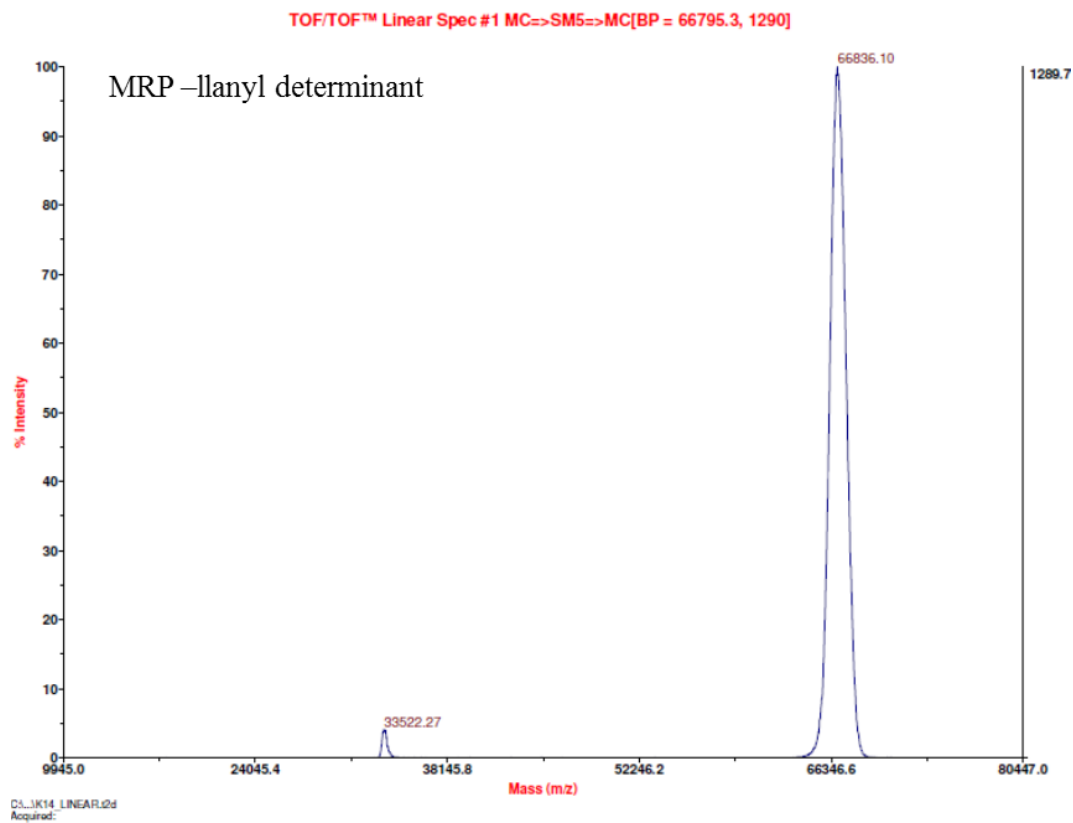
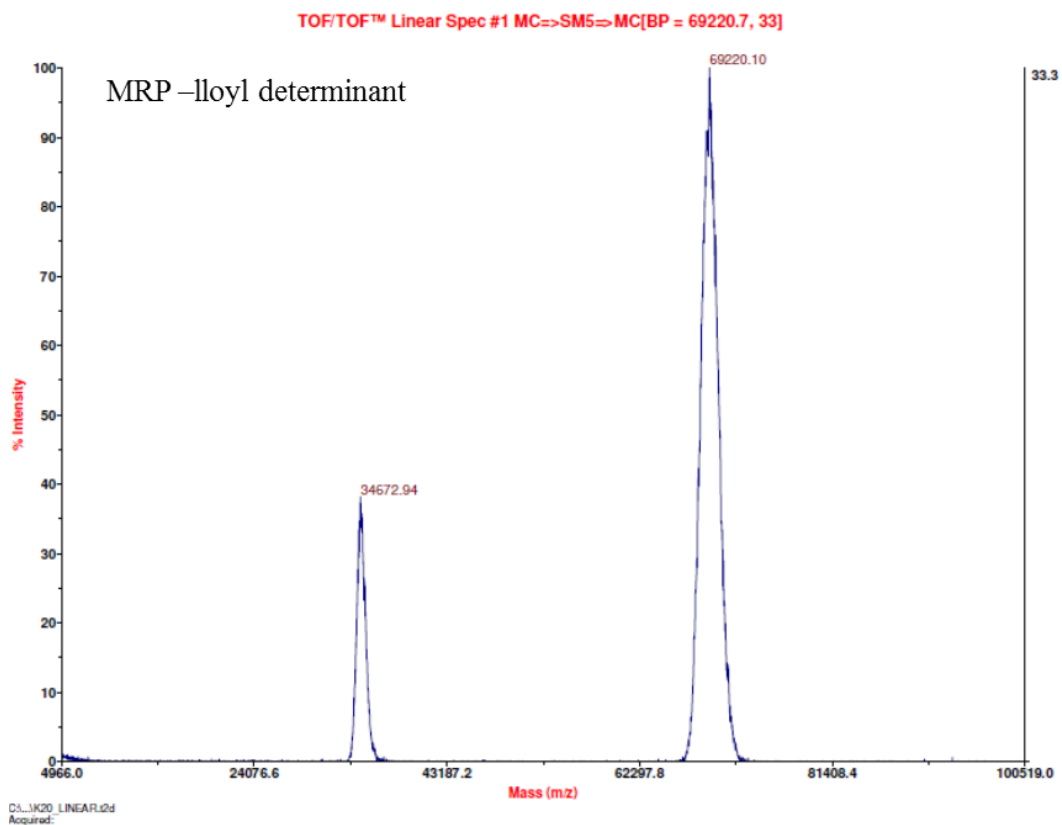
## Control H1



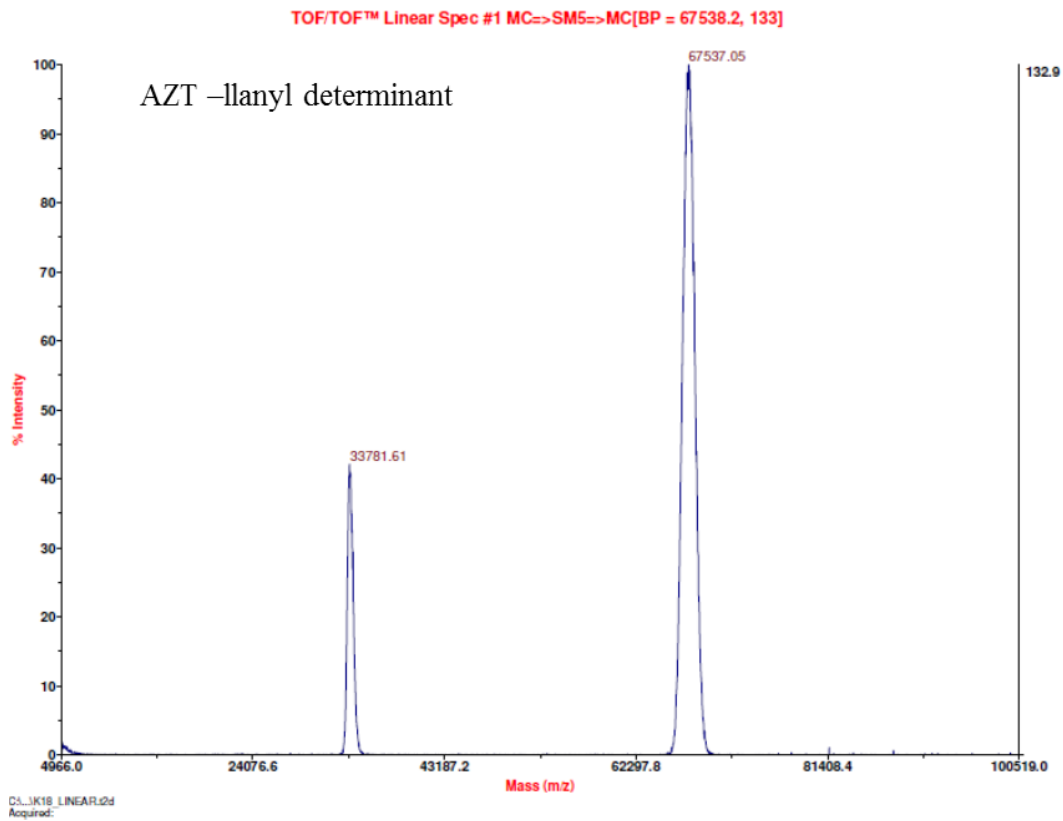
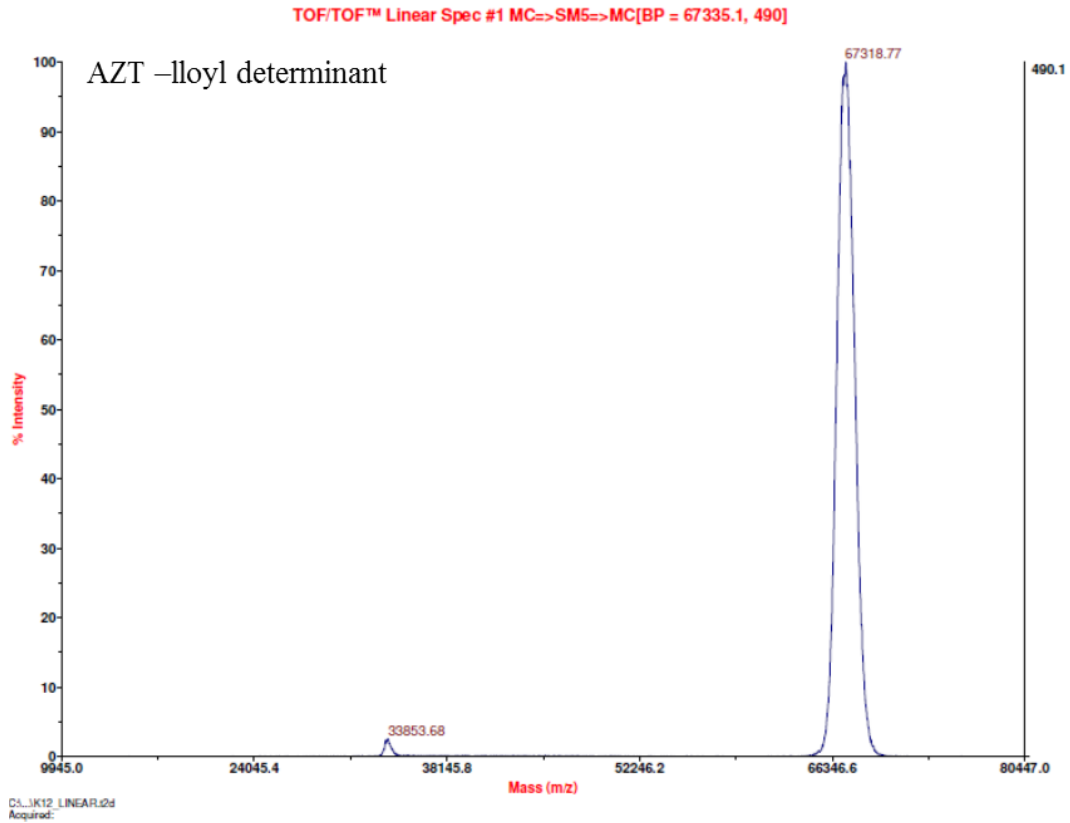
Ceftriaxone determinants



## Meropenem determinants



Aztreonam determinants



## 5. Control samples

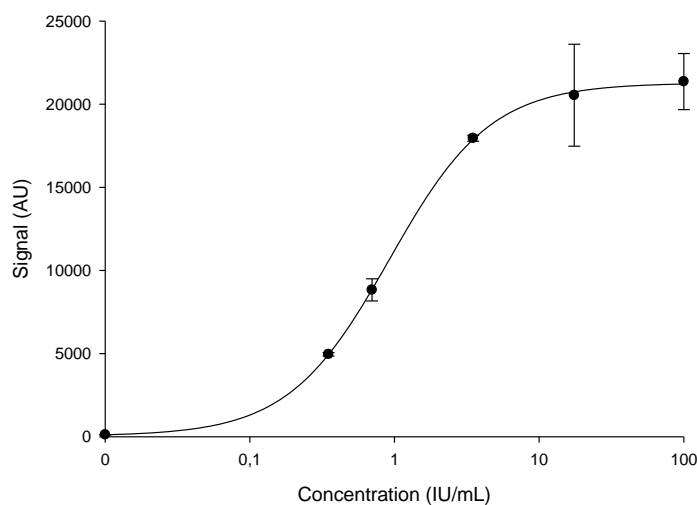
**Table 6.3.S1.** Clinical characteristics of the cohort of control patients

Control Number	Sex	Age (Years)	Control Number	Sex	Age (Years)	Control Number	Sex	Age (Years)
01	M	21	14	M	56	27	M	68
02	F	32	15	M	46	28	F	58
03	M	55	16	F	69	29	F	45
04	F	35	17	F	35	30	F	72
05	F	79	18	F	42	31	F	49
06	F	71	19	M	31	32	F	55
07	F	64	20	F	61	33	F	49
08	F	60	21	F	71	34	F	50
09	M	82	22	M	63	35	F	61
10	F	56	23	F	66	36	F	31
11	M	62	24	F	49	37	F	76
12	M	63	25	F	50			
13	M	76	26	F	22			

## 6. Calibration curve for IgE

The calibration curve obtained for IgE determination using 3<sup>rd</sup> WHO International Standard for serum IgE and the capture antibody Omalizumab is shown in Figure 4. Calibrators used are 0, 0.35, 0.70, 3.50, 17.5 and 100. IU/mL. The standard data points (signal vs. semi-log concentration) were the mean of 5 curves performed in different days and on different disks. A four-parameter logistic (4PL) curve was fitted through the points, using SigmaPlot 11 software.

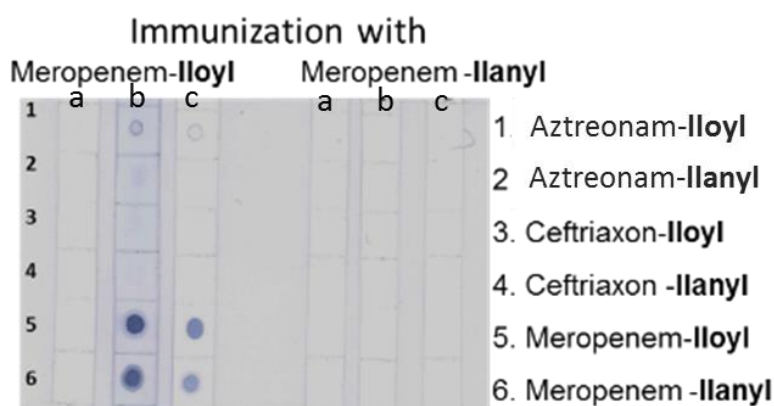
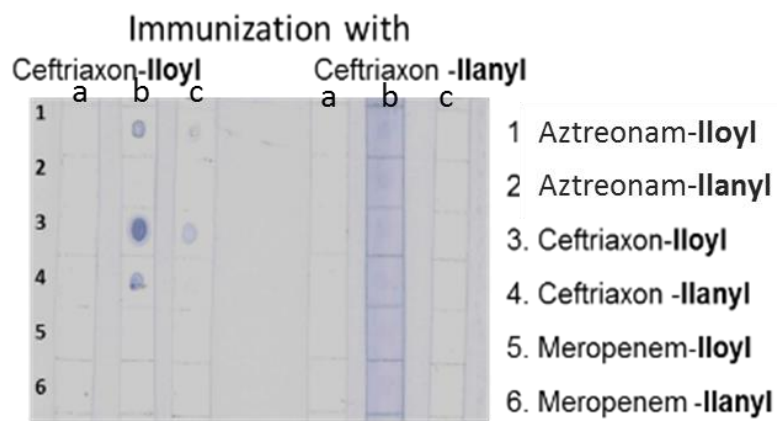
The data obtained shows a sensitivity ( $EC_{50}$ ) of  $0.92 \pm 0.03$  IU/mL, a slope of 1.24 and a regression coefficient ( $r^2$ ) of 0.9998. Limit of detection (LOD) was calculated as the concentration corresponding to the signal of the blank plus 3 times its relative standard deviation, 0.008 IU/mL. The limit of quantification was 0.06 IU/mL, with a relative standard deviation ranging from 3 to 15 %. The concentration of specific IgE for human serum samples was calculated using the calibration curve.



**Figure 6.3.S1.** Calibration curve for IgE



### 7. Dot blot results of rabbit sera for -lloyl and -llanyl determinants



**Figures 6.3.S2-S4.** Dot Blot results using sera raised from immunized rabbit to CFT, MRP and AZT with the corresponding HSA antigens to CFT, MRP and AZT. a) pre-immune sera diluted 1/100 (v/v), b) Sera diluted 1/100 (v/v) and c) 1/500 (v/v) in PBST.

### 8. Affinity parameters of rabbit sera for the different antigenic determinants

**Table 6.3.S2.** Affinity parameters ( $K_d^{app}$  and  $R^2$ ) values of the polyclonal rabbit antibodies raised towards the BLC families of the -lloyl determinants

<b>-lloyl determinants</b>									
<b>HSA</b>									
<b><math>\alpha</math>-IgG-CFT</b>			<b><math>\alpha</math>-IgG-MRP</b>			<b><math>\alpha</math>-IgG-AZT</b>			
	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>
<b>CFR</b>	$9.896 \cdot 10^{-4}$	1/1,011	0.9944	$1.653 \cdot 10^{-3}$	1/605	0.9951	$5.083 \cdot 10^{-3}$	1/197	0.9991
<b>CFT</b>	$1.265 \cdot 10^{-3}$	1/791	0.9989	$4.978 \cdot 10^{-3}$	1/201	0.9825	NR	NR	NR
<b>CFO</b>	$1.201 \cdot 10^{-3}$	1/833	0.9927	$1.898 \cdot 10^{-3}$	1/527	0.9926	$6.044 \cdot 10^{-3}$	1/165	0.9989
<b>MRP</b>	$4.515 \cdot 10^{-2}$	1/22	0.9989	$7.819 \cdot 10^{-3}$	1/128	0.9994	NR	NR	NR
<b>AZT</b>	$9.678 \cdot 10^{-4}$	1/1,033	0.9854	NR <sup>a</sup>	NR	NR	$1.790 \cdot 10^{-4}$	1/5,587	0.9963
<b>H1</b>									
<b><math>\alpha</math>-IgG-CFT</b>			<b><math>\alpha</math>-IgG-MRP</b>			<b><math>\alpha</math>-IgG-AZT</b>			
	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>
<b>CFR</b>	$7.304 \cdot 10^{-4}$	1/1,369	0.9865	$1.151 \cdot 10^{-3}$	1/869	0.9982	$3.985 \cdot 10^{-3}$	1/251	0.9964
<b>CFT</b>	$8.642 \cdot 10^{-4}$	1/1,157	0.9939	$1.362 \cdot 10^{-3}$	1/734	0.9966	$1.166 \cdot 10^{-3}$	1/858	0.9605
<b>CFO</b>	$4.404 \cdot 10^{-4}$	1/2,271	0.9975	$1.871 \cdot 10^{-3}$	1/534	0.9931	$3.163 \cdot 10^{-3}$	1/316	0.9971
<b>MRP</b>	$4.515 \cdot 10^{-2}$	1/22	0.9989	NR	NR	NR	$3.258 \cdot 10^{-3}$	1/307	0.9610
<b>AZT</b>	NR	NR	NR	NR	NR	NR	$2.650 \cdot 10^{-4}$	1/3,774	0.9893

$K_d^{app}$ = Apparent affinity constant (or apparent binding affinity) determined as the concentration of the specific  $\alpha$ -IgG sera to achieve 50 % of the maximum signal (expressed as 1/dilution factor);  $R^2$ = coefficient of determination, R-squared; NR<sup>a</sup>=No recognition.

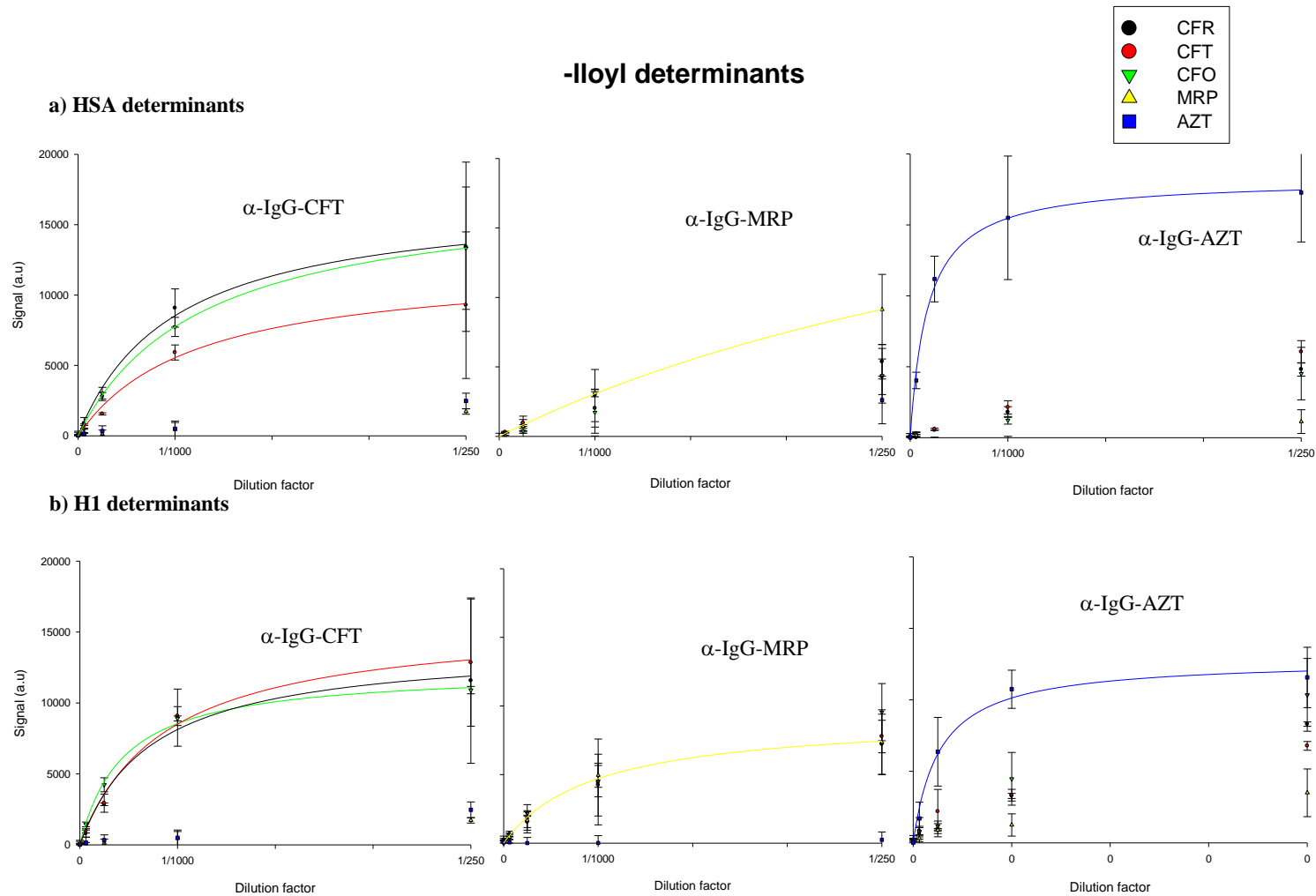
**Table 6.3.S3.** Affinity parameters ( $K_d^{app}$  and  $R^2$ ) values of the polyclonal rabbit antibodies raised towards the BLC families of the -llanyl determinants

<b>-llanyl determinants</b>									
<b>HSA</b>									
<b><math>\alpha</math>-IgG-CFT</b>			<b><math>\alpha</math>-IgG-MRP</b>			<b><math>\alpha</math>-IgG-AZT</b>			
	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>
<b>CFR</b>	$3.028 \cdot 10^{-4}$	1/3,303	0.8971	$2.619 \cdot 10^{-4}$	1/3,818	0.6965	$4.883 \cdot 10^{-4}$	1/2,048	0.6864
<b>CFT</b>	$2.314 \cdot 10^{-3}$	1/432	0.9966	$2.352 \cdot 10^{-4}$	1/4,252	0.6111	NR	NR	NR
<b>CFO</b>	NR <sup>a</sup>	NR	NR	$2.770 \cdot 10^{-2}$	1/36	0.9884	NR	NR	NR
<b>MRP</b>	$9.891 \cdot 10^{-3}$	1/101	0.9475	$1.339 \cdot 10^{-3}$	1/747	0.9953	NR	NR	NR
<b>AZT</b>	NR	NR	NR	NR	NR	NR	$4.488 \cdot 10^{-4}$	1/2,228	0.9984
<b>H1</b>									
<b><math>\alpha</math>-IgG-CFT</b>			<b><math>\alpha</math>-IgG-MRP</b>			<b><math>\alpha</math>-IgG-AZT</b>			
	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>	<b><math>K_d^{app}</math></b>	<b>Dilution factor</b>	<b><math>R^2</math></b>
<b>CFR</b>	$1.245 \cdot 10^{-3}$	1/803	0.9933	NR	NR	NR	$2.230 \cdot 10^{-4}$	1/4,484	0.9689
<b>CFT</b>	$4.612 \cdot 10^{-4}$	1/2,168	0.9910	$7.823 \cdot 10^{-4}$	1/1,278	0.9930	$1.191 \cdot 10^{-4}$	1/8,396	0.9940
<b>CFO</b>	$5.009 \cdot 10^{-4}$	1/1,996	0.9765	$6.620 \cdot 10^{-4}$	1/1,511	0.9799	$9.131 \cdot 10^{-4}$	1/1,095	0.9918
<b>MRP</b>	$2.963 \cdot 10^{-4}$	1/3,375	0.9460	$6.958 \cdot 10^{-4}$	1/1,437	0.9917	$1.090 \cdot 10^{-3}$	1/917	0.9620
<b>AZT</b>	$8.663 \cdot 10^{-4}$	1/1,154	0.9724	$1.508 \cdot 10^{-3}$	1/663	0.9522	$4.712 \cdot 10^{-4}$	1/2,122	0.9659

$K_d^{app}$ = Apparent affinity constant (or apparent binding affinity) determined as the concentration of the specific  $\alpha$ -IgG sera to achieve 50 % of the maximum signal (expressed as 1/dilution factor);  $R^2$ = coefficient of determination, R-squared; NR<sup>a</sup>=No recognition.

1

## 9. Dose-response affinity curves obtained with polyclonal rabbit IgG

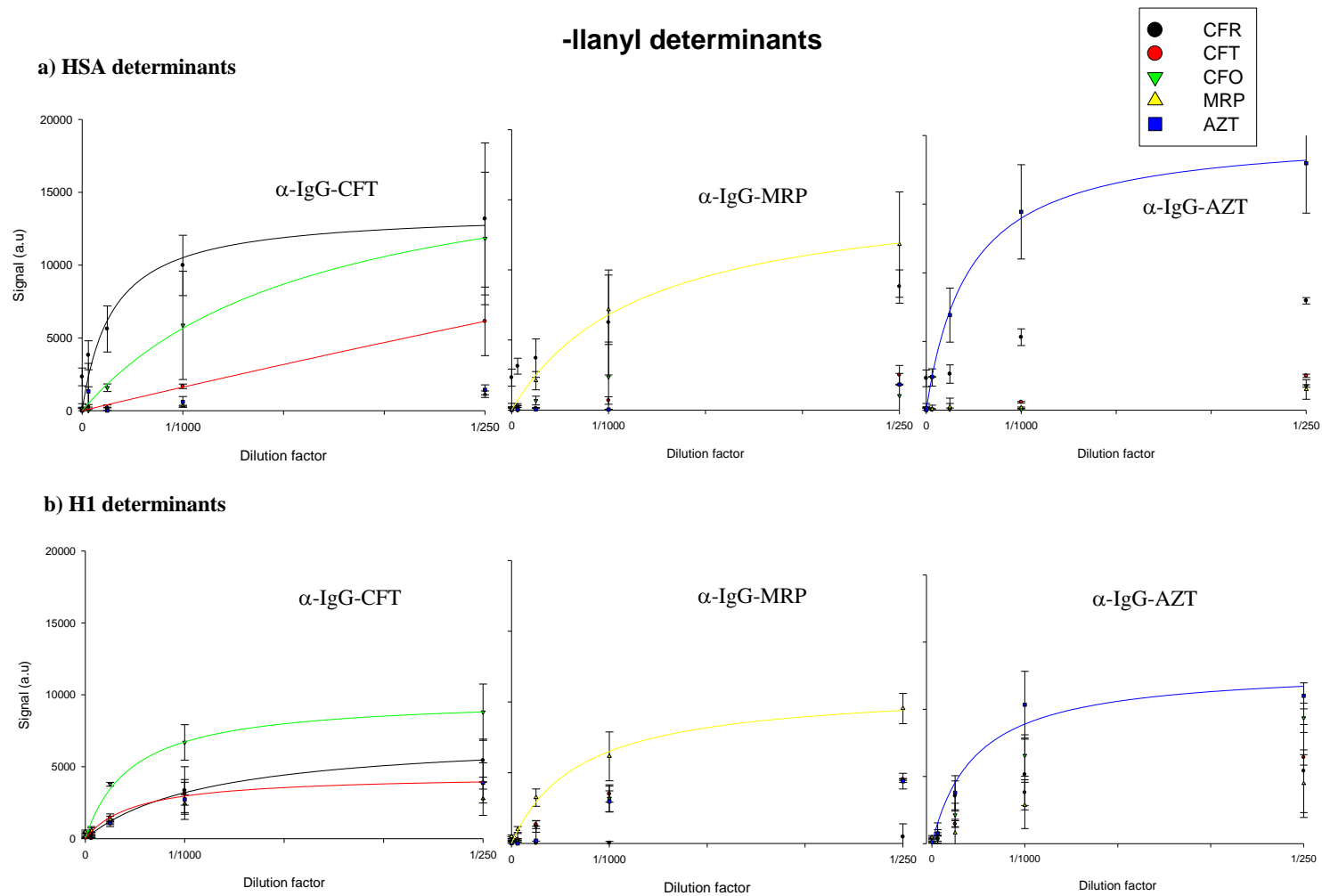


2

3

4

**Figure 6.3.S5.** Dose-response curves obtained for -lloyl determinants with polyclonal rabbit IgG. Dilution factors: 1/250, 1/1,000, 1/4,000 and 1/16,000 and blank, PBS-T. a) HSA determinants, b) H1 determinants



**Figure 6.3.S6.** Dose-response curves obtained for -lanyl determinants with polyclonal rabbit IgG. Dilution factors: 1/250, 1/1,000, 1/4,000 and 1/16,000 and blank, PBS-T. a) HSA determinants, b) H1 determinants



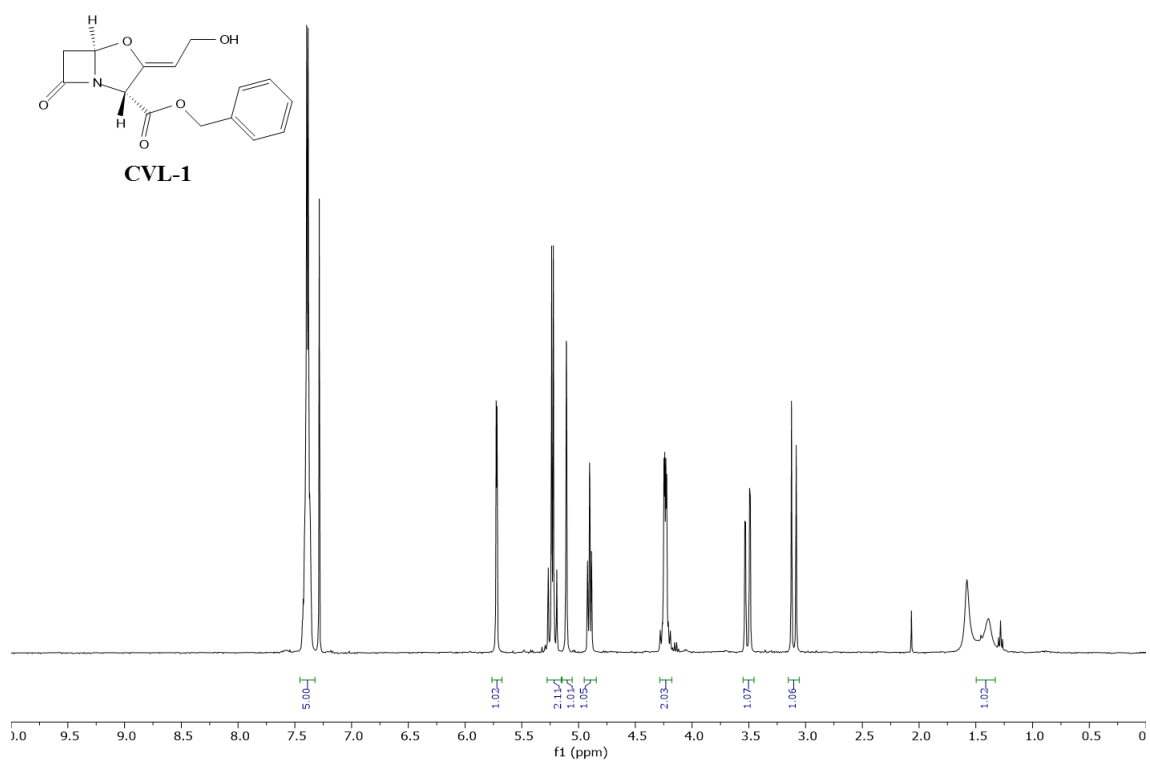
## 6.4. Annex 4

### Supplementary information

#### Hapten synthesis for clavulanic acid

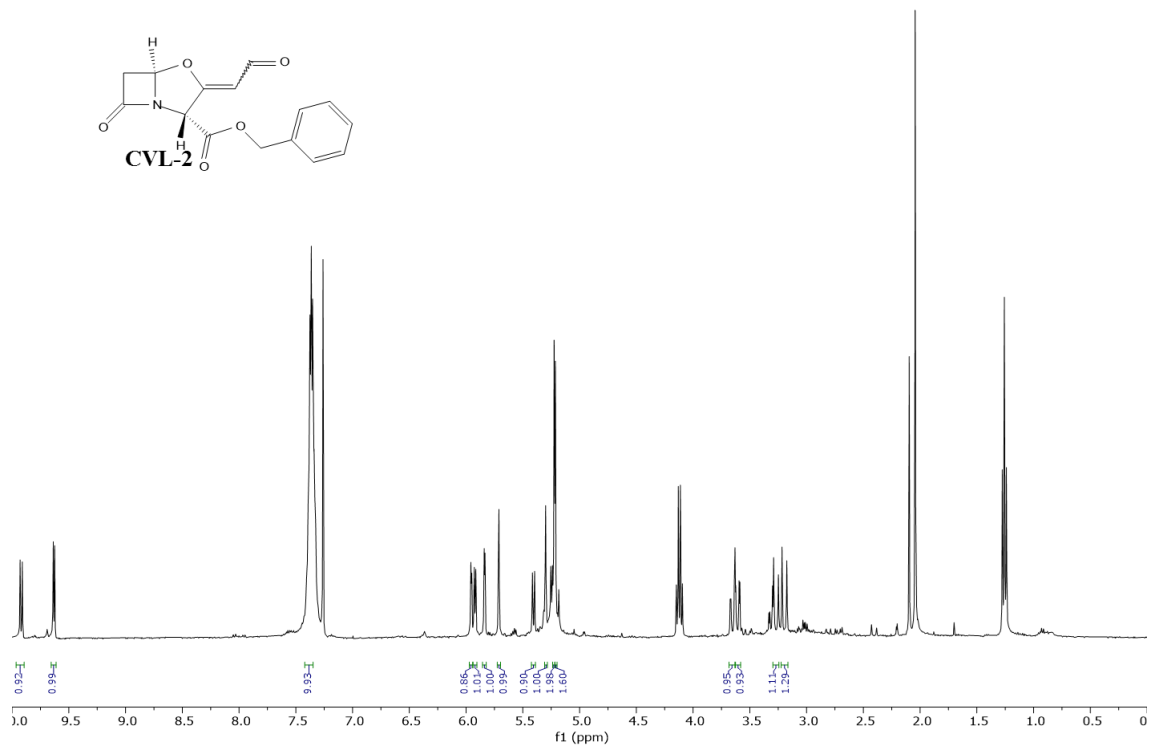
#### Experimental section

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

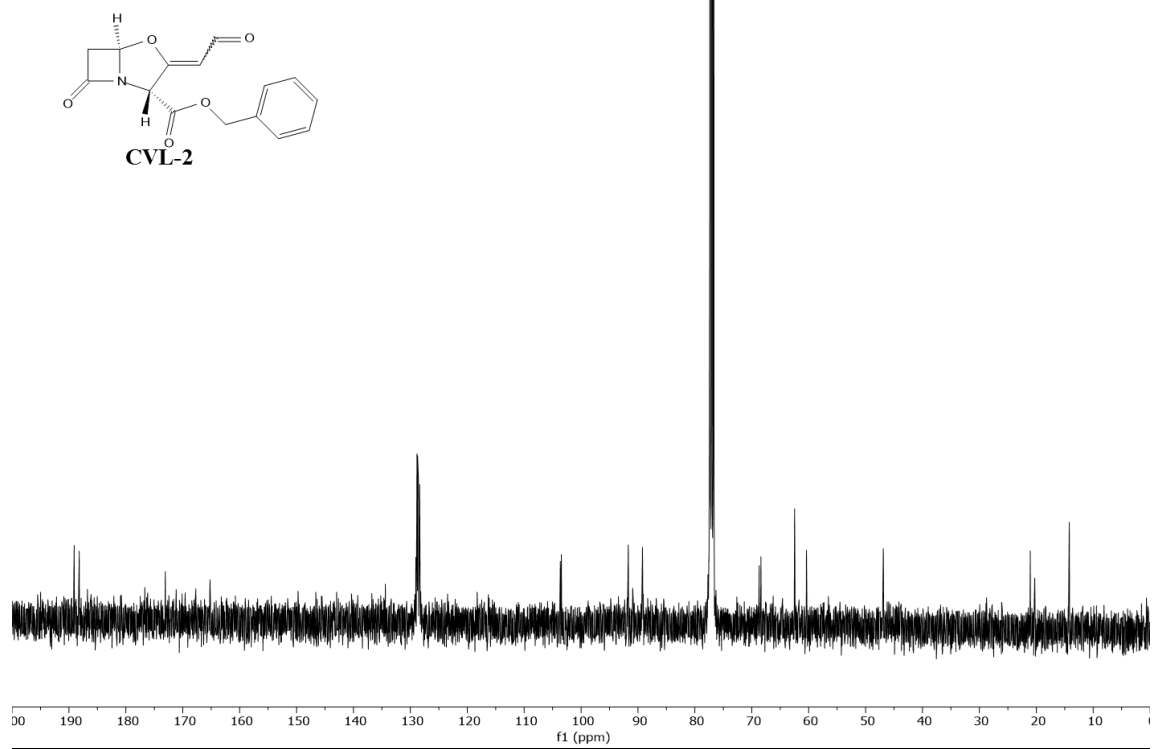


Annex 4

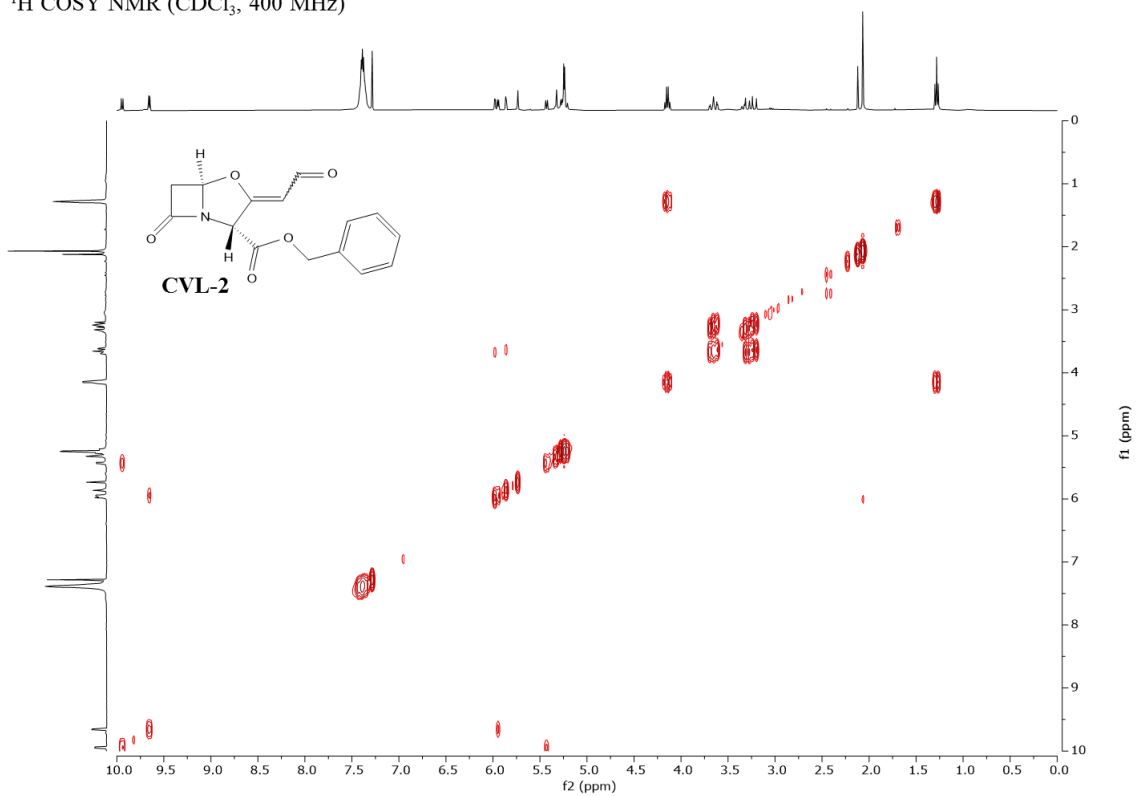
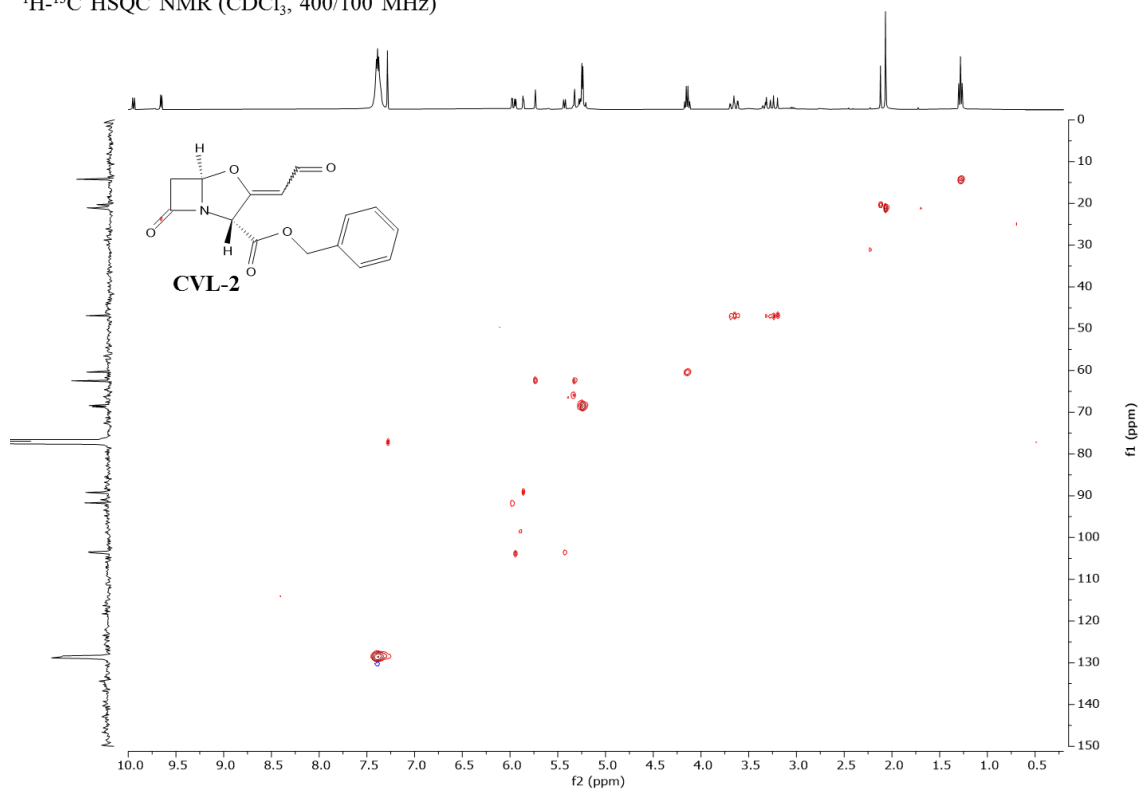
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

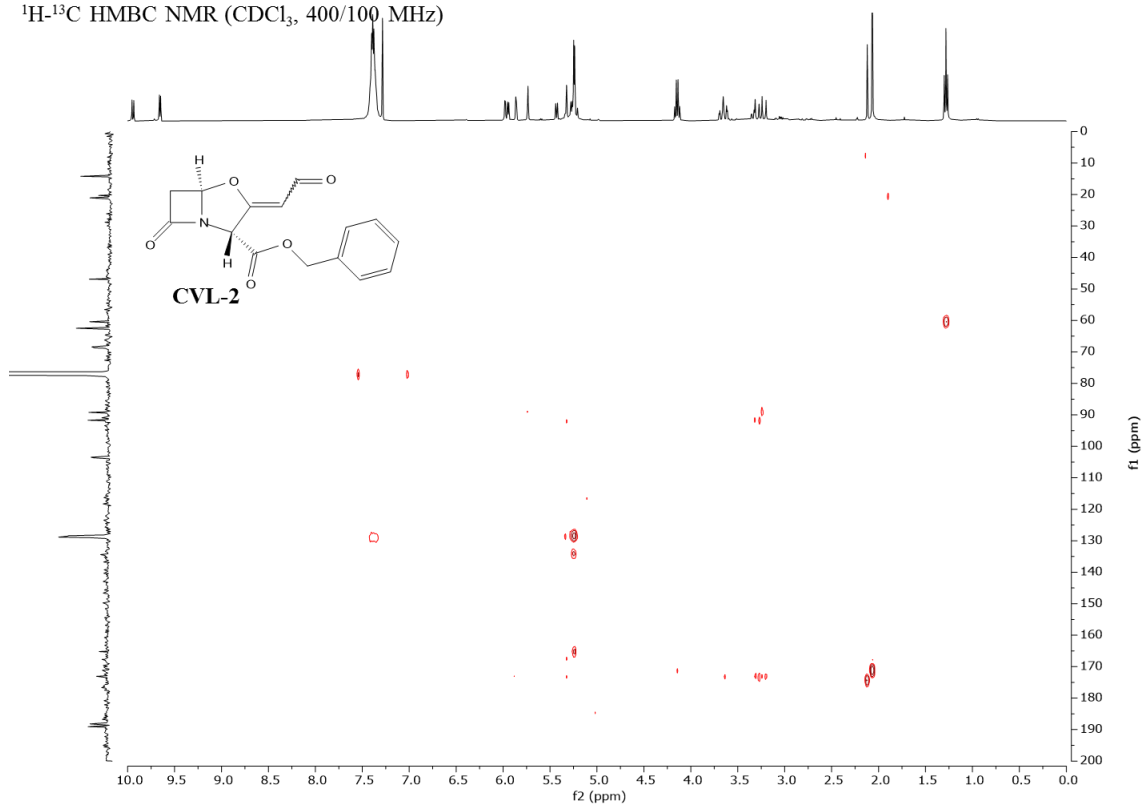
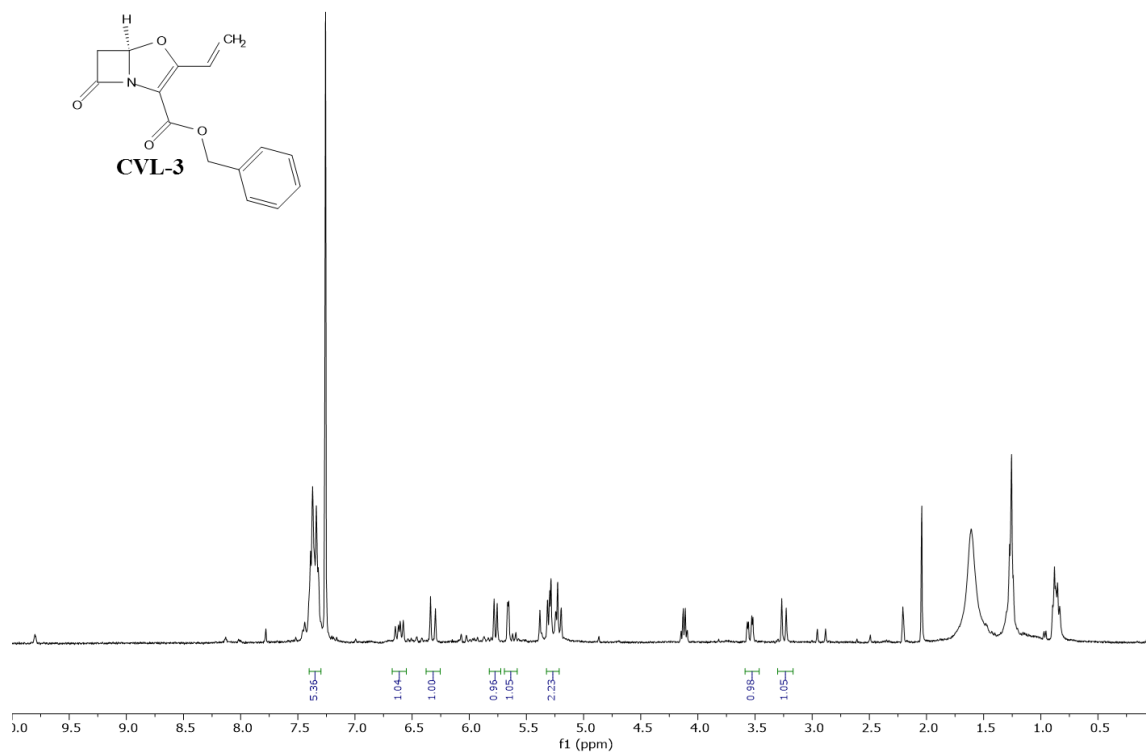


$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)





$^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz) $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

$^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{CDCl}_3$ , 400/100 MHz) $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

**Table 6.4.S1.** Limit of detection (LOD), sensitivity values (EC<sub>50</sub>, dilution factor) and coefficient of determination (R<sup>2</sup>) for the homologous ELISA assays. A) HSA antigens. B) H1 antigens

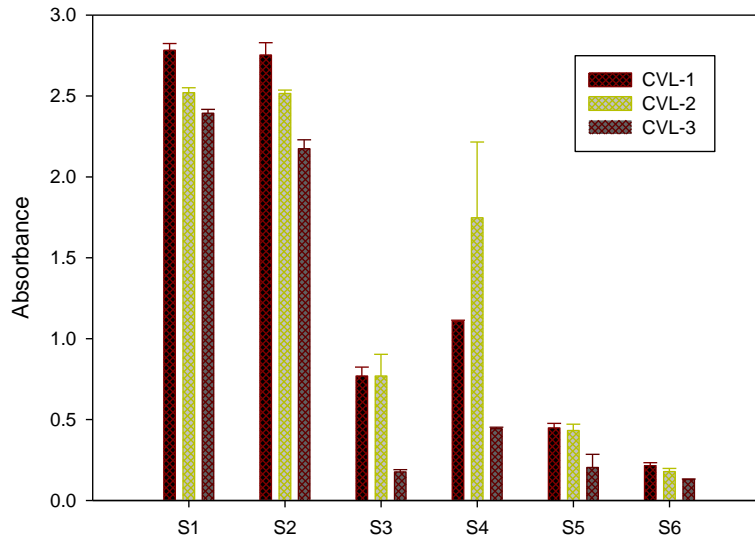
A)	HSA			B)	H1		
	LOD	EC <sub>50</sub>	R <sup>2</sup>		LOD	EC <sub>50</sub>	R <sup>2</sup>
S1	2.450·10 <sup>-5</sup> (or 1/40,816)	1.067·10 <sup>-4</sup> (1/9,372)	0.99717	S1	2.064·10 <sup>-5</sup> (1/40,450)	9.694·10 <sup>-5</sup> (1/10,316)	0.99793
S2	3.059·10 <sup>-5</sup> (1/32,690)	1.354·10 <sup>-4</sup> (1/7,386)	0.99806	S2	2.114·10 <sup>-5</sup> (1/47,304)	1.250·10 <sup>-4</sup> (1/8,000)	0.99795
S3	4.121·10 <sup>-5</sup> (1/24,266)	3.789·10 <sup>-4</sup> (1/2,639)	0.99812	S3	5.065·10 <sup>-5</sup> (1/19,743)	6.329·10 <sup>-4</sup> (1/1,580)	0.99934
S4	2.402·10 <sup>-5</sup> (1/41,632)	1.788·10 <sup>-4</sup> (1/5,593)	0.99715	S4	2.023·10 <sup>-5</sup> (1/49,432)	1.801·10 <sup>-4</sup> (1/5,552)	0.99727
S5	NR <sup>a</sup>	NR	NR	S5	NR	NR	NR
S6	5.906·10 <sup>-4</sup> (1/1,693)	6.665·10 <sup>-4</sup> (1/1,500)	0.93479	S6	8.721·10 <sup>-3</sup> (1/115)	NR	0.97365

The LOD was calculated as the signal of the blank plus 3 times its standard deviation. The half maximum effective concentration (EC<sub>50</sub>) is determined as the concentration of the specific α-IgG serum to achieve 50 % of the maximum signal; <sup>a</sup>NR=No recognition

**Table 6.4.S2.** Values obtained (in arbitrary units, a.u.) for HSA and H1-based antigens using polyclonal IgG raised in serum S1

	Dilution factor			
	4.00·10 <sup>-3</sup>	1.00·10 <sup>-3</sup>	2.50·10 <sup>-4</sup>	6.25·10 <sup>-5</sup>
<b>CVL HSA</b>	15,909	15,533	10,773	3,908
<b>CVL H1</b>	15,749	15,553	11,306	6,918
<b>CVL-1 HSA</b>	12,887	11,803	8,464	2,656
<b>CVL-1 H1</b>	15,059	13,708	11,210	7,158
<b>CVL-2 HSA</b>	15,812	14,137	11,718	4,312
<b>CVL-2 H1</b>	15,027	12,654	9,645	3,312

**Figure 6.4.S1.** Cross-reactivity signals obtained with polyclonal rabbit IgG by heterogeneous ELISA assays for H1 coating antigens. Dilution factor represented:  $2.50 \cdot 10^{-4}$  (1/4,000).



## 6.5. Annex 5

### Electronic supplementary information (ESI†)

#### Synthesis of skeletally diverse $\beta$ -lactam haptens for the *in vitro* diagnosis of IgE-mediated drug allergy

Edurne Peña-Mendizabal, Bruce K. Hua, Ethel Ibañez-Echevarria, Dolores Hernández-Fernández de Rojas, Ángel Maquieira, Stuart L. Schreiber, and Sergi Morais

#### Experimental section

##### 1. General methods

Oxygen- and/or moisture-sensitive reactions were carried out in flame-dried glassware under nitrogen atmosphere. All reagents and solvents were purchased and used as received from commercial vendors or synthesized according to cited procedures.

All yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Reaction progress was monitored by analytical thin-layer chromatography (TLC) and  $^1\text{H-NMR}$  spectroscopy. TLC analyses were performed using E. Merck silica gel 60 F254 pre-coated plates (250  $\mu\text{m}$ ). A handheld 254 nm UV lamp and potassium permanganate staining solution (with light heating) were used for detection. Flash column chromatography was performed using a Teledyne ISCO CombiFlash Rf+ purification system with RediSep Rf Gold Normal-Phase Silica columns.

Known compounds were characterized by, at minimum,  $^1\text{H NMR}$  spectroscopy. Novel synthetic intermediates and final compounds were characterized by, at minimum,  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  spectroscopy and HRMS. Further NMR experiments were performed as needed to confirm structural assignment.

NMR spectra were recorded on Bruker UltraShield 300 MHz and Ascend 400 MHz spectrometers.  $^1\text{H}$  and  $^{13}\text{C}$ -NMR chemical shifts ( $\delta\text{H}$ ,  $\delta\text{C}$ ) are reported in parts per million (ppm) relative to the appropriate solvent— $\text{CDCl}_3$ : 7.26 ( $^1\text{H}$ ), 77.16 ( $^{13}\text{C}$ ) ppm.  $(\text{CD}_3)_2\text{SO}$ : 2.50 ( $^1\text{H}$ ), 39.52 ( $^{13}\text{C}$ ) ppm.  $\text{C}_6\text{D}_6$ : 7.16 ( $^1\text{H}$ ), 128.06 ( $^{13}\text{C}$ ) ppm. Resonances are described using the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), quin. (quintet), m (multiplet), br (broad), dd (doublet of doublets), etc. Coupling constants (J) are given in Hz and are rounded to the nearest 0.1 Hz. All NMR data were collected at 25 °C. All deuterated solvents were purchased from Cambridge Isotope Laboratories.

Low-resolution mass spectra (LRMS) were acquired on a Waters 2795 separations module coupled to a 3100 mass detector operating in ESI+ mode.

## 2. Experimental procedures

(*E*)-2-Cyclopropylboronic acid (**3**) was purchased from Combi-Blocks and dried over 3Å molecular sieves.

### General Procedure A: Trimethylsilyldiazomethane methylation.

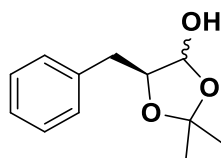
A flame-dried round-bottom flask was charged with a solution of corresponding BLC (1 equiv.) in methanol (1 mL) and DCM (0.2 M) under N<sub>2</sub> atmosphere. Then, (diazomethyl)trimethylsilane (2 M, 1 equiv.) was added dropwise and the reaction was allowed to stir at room temperature until the yellow solution turned clear. The solution was concentrated by rotary evaporation and purified by flash column chromatography (ISCO) to give the desired compound.

### General Procedure B: Petasis reaction.

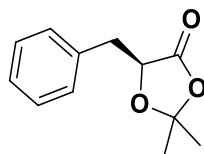
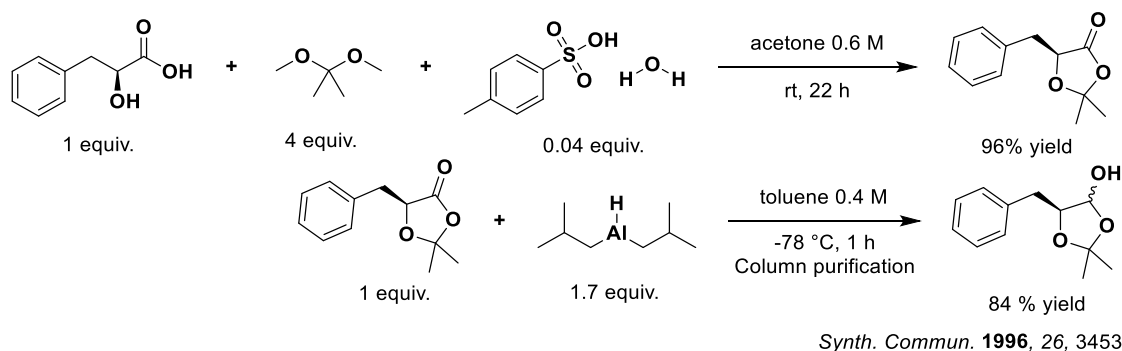
A flame-dried round-bottom flask was charged with (4*R*,5*S*)-5-benzyl-2,2-dimethyl-4-hydroxy-1,3-dioxolane **2** (1 equiv., 0.2 M) in the solvent system of 9:1 EtOH–hexafluoroisopropanol (v/v) and 3Å molecular sieves. To this stirred solution were added the corresponding BLC methyl ester **1a-1f** (1.5 equiv) and (*E*)-2-cyclopropylvinylboronic acid **3** (1.5 equiv), successively. After stirring for 72 h at 40 °C, the resulting mixture was concentrated by rotary evaporation and the residue was purified by flash column chromatography (ISCO) to give the desired compound.

### General Procedure C: N-alkylation of the Petasis product.

A solution of the Petasis product (1 equiv., 0.2 M) and NaHCO<sub>3</sub> (10 equiv.) in DMF was stirred for 30 min at 0°C. Then, propargyl bromide (10 equiv., 80 % in toluene) was added under N<sub>2</sub>. After stirring for 24 h at 70 °C, the reaction mixture was cooled to room temperature and quenched with NH<sub>4</sub>Cl solution (50 mL). The resulting mixture was extracted with ether (3x50 mL), and combined organic layers were washed with brine, and dried over MgSO<sub>4</sub>. The solution was concentrated by rotary evaporation and the residue was purified by flash column chromatography (ISCO) to give the desired compound.



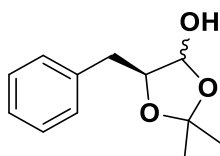
**(5S)-5-benzyl-2,2-dimethyl-1,3-dioxolan-4-ol (2)**



**1. (S)-5-Benzyl-2,2-dimethyl-4-oxo-1,3-dioxolane**

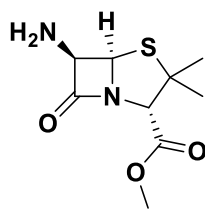
A flame-dried round-bottom flask was charged with a mixture of (S)-2-hydroxy-3-phenylpropanoic acid (10.00 g, 60.00 mmol) and p-TsOH·H<sub>2</sub>O (500 mg) in 2,2-dimethoxypropane (30 mL) and acetone (100 mL) and was stirred at room temperature for 22 h. After concentration by rotary evaporation, the resulting residue was dissolved in ethyl acetate (150 mL), washed with sat. NaHCO<sub>3</sub> (100 mL), brine (100 mL), dried (MgSO<sub>4</sub>), passed through a short pad of silica gel, and concentrated to provide a white solid (11.70 g, 96 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.28 – 7.23 (m, 5H), 4.65 (dd, 1H), 3.12 (ABq, 2H), 1.49 (s, 3H), 1.35 (s, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 172.49, 135.79, 129.81, 128.38, 127.03, 110.84, 75.04, 37.65, 26.93, 26.16. Characterization data consistent with those reported in the literature.





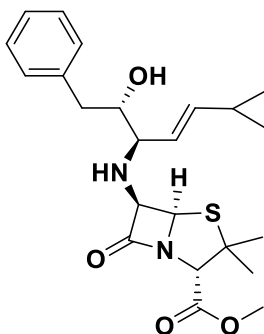
## 2. (4R,5S)-5-Benzyl-2,2-dimethyl-4-hydroxy-1,3-dioxolane (2)

To a stirred solution of (5S)-5-benzyl-2,2-dimethyl-1,3-dioxolan-4-one (1.83 g, 8.89 mmol) in toluene (37.5 mL) was added DIBAL-H (9.8 mL of a 1M solution in toluene, 9.78 mmol) at  $-78\text{ }^{\circ}\text{C}$ . The resulting solution was stirred at the same temperature for 60 min and to this solution was added 1N HCl (9.8 mL) very slowly. After warming to room temperature, the solution was extracted with ethyl acetate (3 x 10 mL), filtered with  $\text{MgSO}_4$ , and concentrated to provide a pale yellow oil **2** (1.53 g, 84 % yield) after purification by flash chromatography (ISCO).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.36–7.20 (m, 5H), 5.28–5.21 (m, 1H), 4.31–4.26 (m, 1H), 3.06 (d,  $J = 6.8\text{ Hz}$ , 1H), 2.94 (qd,  $J = 14.0, 6.6\text{ Hz}$ , 2H), 2.82 (br, 1H), 1.46 and 1.51 (two s, 6H,  $\text{C}(\text{CH}_3)_2$  for major isomer), 1.35 and 1.57 (two s, 6H,  $\text{C}(\text{CH}_3)_2$  for minor isomer); ratio of diastereomers was approximately 2: 1. Characterization data consistent with those reported in the literature.



### 6-aminopenicillanic acid (6-APA) methyl ester (**1a**)

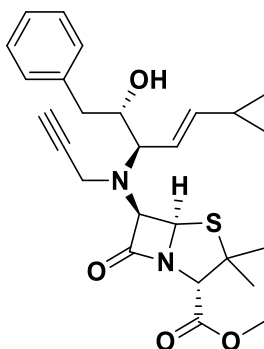
General procedure A was followed using 6-APA (2.00 g, 9.25 mmol), methanol (1 mL), and (diazomethyl)trimethylsilane (4.6 mL, 9.25 mmol) to give the desired compound **1a** as a viscous yellow oil (2.07 g, 97 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 5.51 (d, *J* = 4.3 Hz, 1H), 4.57 (d, *J* = 4.3 Hz, 1H), 4.40 (s, 1H), 3.77 (s, 3H), 1.79 (br, 2H), 1.66 (s, 3H), 1.50 (s, 3H).



### (2*S*,3*R*,*E*)-5-Cyclopropyl-2-hydroxy-3-((2*S*,5*R*,6*R*)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)amino-1-phenyl-4-pentene (**4a**)

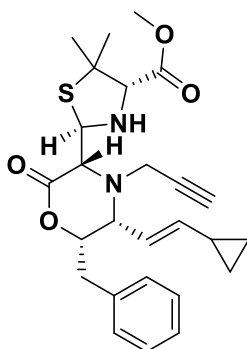
General procedure B was followed using (4*R*,5*S*)-5-benzyl-2,2-dimethyl-4-hydroxy-1,3-dioxolane **2** (759 mg, 3.64 mmol, 0.2 M) in 18.2 mL of the solvent system of 9:1 EtOH–hexafluoroisopropanol (v/v), 6-APA methyl ester **1a** (1.26 g, 5.47 mmol, 1.5 equiv.) and (*E*)-2-cyclopropylvinylboronic acid **3** (590 mg, 5.27 mmol, 1.5 equiv.) to give the desired product **4a** as a pale yellow oil (834 mg, 53 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.31 – 7.19 (m, 5H), 5.47 (dd, *J* = 15.5, 9.0 Hz, 1H), 5.43 (d, *J* = 3.7 Hz, 1H), 5.20 (dd, *J* = 15.3, 8.6 Hz, 1H), 4.44 (d, *J* = 2.9 Hz, 1H), 4.40 (s, 1H), 4.03 – 3.95 (m, 1H), 3.77 (s, 3H), 3.16 (d, *J* = 9.1 Hz, 1H), 2.73 (d, *J* = 6.6 Hz, 2H), 2.42 (br, 2H), 1.65 (s, 3H), 1.49 (s, 3H), 1.47 – 1.42 (m, 1H), 0.77 (d, *J* = 8.0 Hz, 2H), 0.42 (s, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ: 177.1, 168.8, 140.4, 138.4, 129.5, 128.6, 126.5, 124.3, 74.2, 70.6, 70.5, 67.3,

64.7, 64.3, 52.4, 40.1, 31.4, 27.4, 13.8, 7.0, 6.9. LRMS (ESI<sup>+</sup>) m/z calculated for C<sub>23</sub>H<sub>30</sub>N<sub>2</sub>O<sub>4</sub>S ([M+H]<sup>+</sup>): 431.19, found: 431.21.



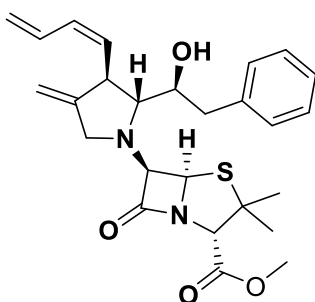
**(2*S*,3*R*,*E*)-5-Cyclopropyl-2-hydroxy-3-(((2*S*,5*R*,6*R*)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)(2-propynyl)amino)-1-phenyl-4-pentene (5a)**

General procedure C was followed using **4a** (834 mg, 1.94 mmol, 0.2 M) and NaHCO<sub>3</sub> (1.63 g, 19.35 mmol, 10 equiv.) in DMF (9.7 mL) and propargyl bromide (2.2 mL, 19.35 mmol, 10 equiv., 80 % in toluene) to give the desired compound **5a** (656 mg, 72 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.33 – 7.19 (m, 5H), 5.57 (dd, *J* = 15.4, 9.8 Hz, 1H), 5.30 (d, *J* = 3.7 Hz, 1H), 5.28 (dd, *J* = 15.0, 8.0 Hz, 1H), 4.48 (d, *J* = 4.0 Hz, 1H), 4.47 (s, 1H), 4.21 (d, *J* = 18.0 Hz, 1H), 3.79 (s, 3H), 3.74 (d, *J* = 18.5 Hz, 1H), 3.34 (dd, *J* = 9.7, 2.7 Hz, 1H), 2.78 (dd, *J* = 13.9, 8.0 Hz, 1H), 2.62 (dd, *J* = 14.0, 5.4 Hz, 1H), 2.22 (s, 1H), 1.67 (s, 3H), 1.57 – 1.49 (m, 1H), 1.48 (s, 3H), 0.83 (d, *J* = 8.1 Hz, 2H), 0.53 – 0.42 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ: 172.6, 168.7, 143.0, 138.8, 129.4, 128.5, 126.4, 121.1, 100.1, 74.3, 70.7, 70.5, 70.4, 69.3, 68.5, 63.8, 52.5, 39.9, 38.9, 31.2, 26.8, 13.9, 7.2, 7.0. LRMS (ESI<sup>+</sup>) m/z calculated for C<sub>26</sub>H<sub>32</sub>N<sub>2</sub>O<sub>4</sub>S ([M+H]<sup>+</sup>): 469.21, found: 469.51.



**(3*R*,5*R*,6*S*)-6-Benzyl-5-((*E*)-2-cyclopropylvinyl)-3-((2*R*,4*R*)-4-methoxycarbonyl-5,5-dimethylthiazolidin-2-yl)-4-propynylmorpholin-2-one (6a)**

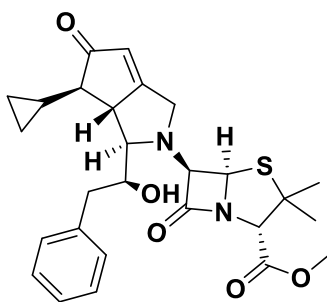
To a stirred solution of **5a** (47.1 mg, 0.10 mmol) in toluene (1.0 mL), was added NaH (ca 60 % mineral oil dispersion, 6.2 mg, 0.15 mmol, 1.5 equiv.) at 0 °C. After stirring at room temperature for 14 h under N<sub>2</sub>, 2 mL sat. NaHCO<sub>3</sub> aq. was added to the reaction mixture. The resulting mixture was extracted with ethyl acetate (3x2 mL), and combined organic layers were washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. The solution was concentrated by rotary evaporation and the residue was purified by flash column chromatography (ISCO) to give **6a** as a colorless oil (23.4 mg, 50 % yield). <sup>1</sup>H-NMR (400 MHz, C<sub>6</sub>D<sub>6</sub>) δ: 7.32 – 7.18 (m, 5H), 5.37 (t, 1H), 5.29 (dd, *J* = 15.2, 9.8 Hz, 1H), 5.19 (dd, *J* = 15.2, 8.5 Hz, 1H), 4.96 (d, *J* = 3.6 Hz, 1H), 4.26 (s, 1H), 3.69 – 3.58 (m, 2H), 3.54 (d, *J* = 3.5 Hz, 1H), 3.25 (d, *J* = 2.5 Hz, 1H), 3.20 (d, *J* = 2.3 Hz, 1H), 3.19 (s, 3H), 2.98 (dd, *J* = 14.2, 7.2 Hz, 1H), 2.65 (dd, *J* = 14.2, 7.4 Hz, 1H), 1.73 (s, 1H), 1.60 (s, 3H), 1.23 – 1.11 (m, 1H), 1.09 (s, 3H), 0.52 (d, *J* = 7.8 Hz, 2H), 0.28 – 0.24 (m, 2H).



**(2*R*,3*R*)-3-((1*E*)-Buta-1,3-dienyl)-2-((*S*)-1-hydroxy-2-phenylethyl)-*N*-((2*S*,5*R*,6*R*)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)-4-methylenepyrrolidine (7a)**

To a stirred solution of **5a** in benzene (76.3 mg, 0.16 mmol, 0.05 M), was added bis(triphenylphosphine)palladium acetate (24.0 mg, 0.03 mmol, 0.2 equiv.) at room

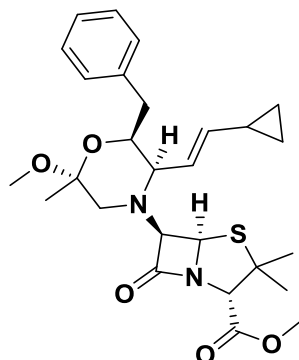
temperature under N<sub>2</sub>. After stirring for 1 h at 80 °C, the resulting solution was concentrated by rotary evaporation and the residue was purified by flash column chromatography (ISCO) to give **7a** as a colorless oil (31.0 mg, 39 % yield, 5/1 inseparable diastereomixture). Selected spectroscopic data for major diastereomer: <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.35 – 7.15 (m, 5H), 6.36 (dd, *J* = 11.3, 7.2 Hz, 1H), 6.18 (t, *J* = 11.6, 10.1 Hz, 1H), 5.60 (dd, *J* = 8.4, 7.5 Hz, 1H), 5.34 (d, *J* = 3.9 Hz, 1H), 5.21 (d, *J* = 16.8 Hz, 1H), 5.08 (d, *J* = 10.1 Hz, 1H), 5.00 (s, 1H), 4.90 (s, 1H), 4.51 (d, *J* = 4.0 Hz, 1H), 4.42 (s, 1H), 4.31 – 4.22 (m, 1H), 3.87 (d, *J* = 13.8 Hz, 1H), 3.78 (s, 3H), 3.55 (d, *J* = 13.1 Hz, 2H), 3.01 (d, *J* = 5.5 Hz, 1H), 2.82 (d, *J* = 7.3 Hz, 1H), 2.76 (d, *J* = 14.0 Hz, 1H), 1.69 (s, 3H), 1.48 (s, 3H). <sup>13</sup>C-NMR (100 MHz, C<sub>6</sub>D<sub>6</sub>) δ: 173.1, 168.1, 149.5, 139.8, 136.9, 135.8, 132.4, 129.7, 128.5, 126.2, 116.2, 107.1, 72.4, 72.3, 71.6, 69.9, 67.0, 64.4, 56.9, 51.4, 47.2, 39.6, 30.6, 26.5.



**(1R,2R,5E,8R)-8-Cyclopropyl-2-((S)-1-hydroxy-2-phenylethyl)-3-((2S,5R,6R)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)-3-azabicyclo[3.3.0]oct-5-en-6-one (**8a**)**

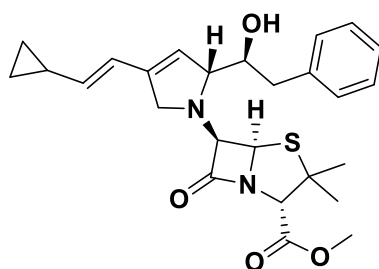
To a stirred solution of **5a** in benzene (47.8 mg, 0.10 mmol, 0.1 M), was added Co<sub>2</sub>(CO)<sub>8</sub> (52.3 mg, 0.15 mmol, 1.5 equiv.) and 3Å molecular sieves (activated) at room temperature under N<sub>2</sub>. After stirring for 90 min at the same temperature, trimethylamine *N*-oxide (76.6 mg, 1.02 mmol, 10 equiv.) was added at 0 °C and the resulting indigo suspension was warmed to room temperature, opened to air and stirred for 4 h at room temperature. The suspension was filtered under vacuum, concentrated by rotary evaporation and the resulting residue was purified by flash column chromatography (ISCO) to give **8a** as a colorless oil (14.4 mg, 29 % yield). <sup>1</sup>H-NMR (400 MHz, C<sub>6</sub>D<sub>6</sub>) δ: 7.37 – 7.19 (m, 4H), 7.14 – 7.06 (m, 1H), 5.65 (s, 1H), 5.00 (d, *J* = 3.7 Hz, 1H), 4.43 (s, 1H), 4.13 (d, *J* = 9.8 Hz, 1H), 4.09 (d, *J* = 3.6 Hz, 1H), 3.81 (d, *J* = 14.0 Hz, 1H), 3.37 (d, *J* = 15.7 Hz, 1H), 3.16 (s, 3H), 2.93 (dd, *J* = 13.8, 3.6 Hz, 1H), 2.88 (s, 1H), 2.66 (dd, *J* = 14.0, 3.1 Hz, 1H),

2.59 (dd,  $J = 9.0, 2.0$  Hz, 1H), 1.64 (s, 1H), 1.32 (s, 3H), 1.21 (s, 3H), 0.81 – 0.72 (m, 2H), 0.61 – 0.50 (m, 1H), 0.39 – 0.26 (m, 2H).



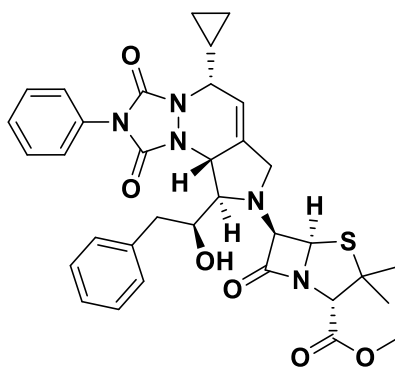
**(2*R*,5*R*,6*S*)-6-Benzyl-5-((*E*)-2-cyclopropylvinyl)-2-methoxy-4-((2*S*,5*R*,6*R*)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)-2-methylmorpholine (9a)**

To a stirred solution of **5a** in MeOH (73.7 mg, 0.16 mmol, 0.05 M), was added NaAuCl<sub>4</sub>·2H<sub>2</sub>O (7.2 mg, 17.00 μmol, 0.1 equiv.) at room temperature under N<sub>2</sub>. After stirring for 1 h at room temperature, reaction mixture was concentrated by rotary evaporation and the resulting residue was purified by flash column chromatography (ISCO) to give **9a** as a colorless oil (28.6 mg, 31 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.26 – 7.15 (m, 5H), 5.87 (dd,  $J = 15.4, 10.1$  Hz, 1H), 5.30 (dd,  $J = 15.4, 8.8$  Hz, 1H), 5.22 (d,  $J = 4.0$  Hz, 1H), 4.43 (d,  $J = 4.0$  Hz, 1H), 4.35 (s, 1H), 4.29 – 4.23 (m, 1H), 3.75 (s, 3H), 3.36 (dd,  $J = 10.0, 2.5$  Hz, 1H), 2.92 (s, 3H), 2.80 (d,  $J = 11.5$  Hz, 1H), 2.67 (dd,  $J = 13.8, 8.8$  Hz, 1H), 2.61 (d,  $J = 11.5$  Hz, 1H), 2.54 (dd,  $J = 13.8, 5.1$  Hz, 1H), 1.70 (s, 3H), 1.54 – 1.47 (m, 1H), 1.44 (s, 3H), 1.25 (s, 3H), 0.77 (d,  $J = 8.0$  Hz, 2H), 0.47 – 0.39 (m, 2H). LRMS (ESI<sup>+</sup>) calculated for C<sub>27</sub>H<sub>36</sub>N<sub>2</sub>O<sub>5</sub>S [M+H]<sup>+</sup>: 501.23, found: 501.57.



**(2R)-4-((E)-2-Cyclopropylvinyl)-2-((S)-1-hydroxy-2-phenylethyl)-N-((2S,5R,6R)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)-3-pyrroline (10a)**

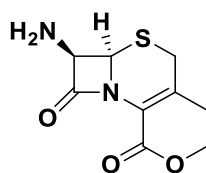
To a stirred solution of **5a** in CH<sub>2</sub>Cl<sub>2</sub> (56.7 mg, 0.12 mmol, 0.05 M), was added Grubbs catalyst second generation (11.0 mg, 12.70 μmol, 0.1 equiv.) at room temperature under N<sub>2</sub>. After stirring for 1 h under reflux, the reaction mixture was concentrated by rotary evaporation and the resulting residue was purified by flash column chromatography (ISCO) to give **10a** as a yellow oil (32.5 mg, 88 % yield, *trans/cis* = 5.6/1, inseparable mixture). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>,) δ: 7.32 – 7.27 (m, 4H), 7.23 – 7.17 (m, 1H), 6.28 (d, *J* = 15.8 Hz, 1H), 5.58 (s, 1H), 5.36 (d, *J* = 3.6 Hz, 1H), 5.03 (dd, *J* = 15.7, 8.9 Hz, 1H), 4.50 (d, *J* = 3.4 Hz, 1H), 4.43 (s, 1H), 4.20-4.07 (s, 2H), 4.15 – 4.09 (m, 1H), 3.77 (s, 3H), 3.50 (d, *J* = 10.3 Hz, 1H), 2.84 (dd, *J* = 13.8, 8.4 Hz, 1H), 2.72 (dd, *J* = 14.0, 5.1 Hz, 1H), 1.67 (s, 3H), 1.47 (s, 3H), 1.45 – 1.40 (m, 1H), 0.79 (d, *J* = 8.4 Hz, 2H), 0.45 – 0.38 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ: 171.4, 168.7, 139.0, 137.0, 129.2, 128.4, 126.2, 121.2, 119.4, 75.08, 72.67, 72.05, 70.12, 65.6, 64.6, 59.4, 52.4, 39.4, 30.8, 26.8, 14.3, 7.6, 7.5.



**Methyl (2S,5R)-6-((5R,9aR)-5-cyclopropyl-9-((R)-1-hydroxy-2-phenylethyl)-1,3-dioxo-2-phenyl-2,3,5,7,9,9a-hexahydro-1H,8H-pyrrolo[3,4-c][1,2,4]triazolo[1,2-a]pyridazin-8-yl)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (11a)**

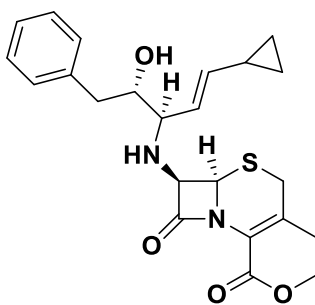
To a stirred solution of **10a** (*trans/cis* = 5.6/1) in CH<sub>2</sub>Cl<sub>2</sub> (28.9 mg, 0.06 mmol, 0.1 M), was added 4-phenyl-1,2,4-triazolin-3,5-dione (21.6 mg, 0.12 mmol, 2 equiv.) at room temperature under N<sub>2</sub>. After stirring for 30 min at room temperature, the reaction mixture was concentrated by rotary evaporation and the resulting residue was purified by flash column chromatography (ISCO) to give **11a** as a colorless oil (14.5 mg, 37 % yield). <sup>1</sup>H-

NMR (400 MHz, CDCl<sub>3</sub>,)  $\delta$ : 7.58 – 7.45 (m, 4H), 7.43 – 7.36 (m, 1H), 7.28 – 7.23 (m, 3H), 7.22 – 7.14 (m, 1H), 5.85 (s, 1H), 5.38 (d,  $J = 3.7$  Hz, 1H), 4.55 (s, 1H), 4.52 (d,  $J = 3.7$  Hz, 1H), 4.47 (s, 1H), 4.18 (dd,  $J = 8.2, 3.8$  Hz, 1H), 4.10 – 4.03 (m, 1H), 3.86 – 3.79 (m, 1H), 3.78 (s, 3H), 3.68 (d,  $J = 13.9$  Hz, 1H), 3.36 (d,  $J = 13.9$  Hz, 1H), 3.20 (d,  $J = 14.0$  Hz, 1H), 2.68 (dd,  $J = 14.0, 10.4$  Hz, 1H), 1.74 (s, 3H), 1.49 (s, 3H), 1.16 – 1.07 (m, 1H), 0.98 – 0.90 (m, 1H), 0.72 – 0.56 (m, 2H), 0.41 – 0.32 (m, 1H). LRMS (ESI+) calculated for C<sub>34</sub>H<sub>37</sub>N<sub>5</sub>O<sub>6</sub>S [M+H<sup>+</sup>]<sup>+</sup>: 644.25, found: 644.70.



### 7-amino-deacetoxycephalosporanic acid (7-ACDA) methyl ester (**1b**)

General procedure A was followed using 7-ACDA (2.00 g, 9.34 mmol), methanol (4 mL), and (diazomethyl)trimethylsilane (4.7 mL, 9.34 mmol) to give the desired compound **1b** as a pale yellow solid (1.35 g, 64 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$ : 4.89 (s, 1H), 4.69 (s, 1H), 3.79 (s, 3H), 3.47 (d,  $J = 18.0$  Hz, 1H), 3.17 (d,  $J = 17.9$  Hz, 1H), 2.11 (s, 3H), 1.82 (s, 2H).

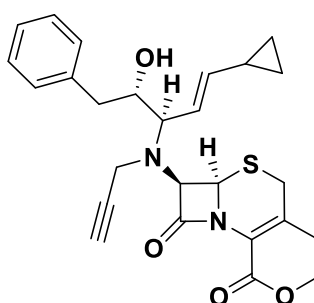


### Methyl (6R,7R)-7-(((4R,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)amino)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (**4b**)

General procedure B was followed using (4R,5S)-5-benzyl-2,2-dimethyl-4-hydroxy-1,3-dioxolane **2** (770 mg, 3.70 mmol, 0.2 M) in 18.5 mL of the solvent system of 9:1 EtOH–hexafluoroisopropanol (v/v), 7-ACDA methyl ester **1a** (1.27 g, 5.55 mmol, 1.5 equiv) and (*E*)-2-cyclopropylvinylboronic acid **3** (621 mg, 5.55 mmol, 1.5 equiv) to give the desired product **4b** as a pale yellow oil (1.03 g, 65 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$ : 7.34

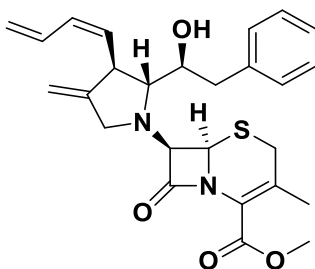


– 7.16 (m, 5H), 5.57 (dd,  $J = 14.4, 8.0$  Hz, 1H), 5.25 (dd,  $J = 15.6, 8.7$  Hz, 1H), 4.85 (d,  $J = 4.1$  Hz, 1H), 4.57 (d,  $J = 5.1$  Hz, 1H), 4.06 – 3.93 (m, 1H), 3.83 (s, 3H), 3.49 (d,  $J = 18.1$  Hz, 1H), 3.23 (s, 1H), 3.20 (d,  $J = 13.4$  Hz, 1H), 2.81 – 2.66 (m, 2H), 2.44 (br, 1H), 2.12 (s, 3H), 1.54 – 1.38 (m, 1H), 0.77 (d,  $J = 6.6$  Hz, 2H), 0.49 – 0.38 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 166.9, 162.7, 142.1, 138.1, 129.4, 128.5, 128.5, 126.4, 122.7, 122.6, 66.4, 64.8, 60.4, 58.5, 52.3, 39.6, 20.1, 13.8, 7.0, 7.0. LRMS (ESI<sup>+</sup>)  $m/z$  calculated for  $\text{C}_{23}\text{H}_{28}\text{N}_2\text{O}_4\text{S}$  ( $[\text{M}+\text{H}^+]^+$ ): 429.18, found: 429.53.



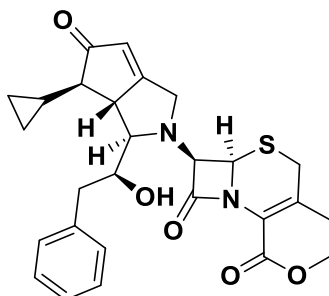
**Methyl (6R,7R)-7-(((4R,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)(prop-2-yn-1-yl)amino)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (5b)**

General procedure C was followed using **4b** (897 mg, 2.09 mmol, 0.2 M) and  $\text{NaHCO}_3$  (1.76 g, 20.93 mmol, 10 equiv.) in DMF (10.5 mL) and propargyl bromide (3.1 mL, 20.93 mmol, 10 equiv., 80 % in toluene) to give the desired compound **5b** (640 mg, 66 % yield).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.35 – 7.19 (m, 5H), 5.73 (dd,  $J = 15.3, 9.5$  Hz, 1H), 5.33 (dd,  $J = 15.5, 8.8$  Hz, 1H), 4.80 (dd,  $J = 4.4$  Hz, 1H), 4.72 (dd,  $J = 4.5$  Hz, 1H), 4.10 – 4.07 (m, 1H), 3.99 (d,  $J = 17.8$  Hz, 1H), 3.85 (s, 3H), 3.71 (d,  $J = 17.7$  Hz, 1H), 3.51 (d,  $J = 18.3$  Hz, 1H), 3.39 (dd,  $J = 9.6, 3.5$  Hz, 1H), 3.23 (d,  $J = 18.3$  Hz, 1H), 2.75 (d,  $J = 6.6$  Hz, 2H), 2.51 (br, 1H), 2.23 (s, 1H), 2.12 (s, 3H), 1.57 – 1.46 (m, 1H), 0.79 (d,  $J = 8.0$  Hz, 2H), 0.52 – 0.42 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 171.1, 162.9, 143.0, 138.6, 129.3, 128.4, 126.3, 122.5, 120.6, 73.6, 71.5, 69.7, 67.5, 60.4, 59.4, 52.4, 40.2, 39.9, 30.7, 20.0, 13.8, 7.1, 7.0. LRMS (ESI<sup>+</sup>)  $m/z$  calculated for  $\text{C}_{26}\text{H}_{30}\text{N}_2\text{O}_4\text{S}$  ( $[\text{M}+\text{H}^+]^+$ ): 467.20, found: 467.53.



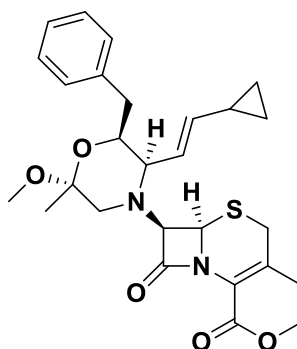
**Methyl (6R,7R)-7-((2R,3R)-3-((Z)-buta-1,3-dien-1-yl)-2-((S)-1-hydroxy-2-phenylethyl)-4-methylenepyrrolidin-1-yl)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (7b)**

To a stirred solution of **5b** in benzene (57.8 mg, 0.12 mmol, 0.05 M), was added bis(triphenylphosphine)palladium acetate (18.4 mg, 25.00  $\mu$ mol, 0.2 equiv.) at room temperature under  $N_2$ . After stirring for 1 h at 80  $^{\circ}C$ , the resulting solution was concentrated by rotary evaporation and the residue was purified by flash column chromatography (ISCO) to give **7b** as a colorless oil (32.4 mg, 56 % yield). Selected spectroscopic data for major diastereomer:  $^1H$ -NMR (400 MHz,  $C_6D_6$ )  $\delta$ : 7.42 – 7.20 (m, 5H), 6.28 (dd,  $J = 10.8, 9.4$  Hz, 1H), 6.06 (dd,  $J = 15.2, 10.4$  Hz, 1H), 5.57 (dd,  $J = 15.1, 8.7$  Hz, 1H), 5.07 (d,  $J = 16.9$  Hz, 1H), 4.95 (d,  $J = 8.4$  Hz, 1H), 4.86 (dd,  $J = 14.6, 2.2$  Hz, 1H), 4.20 (d,  $J = 4.4$  Hz, 1H), 4.14 (d,  $J = 4.4$  Hz, 1H), 3.95 (d,  $J = 13.8$  Hz, 1H), 3.72 (d,  $J = 2.1$  Hz, 1H), 3.63 – 3.56 (m, 1H), 3.54 (s, 3H), 3.25 (dd,  $J = 6.0, 2.5$  Hz, 1H), 2.90 (d,  $J = 8.9$  Hz, 1H), 2.78 (dd,  $J = 13.9, 4.2$  Hz, 1H), 2.61 (d,  $J = 18.5$  Hz, 1H), 2.29 (d,  $J = 18.1$  Hz, 1H), 1.67 (s, 3H), 0.43 (br, 1H).  $^{13}C$ -NMR (100 MHz,  $C_6D_6$ )  $\delta$ : 163.7, 162.3, 149.7, 139.8, 136.9, 135.5, 132.3, 129.5, 128.2, 125.8, 122.8, 115.9, 106.8, 72.6, 71.6, 71.1, 57.4, 57.2, 51.6, 47.0, 39.5, 29.4, 19.2.



**Methyl (6R,7S)-7-((3S,3aS,4S)-4-cyclopropyl-3-((R)-1-hydroxy-2-phenylethyl)-5-oxo-3,3a,4,5-tetrahydrocyclopenta[c]pyrrol-2(1H)-yl)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (8b)**

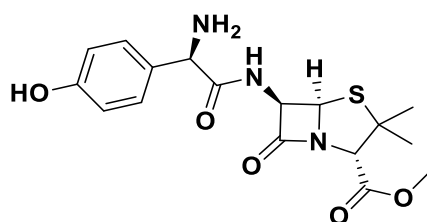
To a stirred solution of **5b** in benzene (80.0 mg, 0.17 mmol, 0.1 M), was added  $\text{Co}_2(\text{CO})_8$  (87.9 mg, 0.26 mmol, 1.5 equiv.) and 3Å molecular sieves (activated) at room temperature under  $\text{N}_2$ . After stirring for 90 min at the same temperature, trimethylamine *N*-oxide (128.78 mg, 1.71 mmol, 10 equiv.) was added at 0 °C and the resulting indigo suspension was warmed to room temperature, opened to air and stirred for 4 h at room temperature. The suspension was filtered under vacuum, concentrated by rotary evaporation and the resulting residue was purified by flash column chromatography (ISCO) to give **8a** as a colorless oil (24.4 mg, 30 % yield).  $^1\text{H-NMR}$  (400 MHz,  $\text{C}_6\text{D}_6$ )  $\delta$ : 7.32 – 7.18 (m, 4H), 7.13 – 7.08 (m, 1H), 5.72 (s, 1H), 4.43 (d,  $J = 4.2$  Hz, 1H), 4.17 (d,  $J = 4.2$  Hz, 1H), 3.99 (d,  $J = 10.0$  Hz, 1H), 3.71 (dd,  $J = 62.8, 15.6$  Hz, 2H), 3.58 (s, 3H), 3.16 (s, 1H), 2.95 – 2.88 (m, 1H), 2.90 – 2.88 (m, 1H), 2.87 (s, 1H), 2.66 (d,  $J = 18.0$  Hz, 1H), 2.64 (s, 1H), 2.31 (d,  $J = 18.2$  Hz, 1H), 1.69 (s, 3H), 0.81 – 0.69 (m, 1H), 0.61 – 0.52 (m, 2H), 0.37 – 0.31 (m, 1H), 0.29 – 0.23 (m, 1H).  $^{13}\text{C-NMR}$  (100 MHz,  $\text{C}_6\text{D}_6$ )  $\delta$ : 178.6, 163.3, 162.8, 129.8, 128.9, 128.8, 128.7, 126.7, 124.2, 122.9, 71.8, 71.5, 70.5, 57.5, 54.2, 52.1, 51.9, 39.0, 38.7, 30.3, 19.5, 11.9, 3.6, 3.3. LRMS (ESI<sup>+</sup>)  $m/z$  calculated for  $\text{C}_{27}\text{H}_{30}\text{N}_2\text{O}_5\text{S}$  ( $[\text{M}+\text{H}]^+$ ): 495.19, found: 495.56.



**Methyl (6R,7R)-7-(((6R)-6-benzyl-5-((E)-2-cyclopropylvinyl)-2-methoxy-2-methylmorpholino)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (9b)**

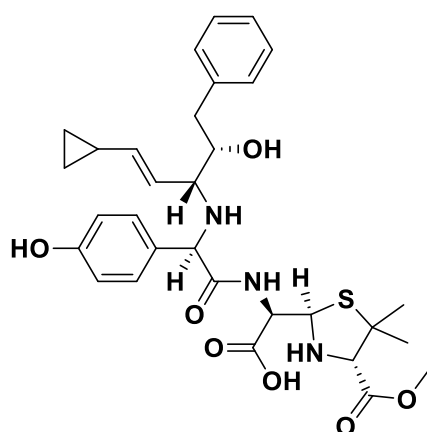
To a stirred solution of **5b** in MeOH (33.7 mg, 0.07 mmol, 0.05 M), was added  $\text{NaAuCl}_4 \cdot 2\text{H}_2\text{O}$  (2.9 mg, 7.00  $\mu\text{mol}$ , 0.1 equiv.) at room temperature under  $\text{N}_2$ . After stirring for 1 h at room temperature, reaction mixture was concentrated by rotary evaporation and the resulting residue was purified by flash column chromatography (ISCO) to give **9b** as a colorless oil (12.0 mg, 33 % yield).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.28 – 7.13 (m, 5H), 5.86 (dd,  $J = 15.5, 9.9$  Hz, 1H), 5.49 (dd,  $J = 15.4, 8.9$  Hz, 1H),

4.78 (d,  $J = 4.4$  Hz, 1H), 4.39 (d,  $J = 4.3$  Hz, 1H), 4.33 – 4.27 (m, 1H), 3.85 (s, 3H), 3.61 (d,  $J = 10.6$  Hz, 1H), 3.45 (d,  $J = 18.1$  Hz, 1H), 3.21 (d,  $J = 18.2$  Hz, 1H), 2.94 (s, 3H), 2.73 (q,  $J = 11.1$  Hz, 2H), 2.66 (d,  $J = 8.4$  Hz, 1H), 2.57 (d,  $J = 5.1$  Hz, 1H), 2.06 (s, 3H), 1.58 – 1.48(m, 1H), 1.28 (s, 3H), 0.83 – 0.73 (m, 2H), 0.52 – 0.43 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 163.1, 162.7, 143.5, 138.8, 129.3, 128.4, 126.3, 122.8, 117.0, 96.8, 72.3, 71.4, 60.0, 59.4, 57.1, 54.2, 52.3, 48.1, 38.7, 30.9, 21.9, 20.2, 14.1, 7.0, 7.0. LRMS (ESI+) calculated for  $\text{C}_{27}\text{H}_{34}\text{N}_2\text{O}_5\text{S}$   $[\text{M}+\text{H}^+]^+$ : 499.22, found: 499.55.



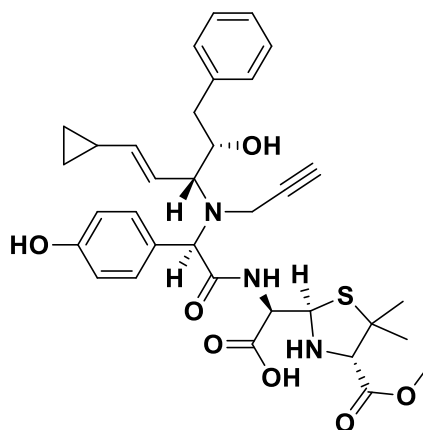
#### Amoxicillin methyl ester (1c)

General procedure A was followed using amoxicillin (500 mg, 1.37 mmol), methanol (1 mL), and (diazomethyl)trimethylsilane (685  $\mu\text{L}$ , 1.37 mmol) to give the desired compound **1c** as a pale yellow solid (383 g, 74 % yield).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 8.10 (br, 1H), 7.06 (d,  $J = 7.8$  Hz, 2H), 6.61 (d,  $J = 7.7$  Hz, 2H), 5.60 (s, 1H), 5.52 (s, 1H), 4.44 (s, 2H), 4.28 (br, 2H), 3.77 (s, 3H), 1.61 (s, 3H), 1.47 (s, 3H).



**(R)-2-((R)-2-(((3R,4S,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)amino)-2-(4-hydroxyphenyl)acetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid (4c)**

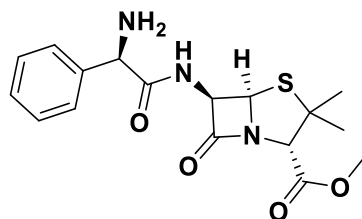
General procedure B was followed using (4R,5S)-5-benzyl-2,2-dimethyl-4-hydroxy-1,3-dioxolane **2** (526 mg, 2.52 mmol, 0.2 M) in 12.5 mL of the solvent system of 9:1 EtOH–hexafluoroisopropanol (v/v), AMX methyl ester **1c** (1.14 g, 3.00 mmol, 1.2 equiv) and (*E*)-2-cyclopropylvinylboronic acid **3** (418 mg, 2.88 mmol, 1.5 equiv) to give the desired product **4c** as a pale yellow solid (671 mg, 46 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.37 (d, *J* = 9.1 Hz, 2H), 7.33 – 7.21 (m, 5H), 7.17 (d, *J* = 6.9 Hz, 2H), 6.66 (d, *J* = 6.8 Hz, 2H), 5.52 (dd, *J* = 15.4, 8.7 Hz, 1H), 5.15 – 5.12 (m, 1H), 5.11 (dd, *J* = 15.4, 8.9 Hz, 1H), 4.58 (dd, *J* = 8.9, 3.3 Hz, 1H), 4.36 (s, 1H), 3.95 – 3.84 (m, 1H), 3.67 (s, 3H), 3.49 (s, 1H), 3.08 (d, *J* = 9.1 Hz, 1H), 2.76 (ddt, *J* = 3.8, 3.1 Hz, 1H), 2.70 (d, *J* = 9.3 Hz, 1H), 1.51 (s, 3H), 1.49 – 1.42 (m, 1H), 1.18 (s, 3H), 0.78 (d, *J* = 7.2 Hz, 2H), 0.42 (d, *J* = 5.1 Hz, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ: 173.0, 169.9, 169.7, 140.6, 129.4, 129.3, 128.5, 126.5, 126.3, 123.1, 116.1, 74.2, 72.7, 65.8, 63.2, 62.6, 58.8, 57.1, 52.3, 39.5, 26.5, 26.2, 13.7, 7.1, 7.0.



**(R)-2-((R)-2-(((3R,4S,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)(prop-2-yn-1-yl)amino)-2-(4-hydroxyphenyl)acetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid (**5c**)**

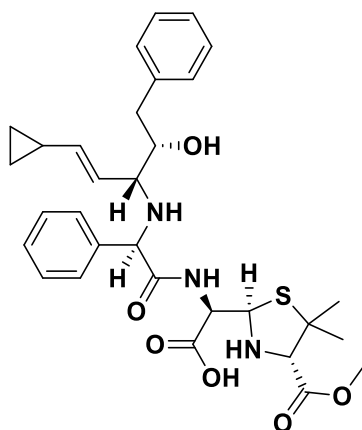
General procedure C was followed using **4c** (671 mg, 1.16 mmol, 0.2 M) and NaHCO<sub>3</sub> (145 mg, 1.74 mmol, 1.5 equiv.) in DMF (5.8 mL) and propargyl bromide (155 μL, 1.39 mmol, 1.2 equiv., 80 % in toluene) at room temperature to give the desired compound **5c** (404 mg, 56 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.99 (d, *J* = 8.8 Hz, 1H), 7.32 – 7.24 (m, 3H), 7.22 – 7.15 (m, 2H), 7.05 (d, *J* = 8.1 Hz, 2H), 6.57 (d, *J* = 8.1 Hz, 2H), 5.78 (dd, *J* = 15.4, 8.9 Hz, 1H), 5.11 (dd, *J* = 15.4, 8.9 Hz, 1H), 5.06 (d, *J* = 5.9 Hz, 1H), 4.71 (s, 1H), 4.67 (d, *J* = 5.9 Hz, 2H), 4.22 – 4.10 (m, 1H), 3.79 (s, 1H), 3.72 (s, 3H), 3.54 (d,

$J = 17.6$  Hz, 1H), 3.33 (s, 1H), 3.28 (d,  $J = 17.5$  Hz, 1H), 2.80 (dd,  $J = 4.1, 3.4$  Hz, 1H), 2.64 (dd,  $J = 13.7, 8.9$  Hz, 1H), 2.15 (s, 1H), 1.54 (s, 3H), 1.52 – 1.43 (m, 1H), 1.19 (s, 3H), 0.76 (d,  $J = 8.1$  Hz, 2H), 0.46 – 0.37 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 173.8, 170.1, 169.8, 142.5, 131.1, 129.5, 128.5, 127.0, 126.3, 120.9, 115.8, 81.3, 73.6, 73.0, 72.6, 68.4, 66.2, 66.2, 58.9, 57.2, 52.3, 41.4, 39.2, 27.1, 26.9, 13.9, 7.1, 7.0.



### Ampicillin methyl ester (**1d**)

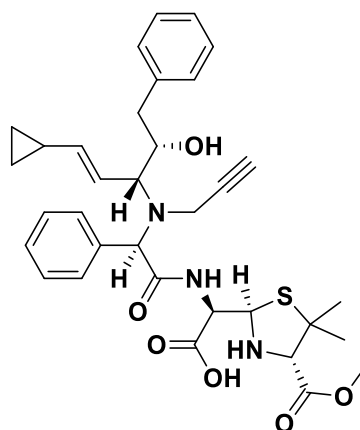
General procedure A was followed using amoxicillin (500 mg, 1.37 mmol), methanol (1 mL), and (diazomethyl)trimethylsilane (685  $\mu\text{L}$ , 1.37 mmol) to give the desired compound **1d** as a pale yellow solid (383 g, 74 % yield).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 8.03 (d,  $J = 9.2$  Hz, 1H), 7.29 – 7.42 (m, 5H), 5.68 (dd,  $J = 9.4, 4.1$  Hz, 1H), 5.56 (d,  $J = 4.2$  Hz, 1H), 4.60 (s, 1H), 4.47 (s, 1H), 3.79 (s, 3H), 2.62 (br, 2H), 1.66 (s, 3H), 1.51 (s, 3H).



### (*R*)-2-((*R*)-2-(((3*R*,4*S*,*E*)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)amino)-2-phenylacetamido)-2-((2*R*,4*S*)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid (**4d**)

General procedure B was followed using (4*R*,5*S*)-5-benzyl-2,2-dimethyl-4-hydroxy-1,3-dioxolane **2** (200 mg, 0.96 mmol, 0.2 M) in 4.8 mL of the solvent system of 9:1 EtOH–

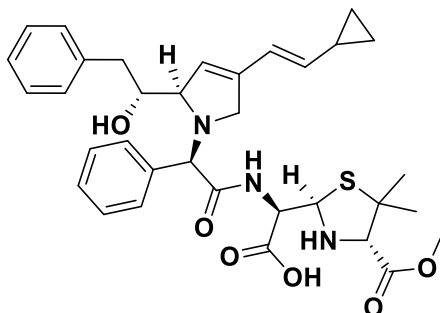
hexafluoroisopropanol (v/v), AMP methyl ester **1d** (418 mg, 1.15 mmol, 1.2 equiv) and (*E*)-2-cyclopropylvinylboronic acid **3** (161 mg, 1.44 mmol, 1.5 equiv) to give the desired product **4d** as a pale yellow solid (234 mg, 41 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.73 (s, 1H), 7.49 – 7.08 (m, 10H), 5.53 (dd, *J* = 15.3, 8.7 Hz, 1H), 5.13 (d, *J* = 3.8 Hz, 1H), 5.08 (dd, *J* = 15.3, 8.7 Hz, 1H), 4.60 (dd, *J* = 9.1, 3.8 Hz, 1H), 4.42 (s, 1H), 3.86 – 3.70 (m, 2H), 3.68 (s, 3H), 3.46 (s, 1H), 3.01 (dd, *J* = 8.8, 3.7 Hz, 1H), 2.78 (dd, *J* = 13.9, 3.8 Hz, 1H), 2.67 (dd, *J* = 13.8, 9.2 Hz, 1H), 2.45 (s, 1H), 2.29 (br, 1H), 1.48 (s, 3H), 1.48 – 1.41 (m, 1H), 1.17 (s, 3H), 0.84 – 0.67 (m, 2H), 0.48 – 0.29 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ: 169.8, 169.7, 164.8, 142.8, 129.3, 129.1, 128.5, 126.3, 122.3, 76.0, 72.7, 65.5, 62.8, 62.7, 58.6, 57.0, 52.2, 39.7, 26.5, 26.3, 13.7, 7.1, 7.0.



**(R)-2-((R)-2-(((3R,4S,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)(prop-2-yn-1-yl)amino)-2-phenylacetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid (**5d**)**

General procedure C was followed using **4d** (108 mg, 0.19 mmol, 0.2 M) and NaHCO<sub>3</sub> (24.1 mg, 0.29 mmol, 1.5 equiv.) in DMF (1.0 mL) and propargyl bromide (25 μL, 0.23 mmol, 1.2 equiv., 80 % in toluene) at room temperature to give the desired compound **5d** (44.0 mg, 38 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.73 (s, 1H), 7.36 – 7.16 (m, 10H), 5.81 (dd, *J* = 15.3, 9.5 Hz, 1H), 5.18 – 5.07 (m, 1H), 5.05 (d, *J* = 5.9 Hz, 1H), 4.84 (s, 1H), 4.71 (dd, *J* = 9.1, 6.0 Hz, 1H), 4.16 – 4.11 (m, 1H), 3.75 (s, 1H), 3.74 (s, 3H), 3.64 – 3.52 (m, 1H), 3.43 – 3.30 (m, 2H), 2.84 – 2.74 (m, 1H), 2.73 – 2.63 (m, 1H), 2.16 (br, 1H), 1.69 – 1.57 (m, 1H), 1.51 (s, 3H), 1.19 (s, 3H), 0.82 – 0.74 (m, 2H), 0.49 – 0.39 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ: 173.4, 170.2, 169.8, 148.6, 129.8, 129.3, 128.4,

126.3, 121.5, 78.5, 73.5, 73.2, 72.4, 68.4, 66.2, 66.1, 58.7, 56.7, 52.2, 41.4, 39.4, 27.0, 26.8, 13.8, 7.0, 6.9.



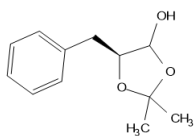
**(2R)-2-((2R)-2-(4-((E)-2-cyclopropylvinyl)-2-((R)-1-hydroxy-2-phenylethyl)-2,5-dihydro-1H-pyrrol-1-yl)-2-phenylacetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid (10d)**

To a stirred solution of **5d** in CH<sub>2</sub>Cl<sub>2</sub> (20.0 mg, 0.03 mmol, 0.05 M), was added Grubbs catalyst second generation (2.8 mg, 3.30 μmol, 0.1 equiv.) at room temperature under N<sub>2</sub>. After stirring for 1 h under reflux, the reaction mixture was concentrated by rotary evaporation and the resulting residue was purified by flash column chromatography (ISCO) to give **10a** as a yellow oil (11.9 mg, 60 % yield). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.47 – 7.15 (m, 10H), 7.04 – 6.91 (m, 1H), 6.29 (d, *J* = 15.8 Hz, 1H), 5.60 (d, *J* = 51.8 Hz, 1H), 5.22 – 5.13 (m, 1H), 5.10 – 5.00 (m, 1H), 4.83 (s, 1H), 4.70 (dd, *J* = 8.9, 5.4 Hz, 1H), 4.20 – 4.07 (m, 2H), 4.15 – 4.09 (m, 1H), 3.77 (s, 3H), 3.58 – 3.48 (m, 1H), 3.37 (s, 1H), 2.71 – 2.62 (m, 1H), 2.52 (dd, *J* = 13.7, 5.3 Hz, 1H), 1.51 (s, 3H), 1.50 – 1.38 (m, 1H), 1.22 (s, 3H), 0.82 – 0.74 (m, 2H), 0.47 – 0.38 (m, 2H).

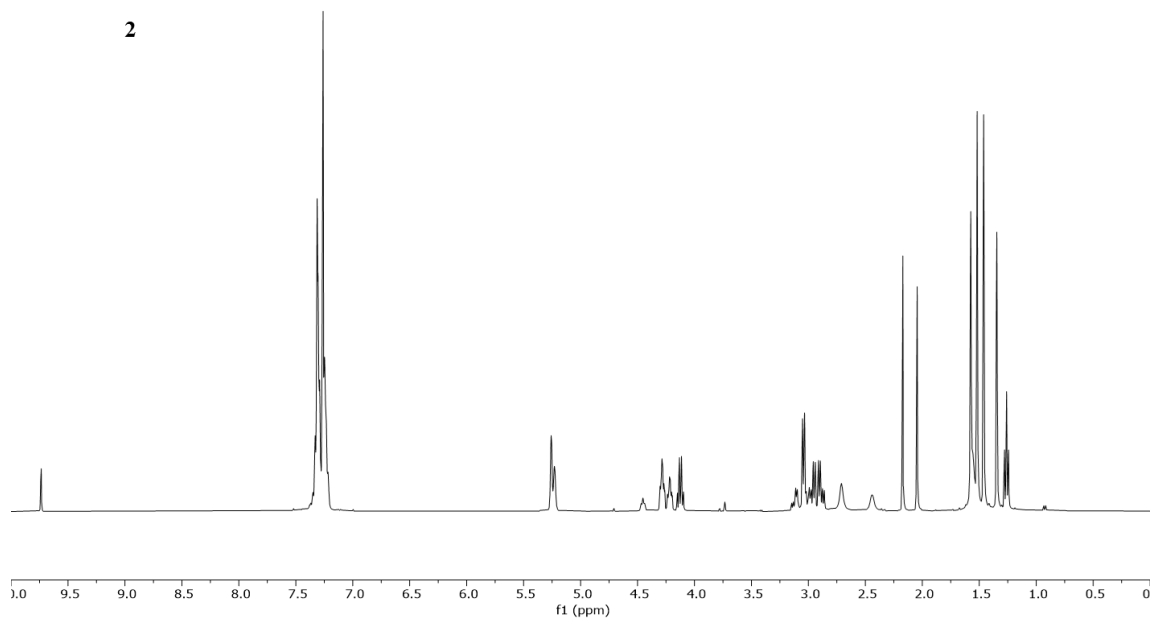


### 3. NMR spectrum

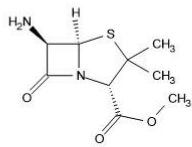
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



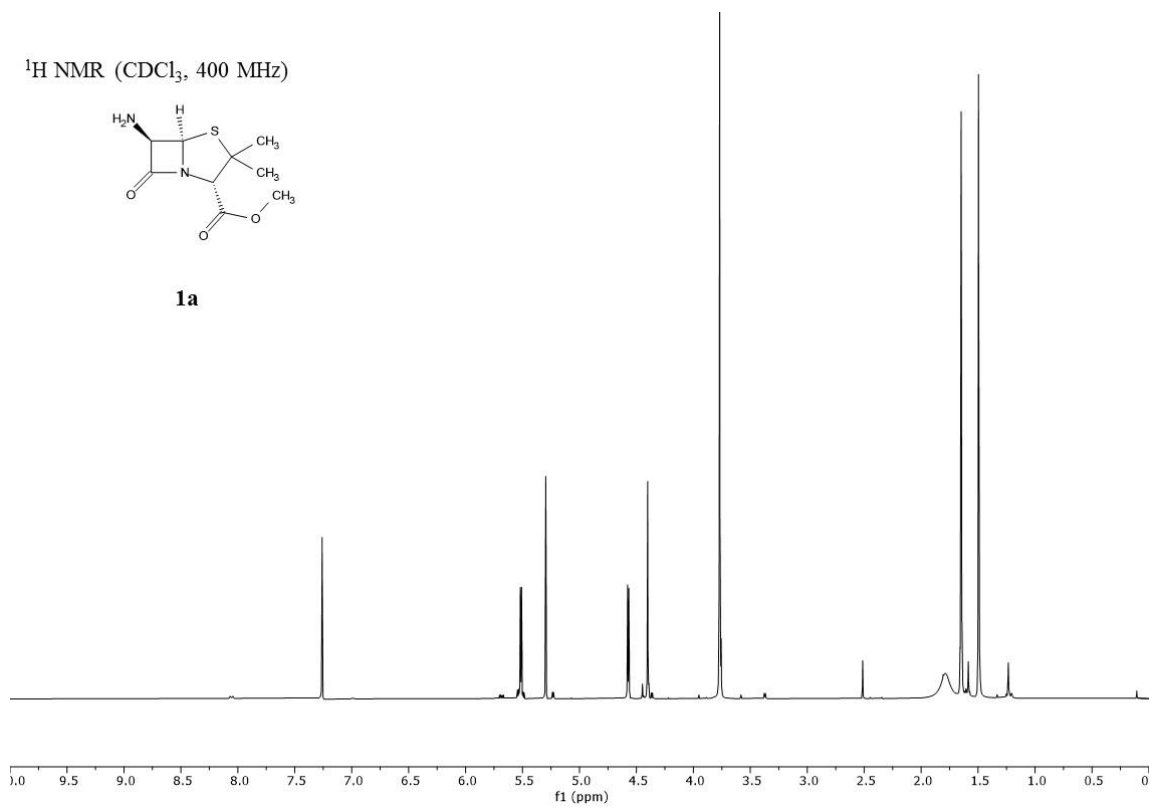
**2**

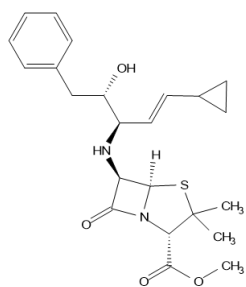
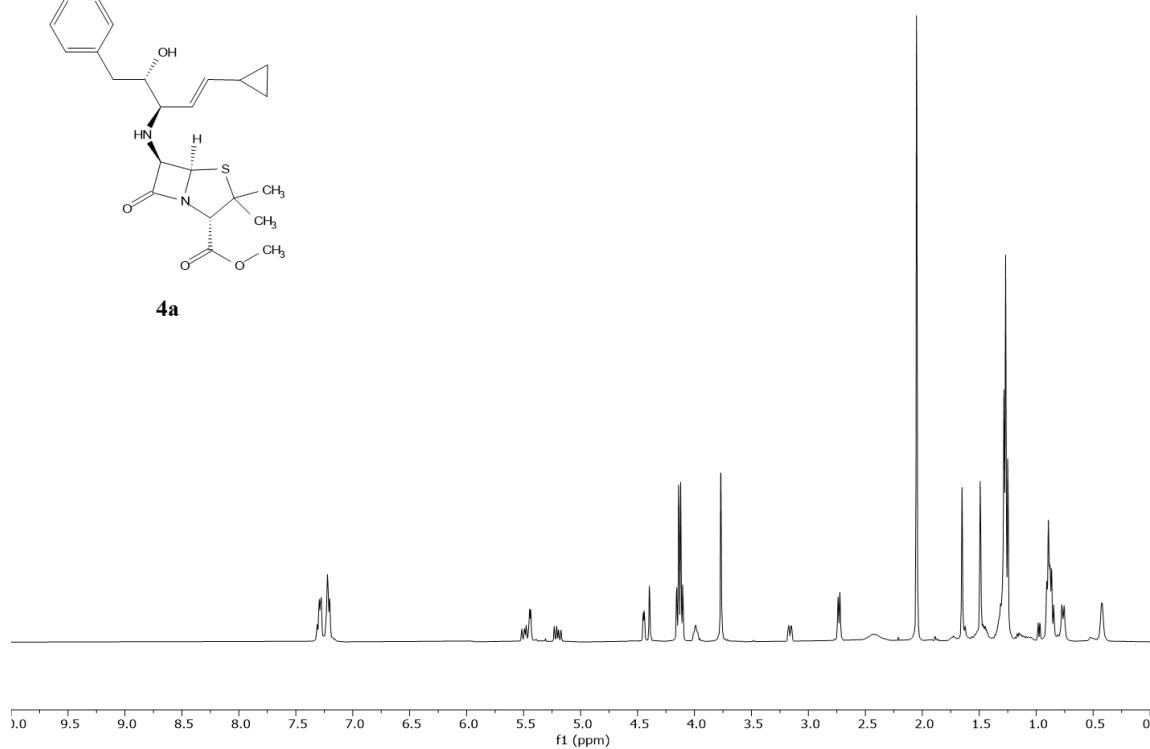
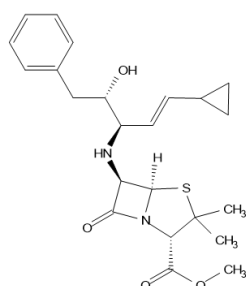
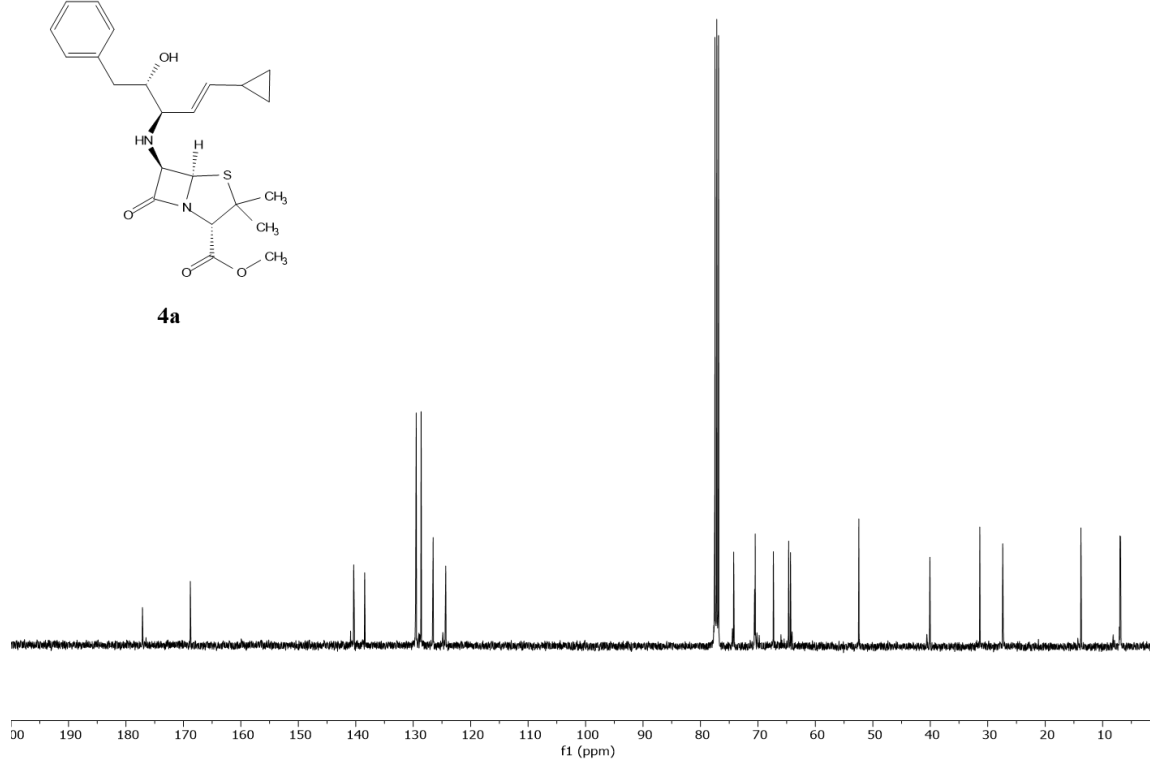


$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



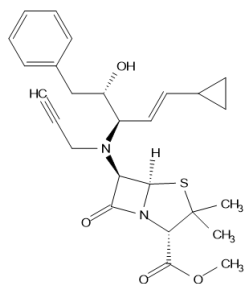
**1a**



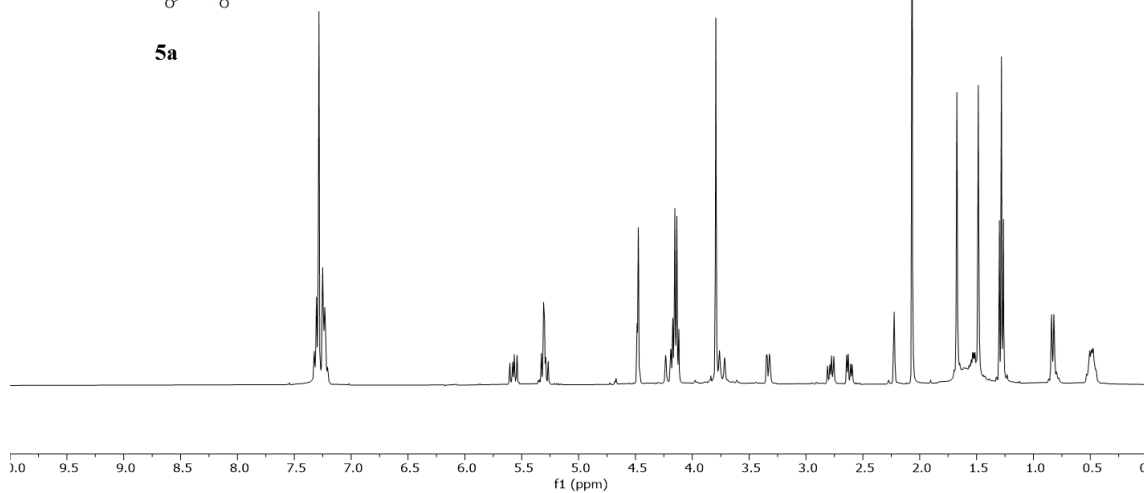
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)**4a** $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)**4a**

Annex 5

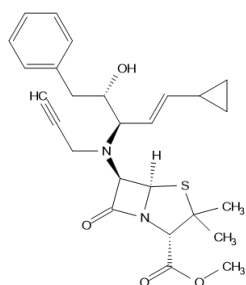
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



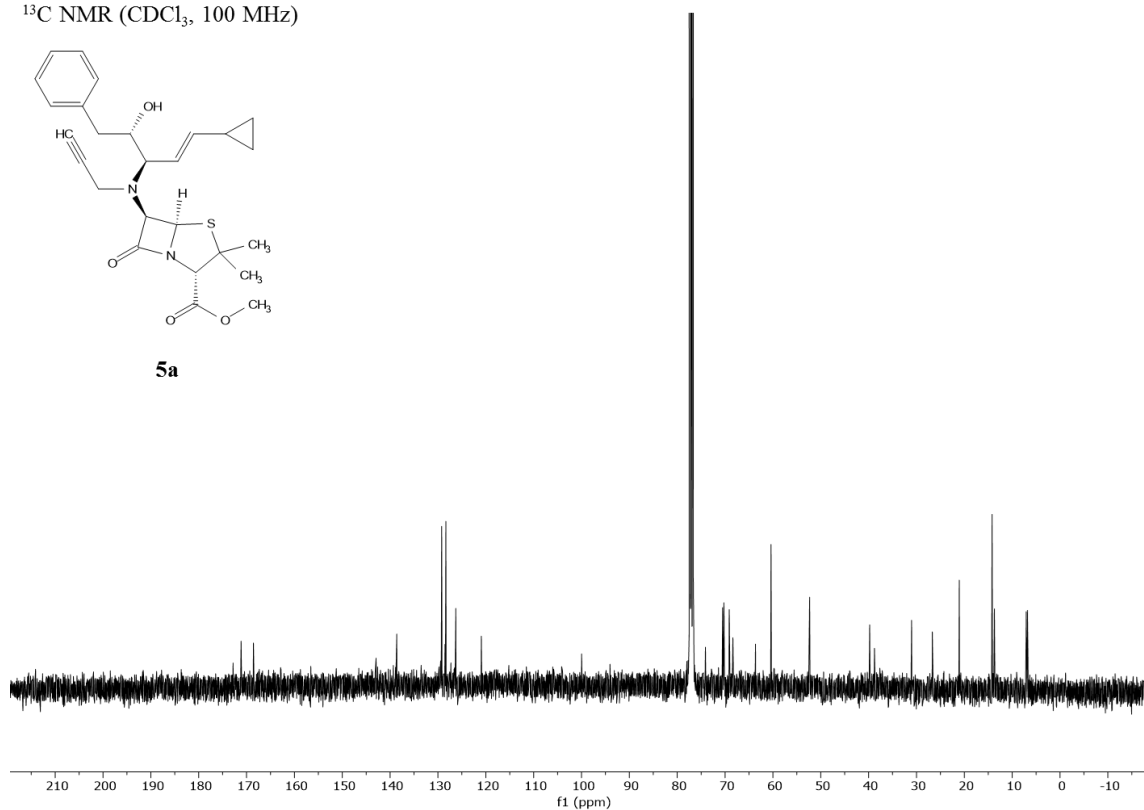
**5a**

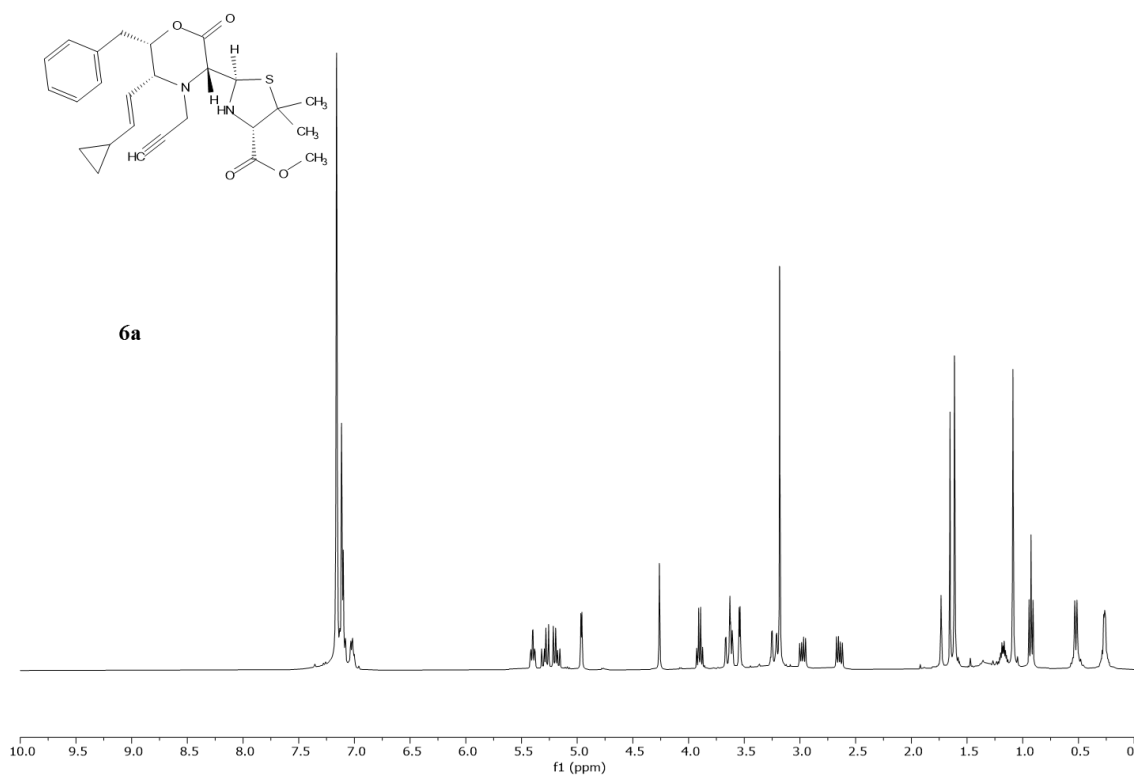


$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)



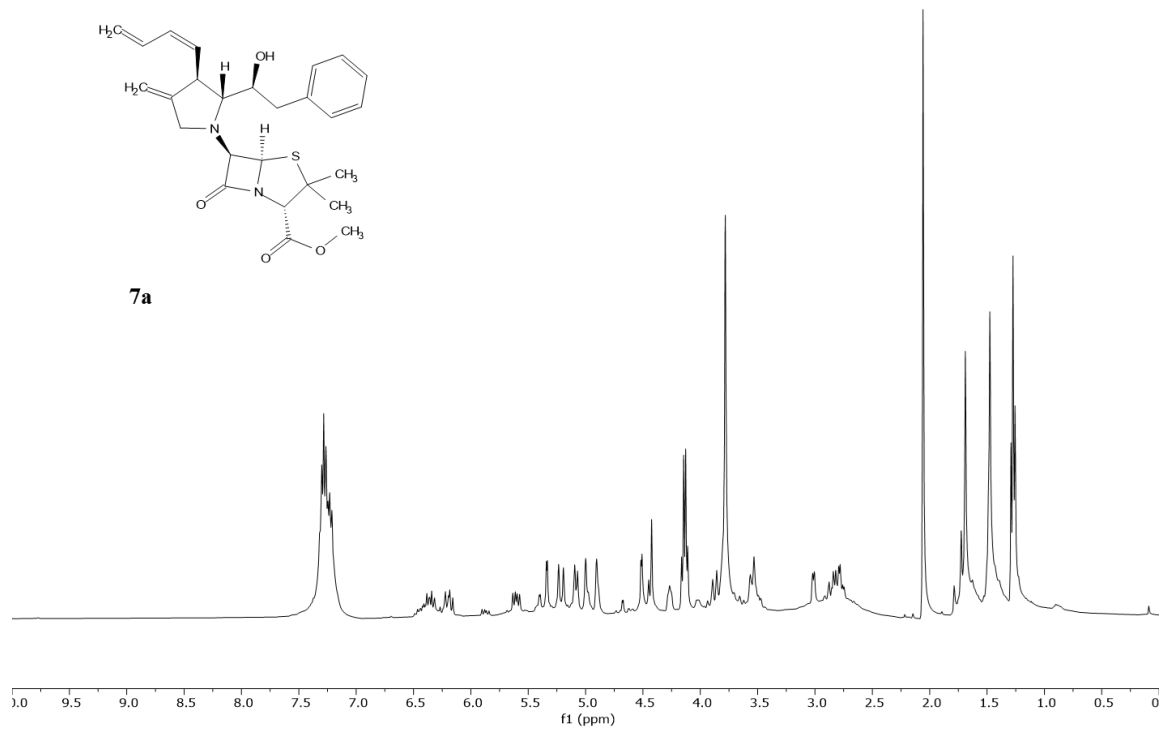
**5a**



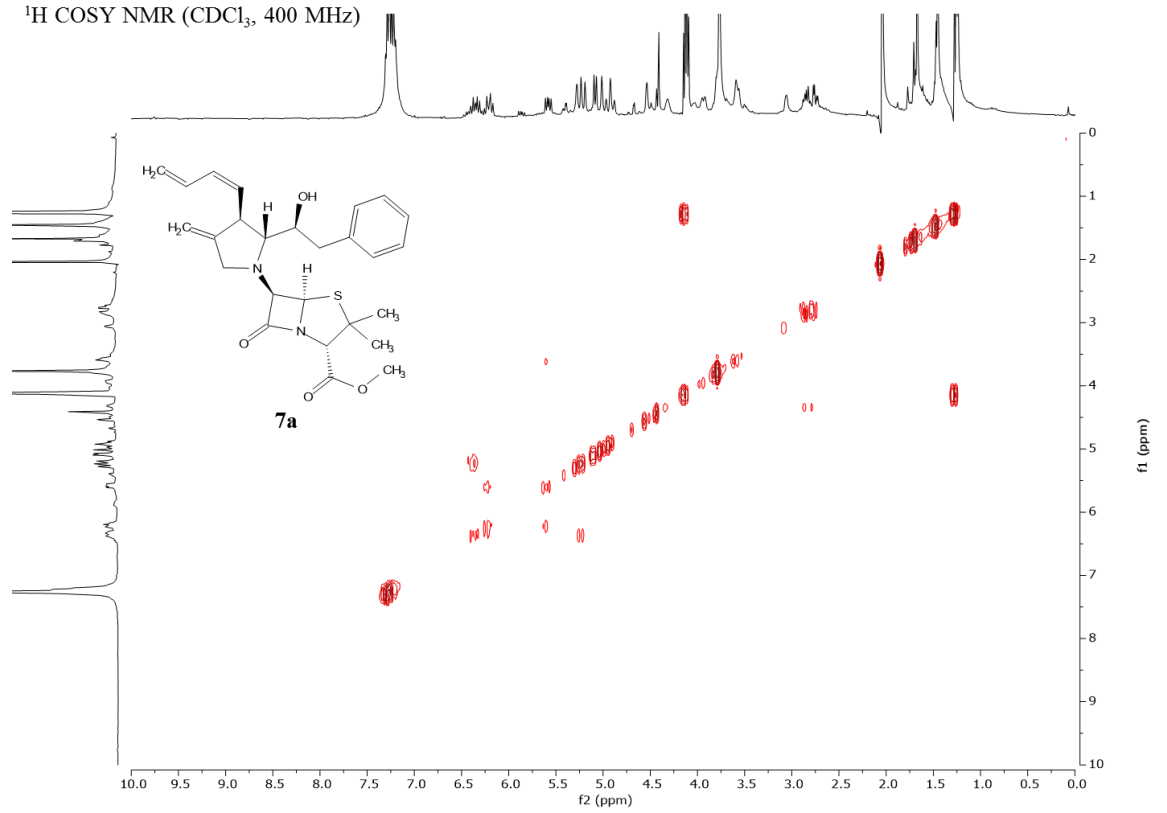
$^1\text{H}$  NMR ( $\text{C}_6\text{D}_6$ , 400 MHz)

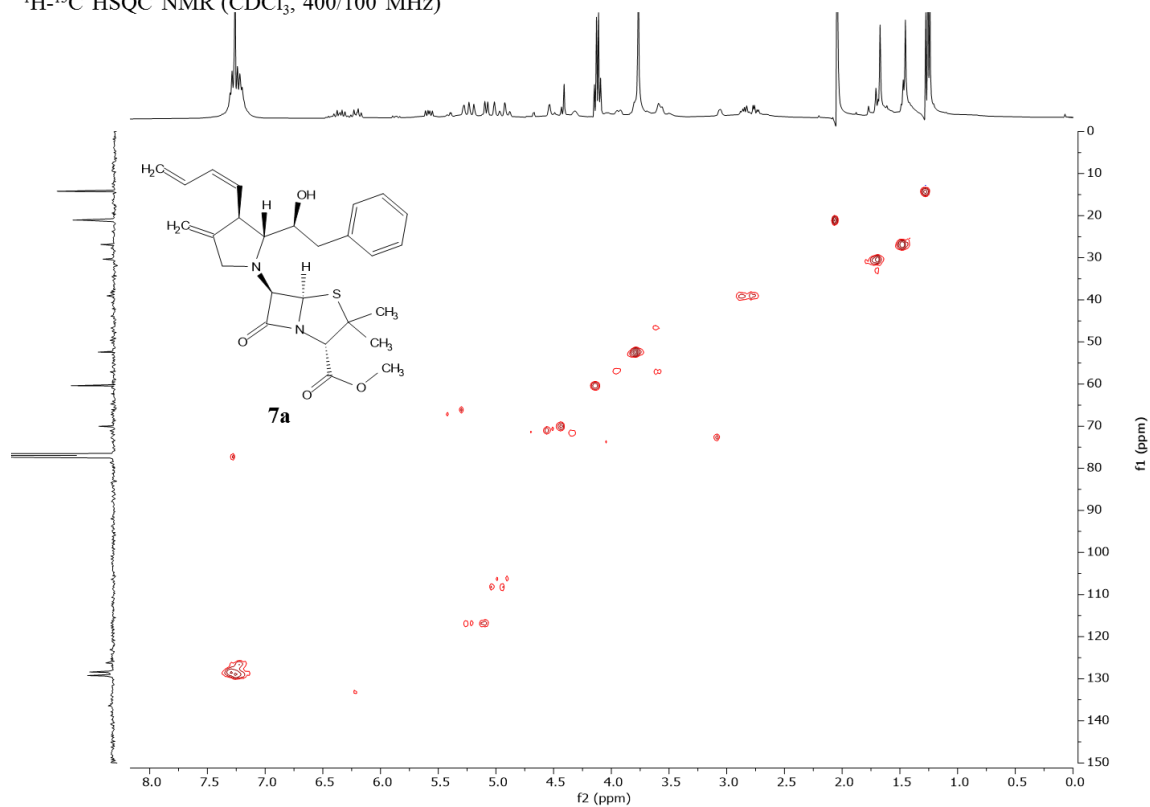
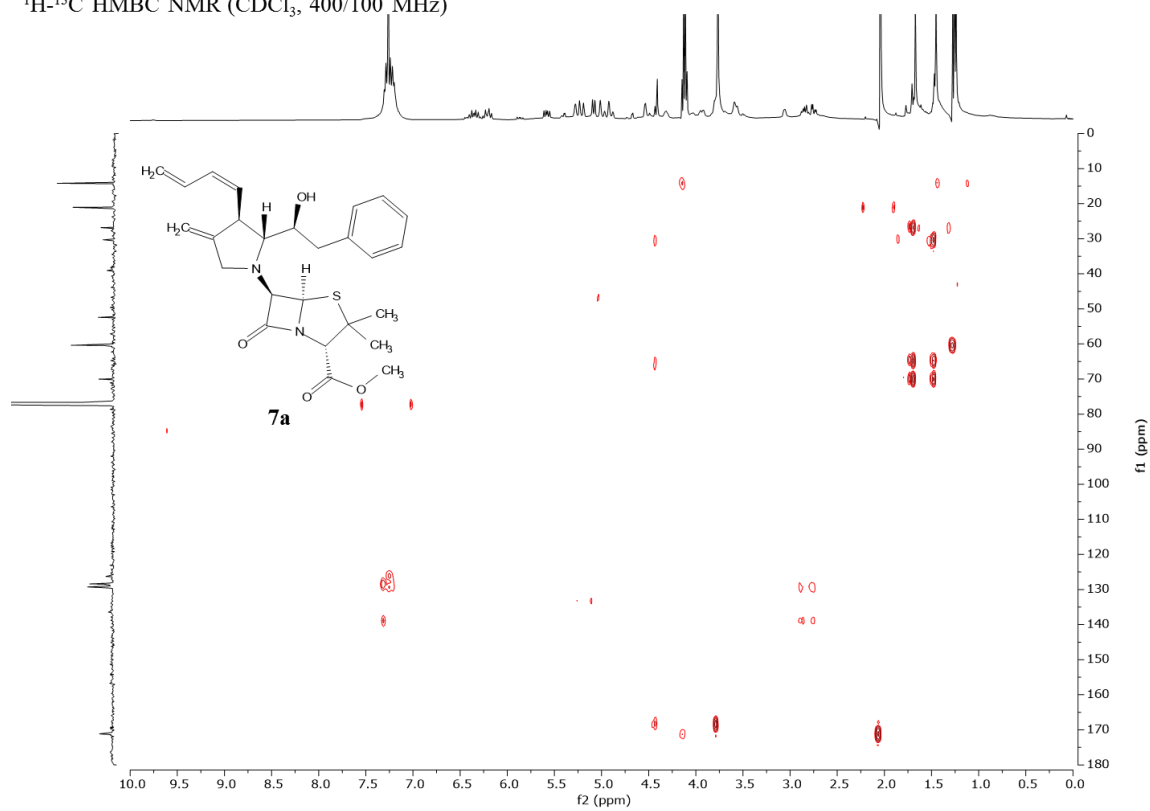
Annex 5

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



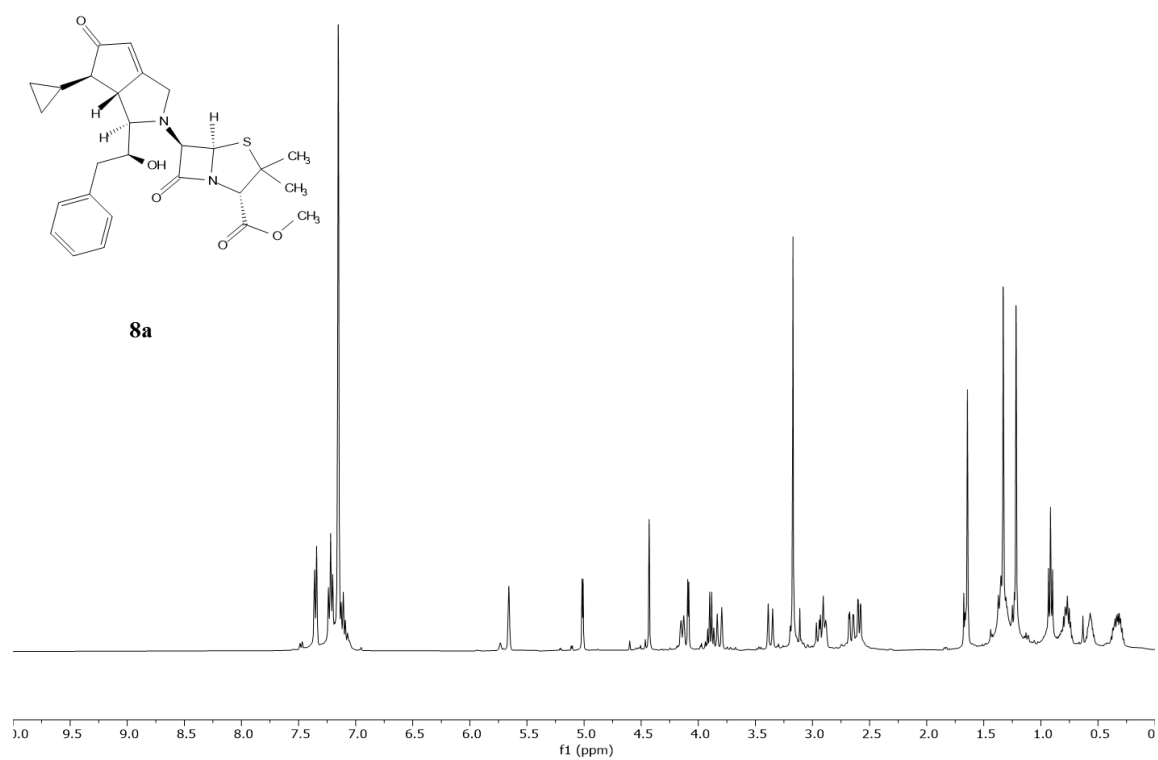
$^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz)



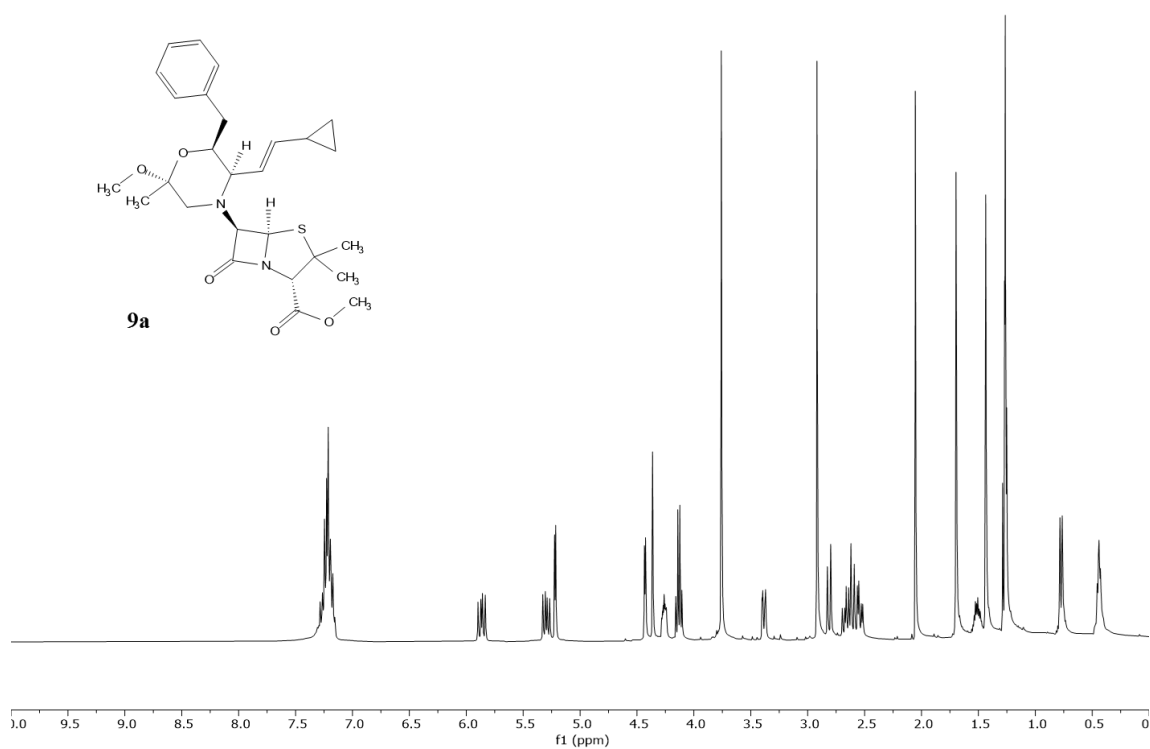
$^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{CDCl}_3$ , 400/100 MHz) $^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

Annex 5

$^1\text{H}$  NMR ( $\text{C}_6\text{D}_6$ , 400 MHz)

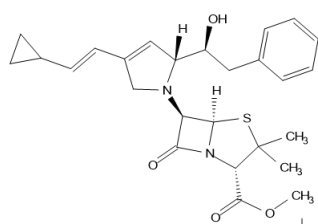




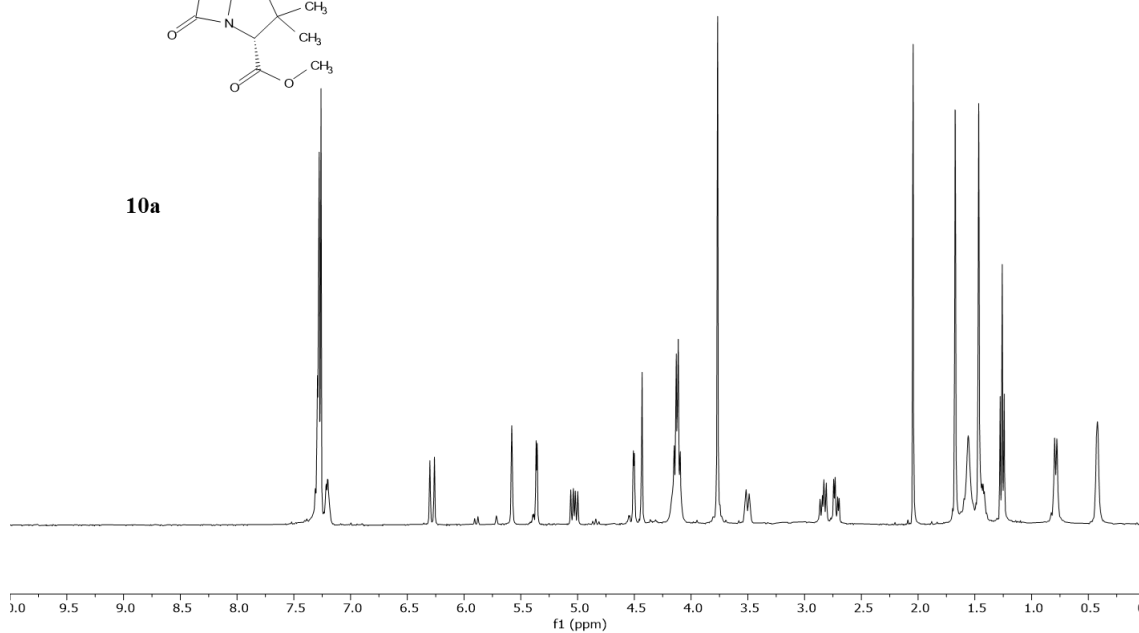
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

Annex 5

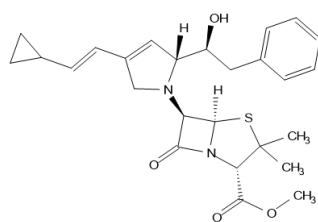
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



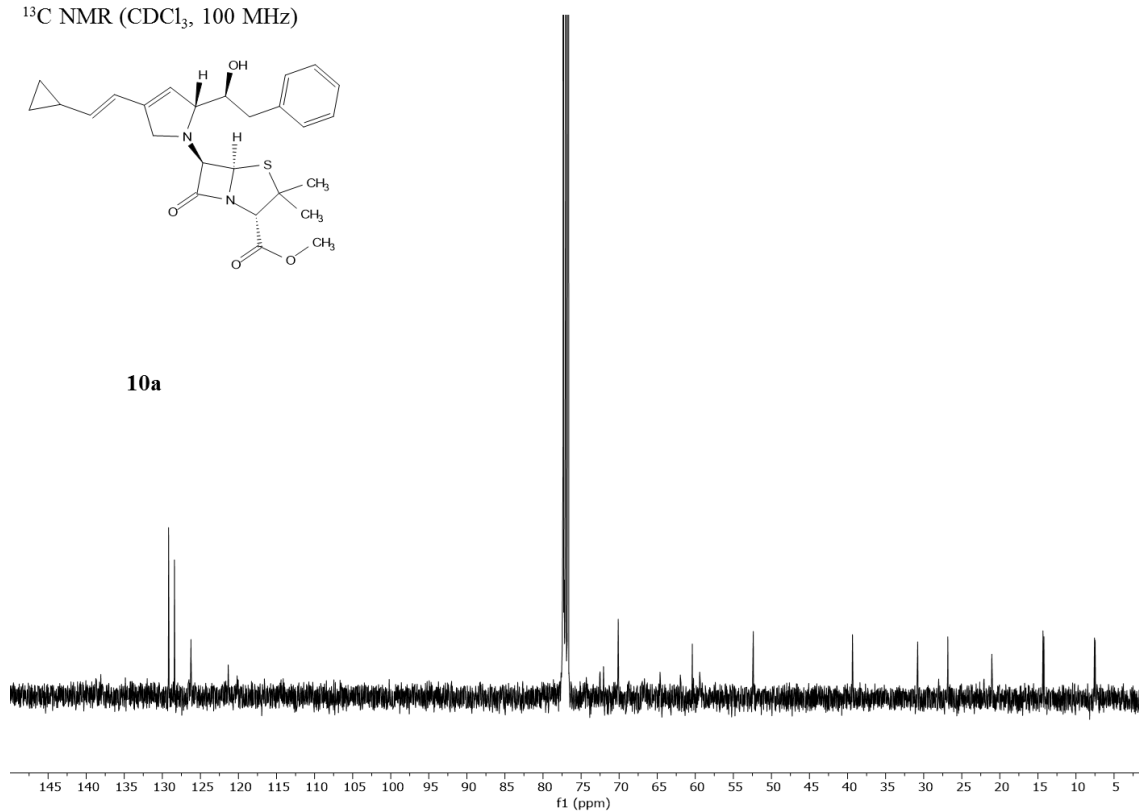
10a

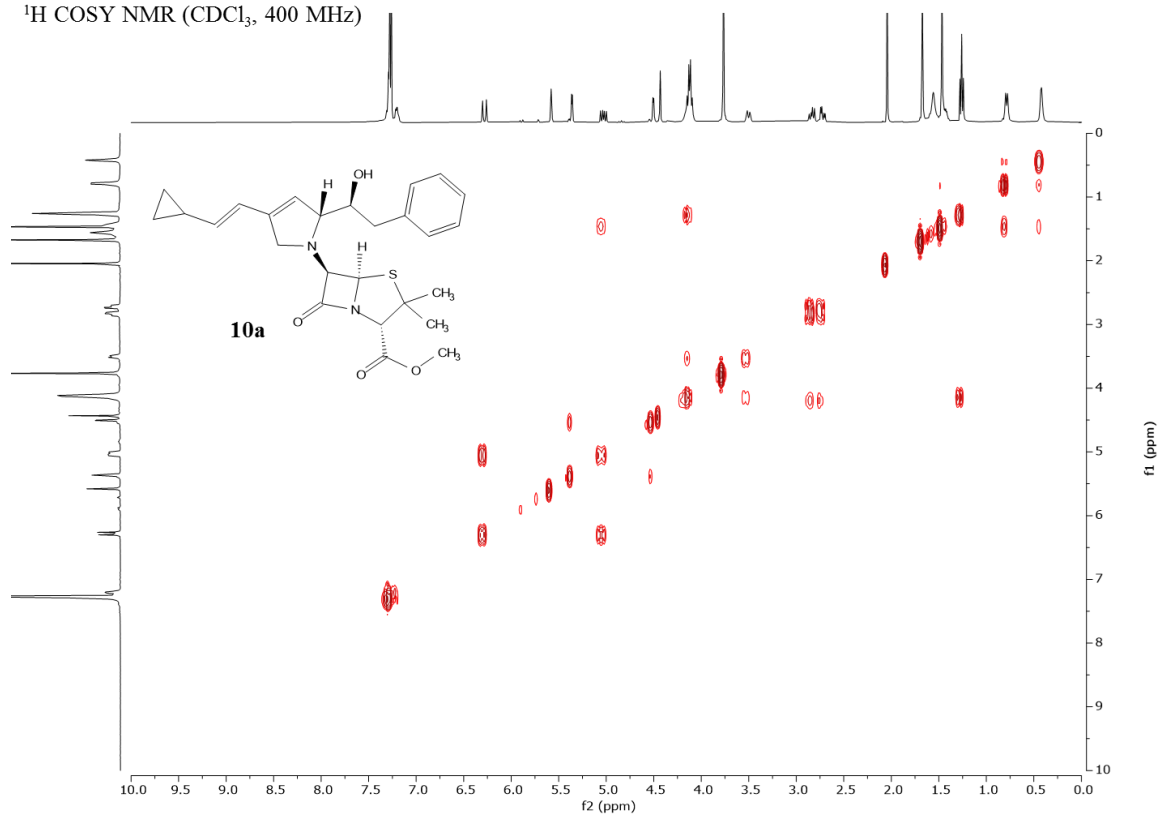
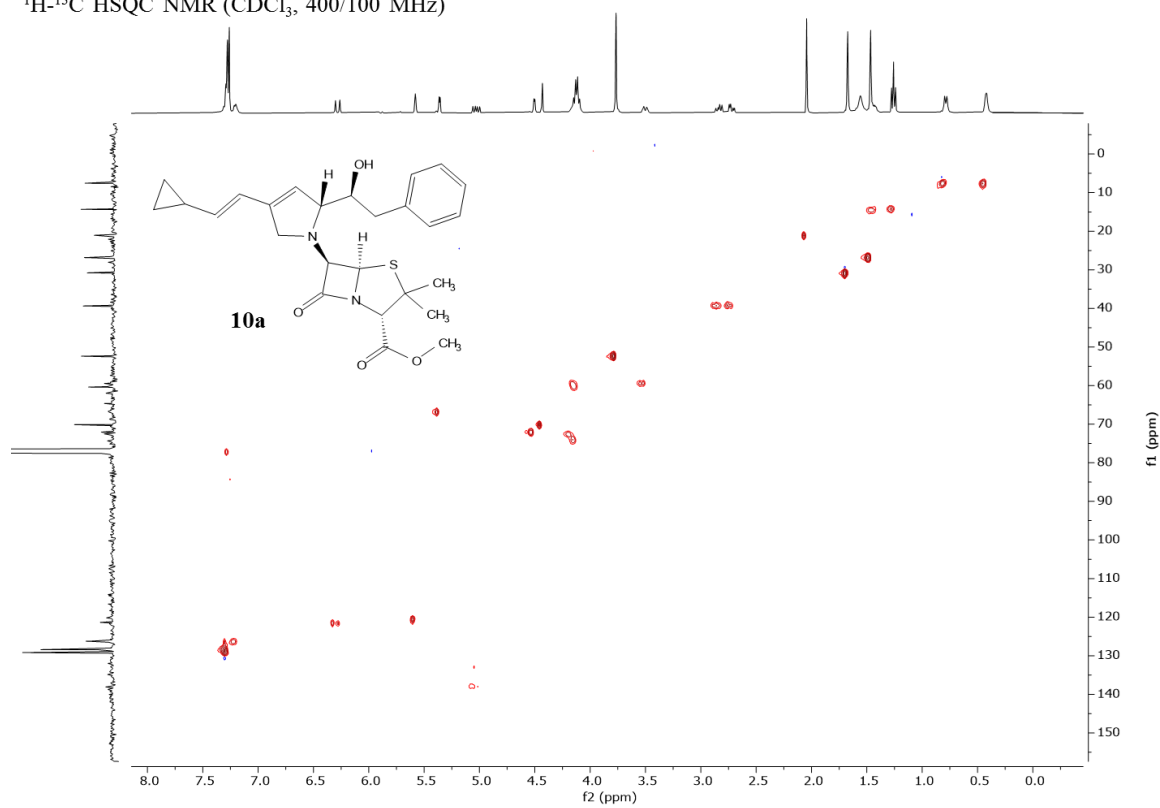


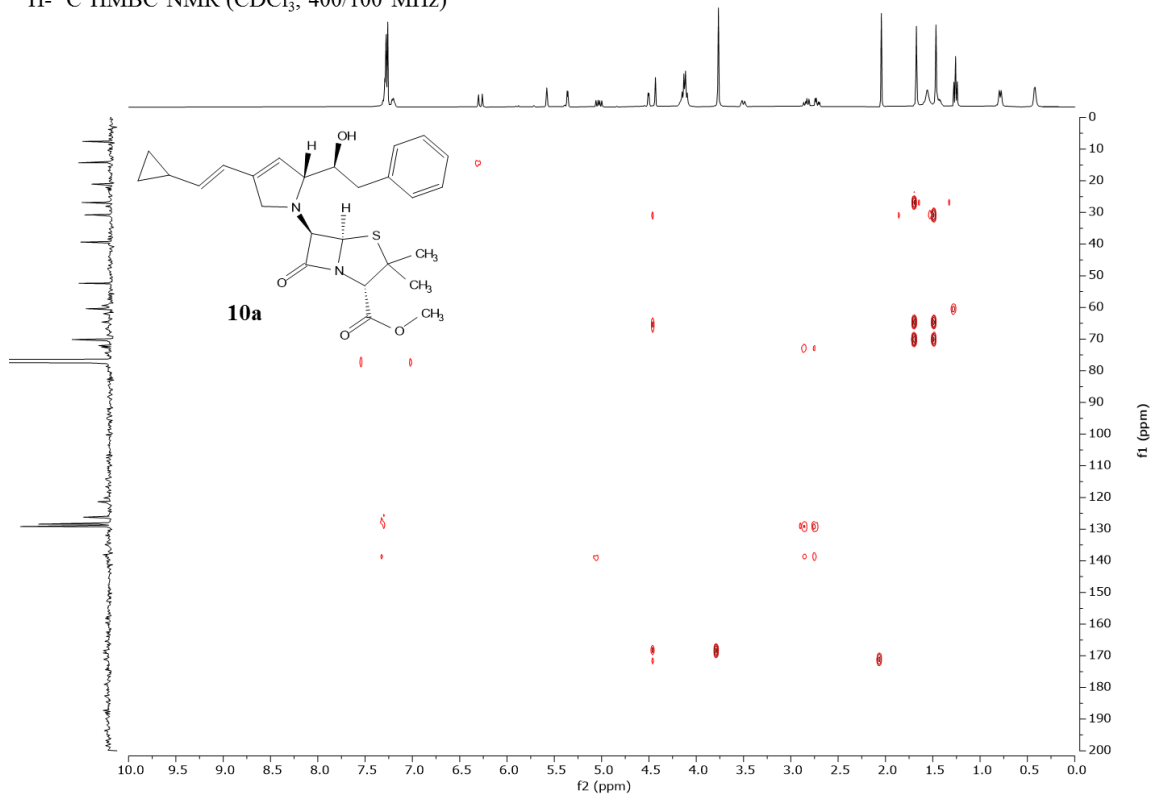
$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)

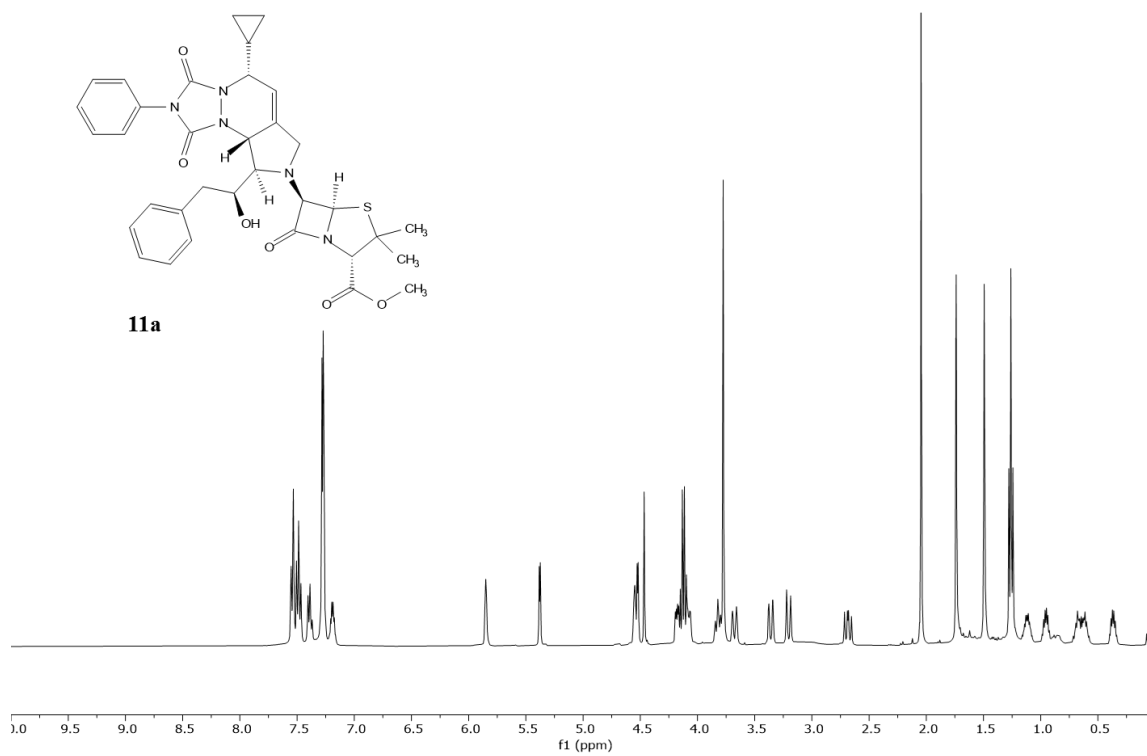


10a



$^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz) $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

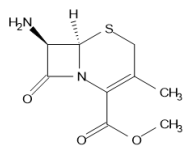
$^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

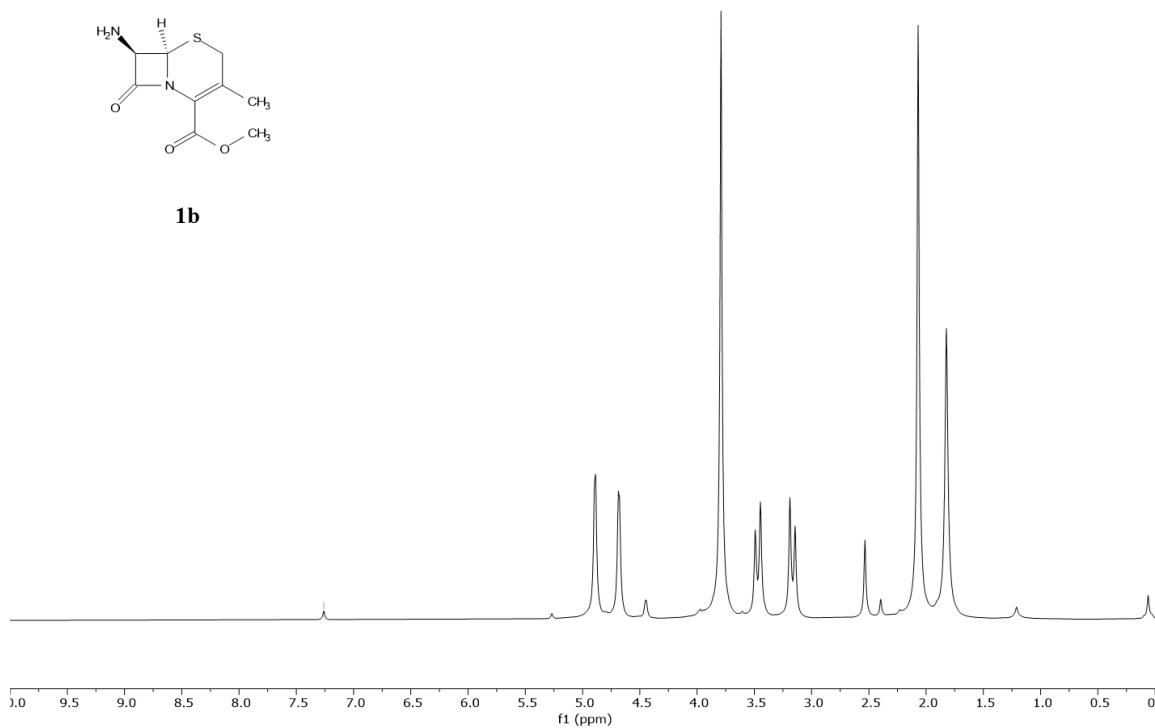
Annex 5

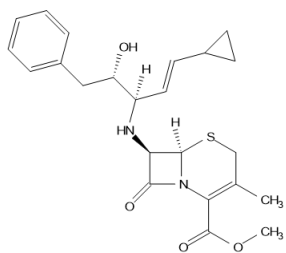
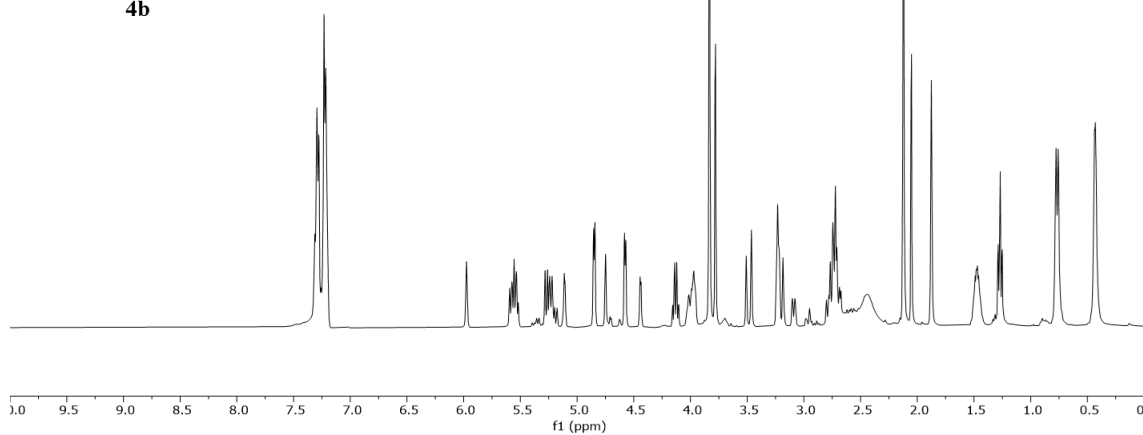
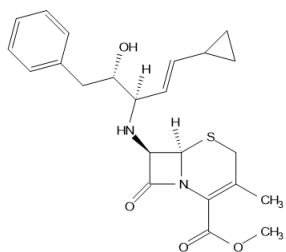
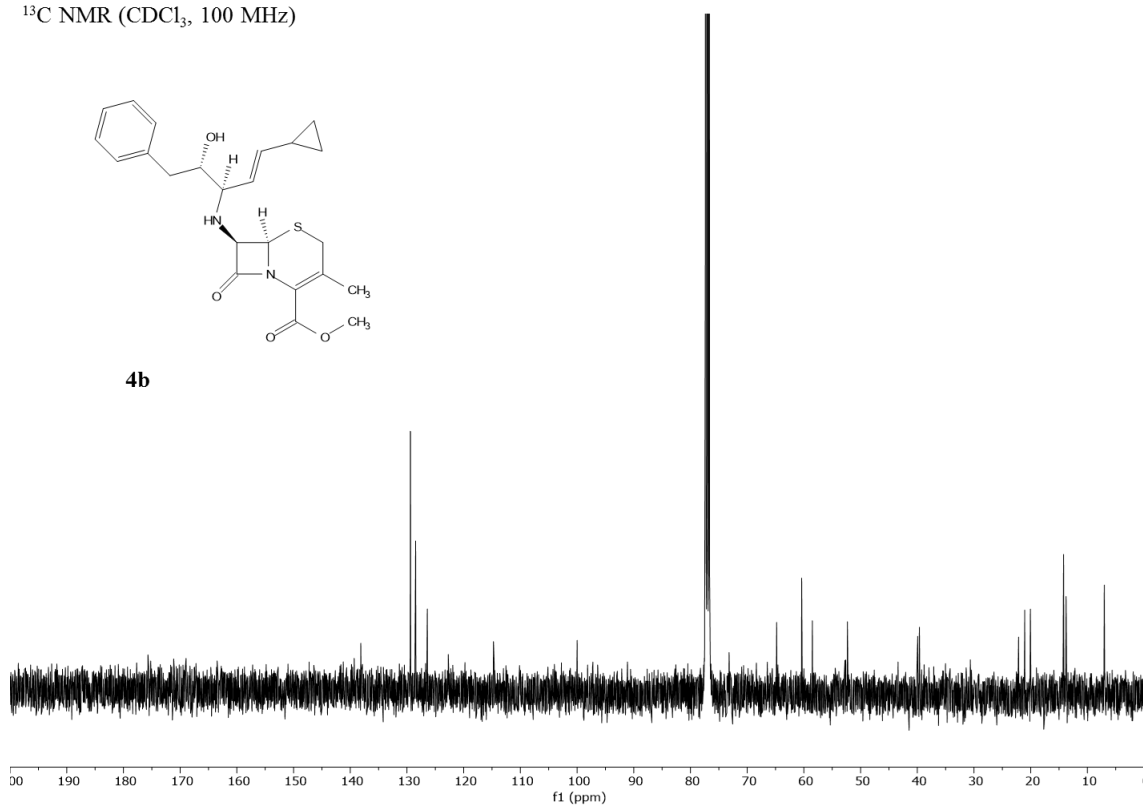
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

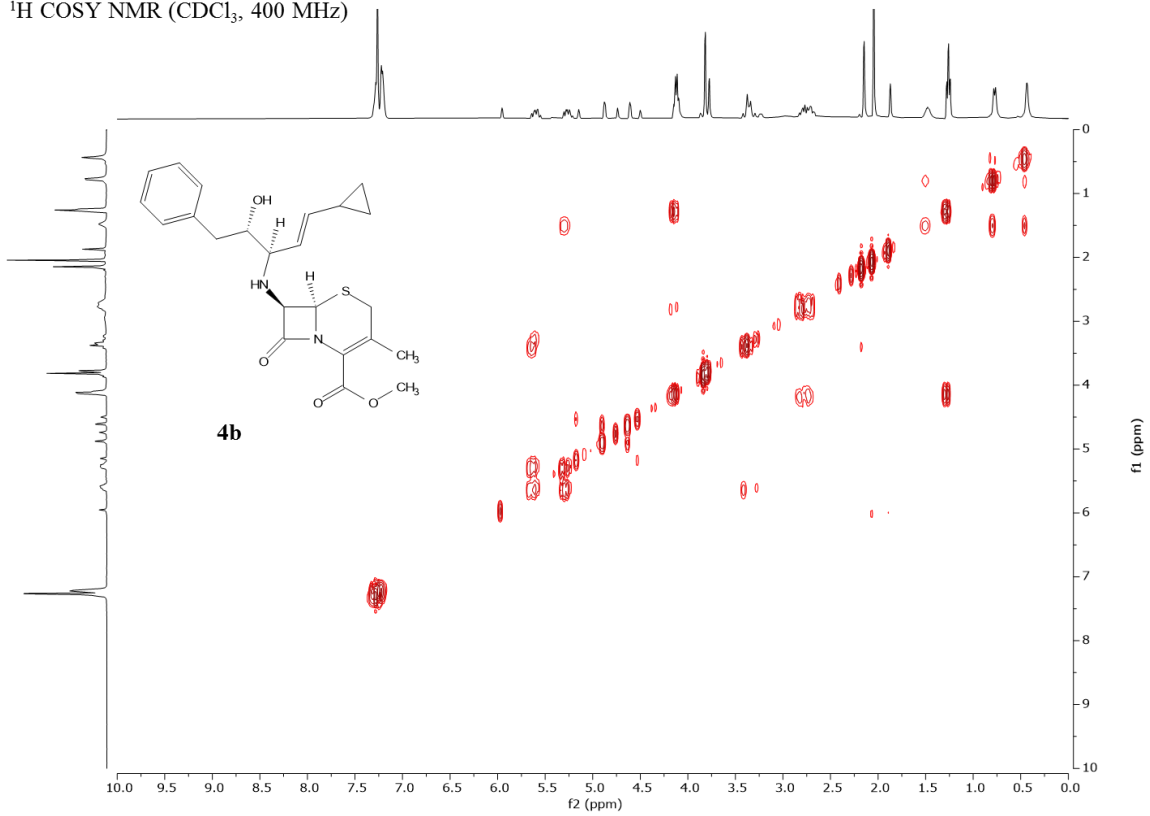
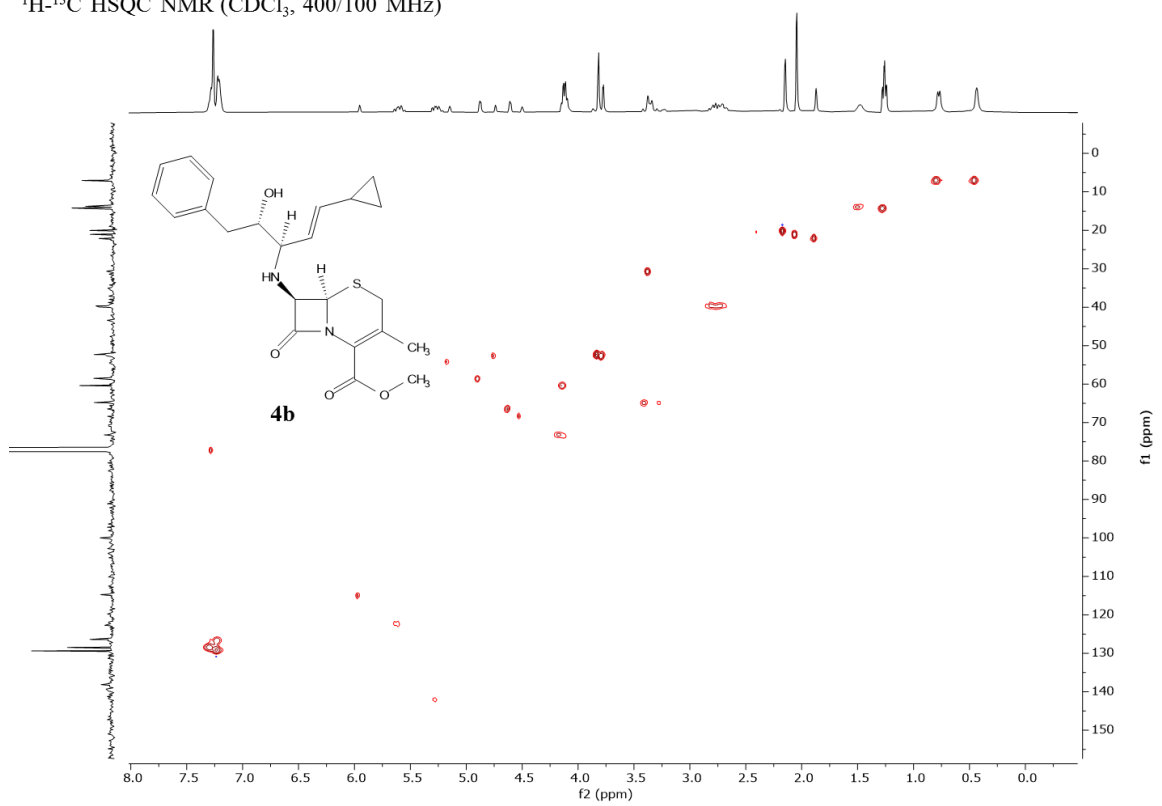
$\text{CDCl}_3$



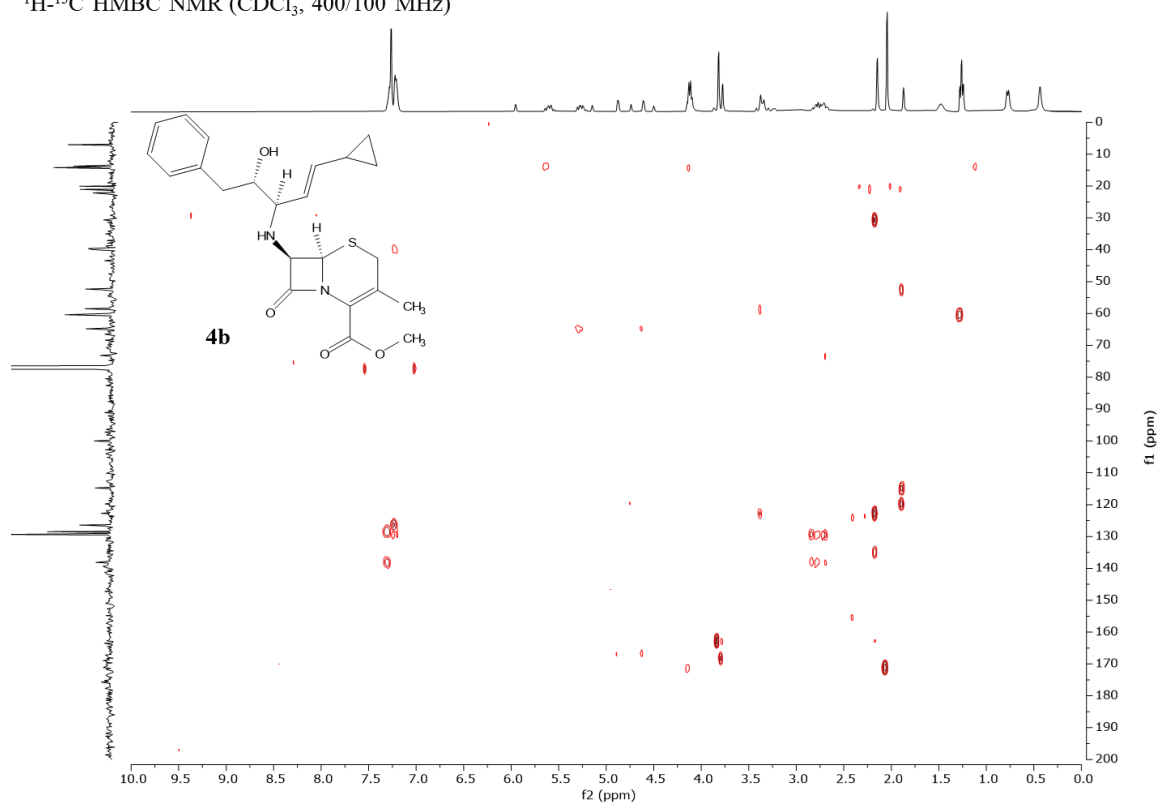
**1b**



$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)**4b** $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)**4b**

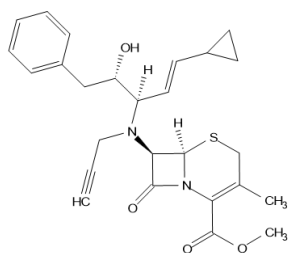
$^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz) $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{CDCl}_3$ , 400/100 MHz)



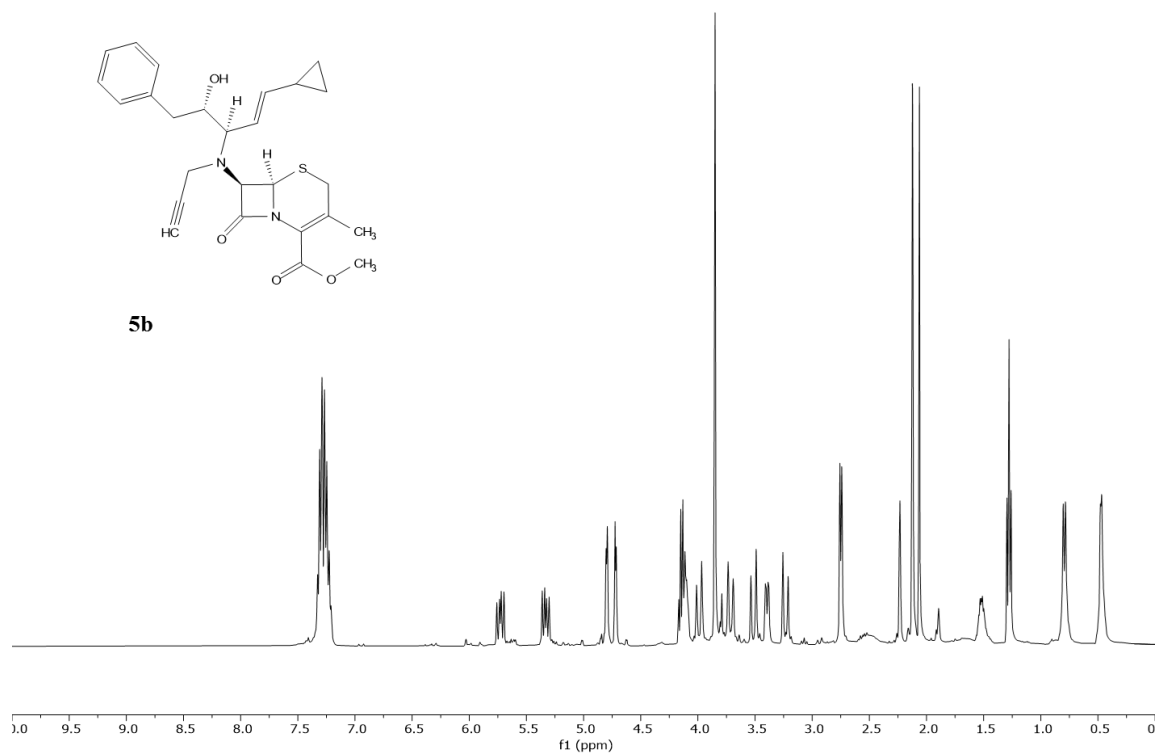
$^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

Annex 5

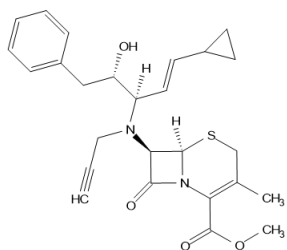
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



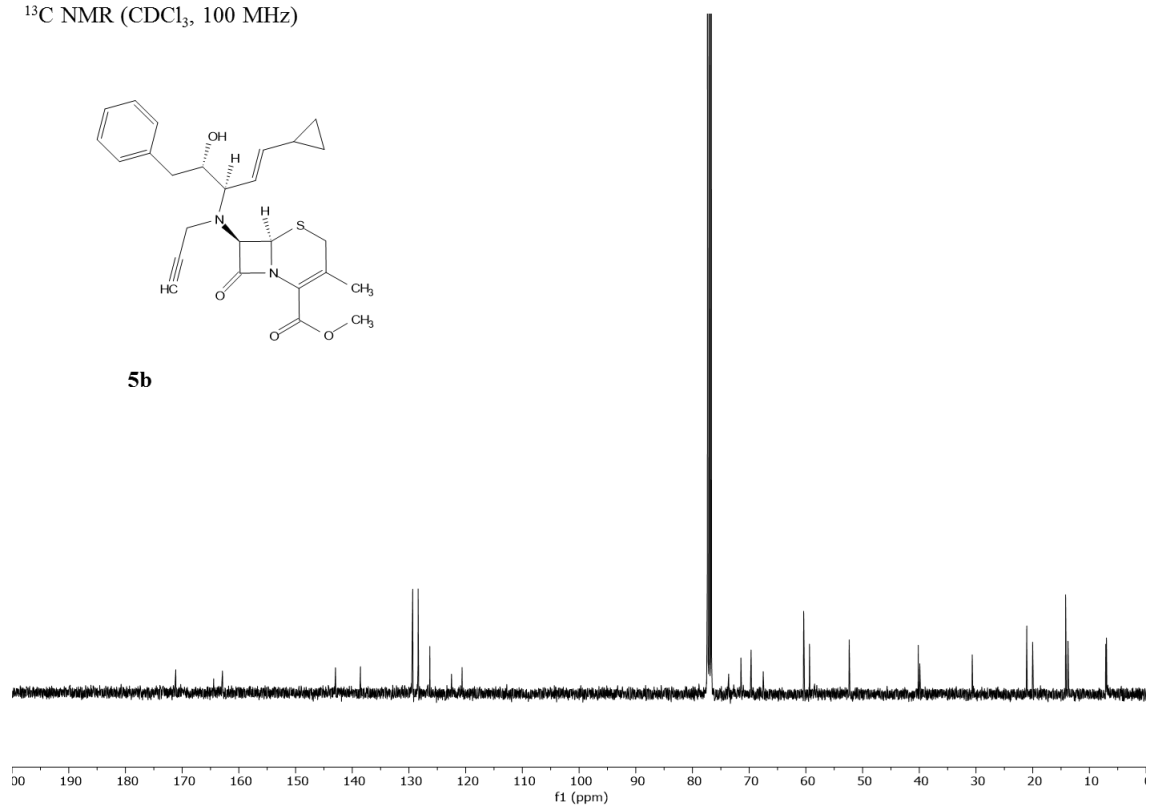
**5b**

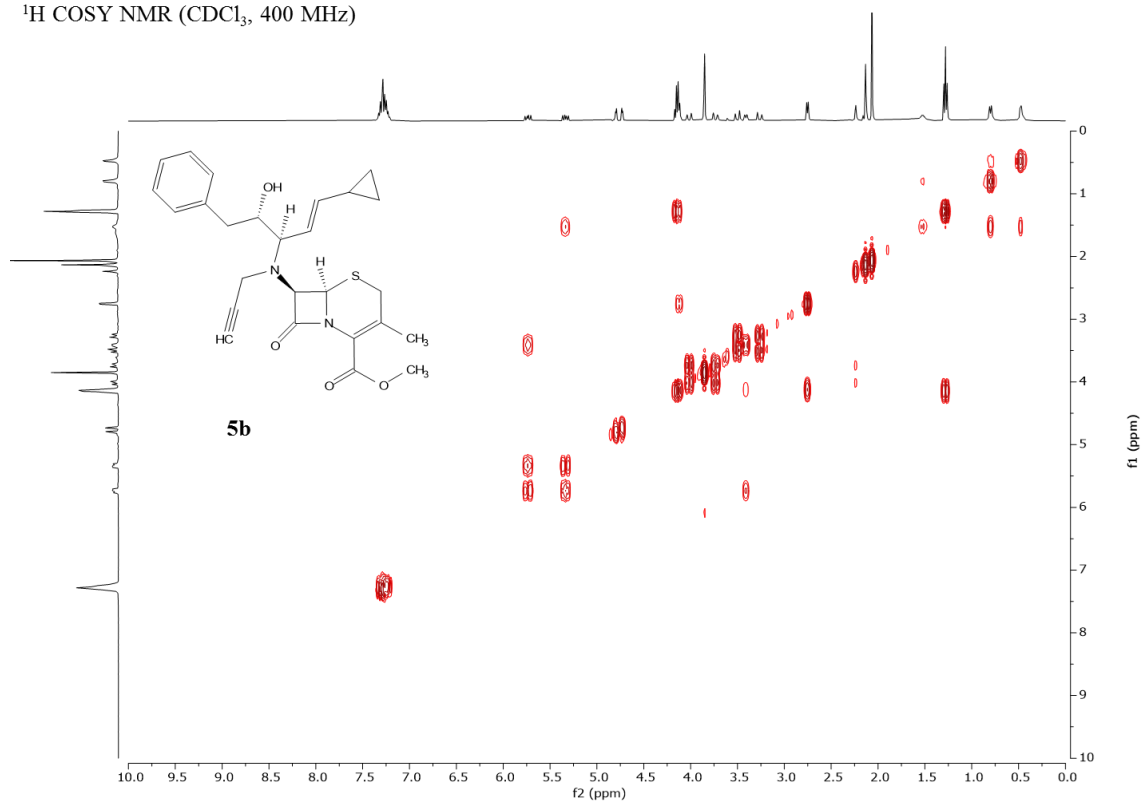
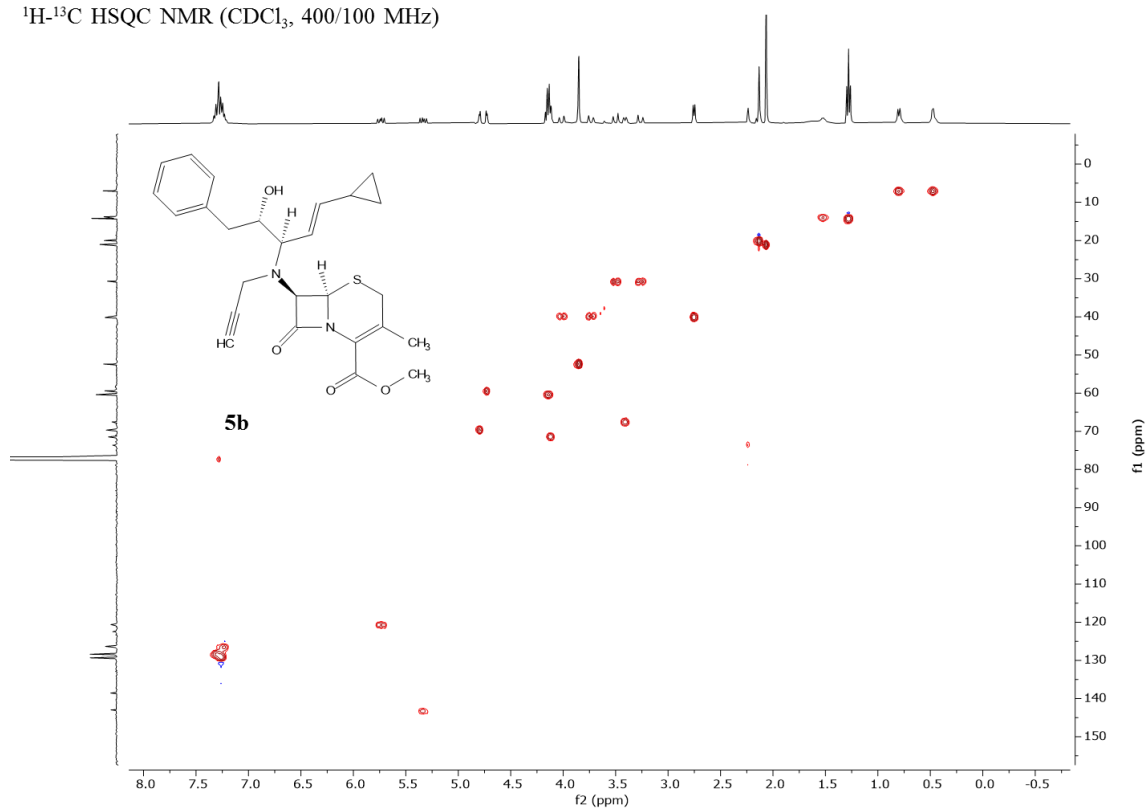


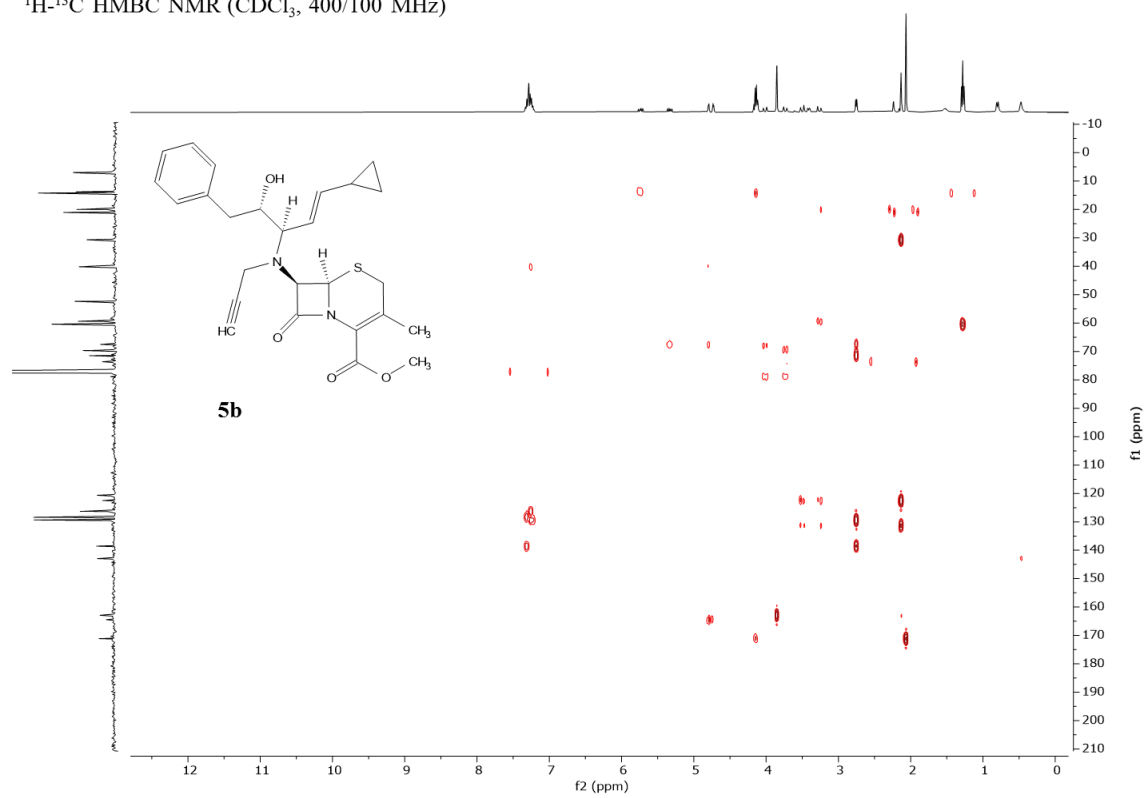
$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)

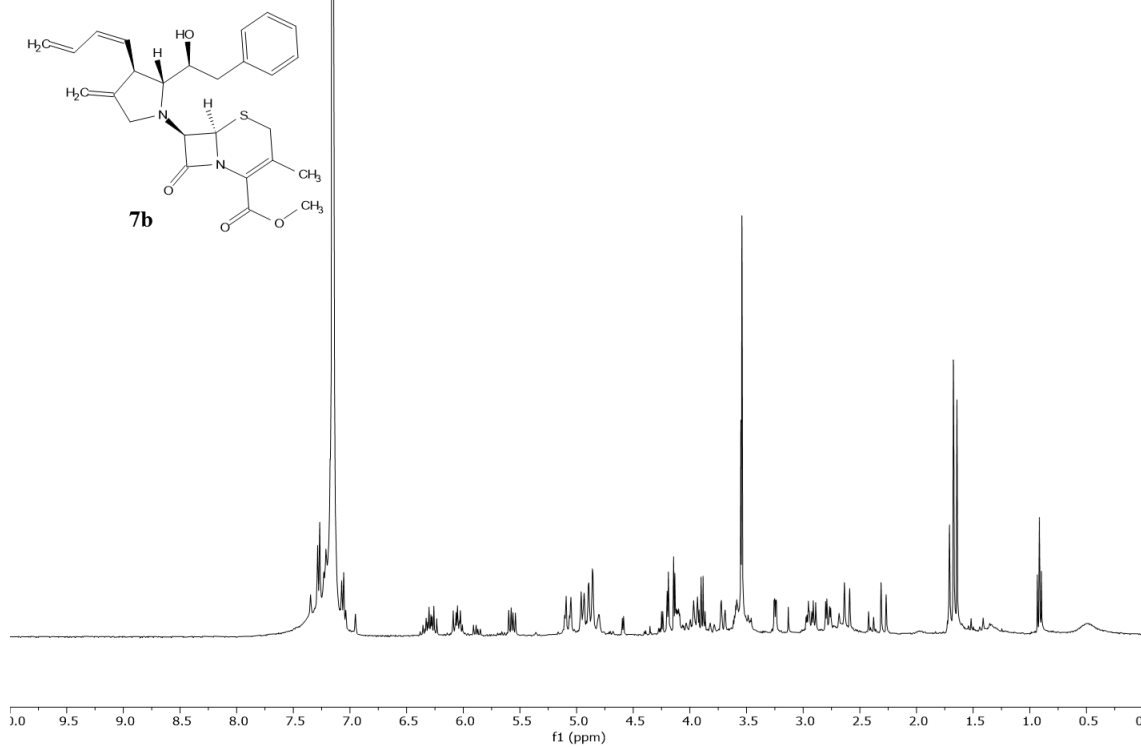
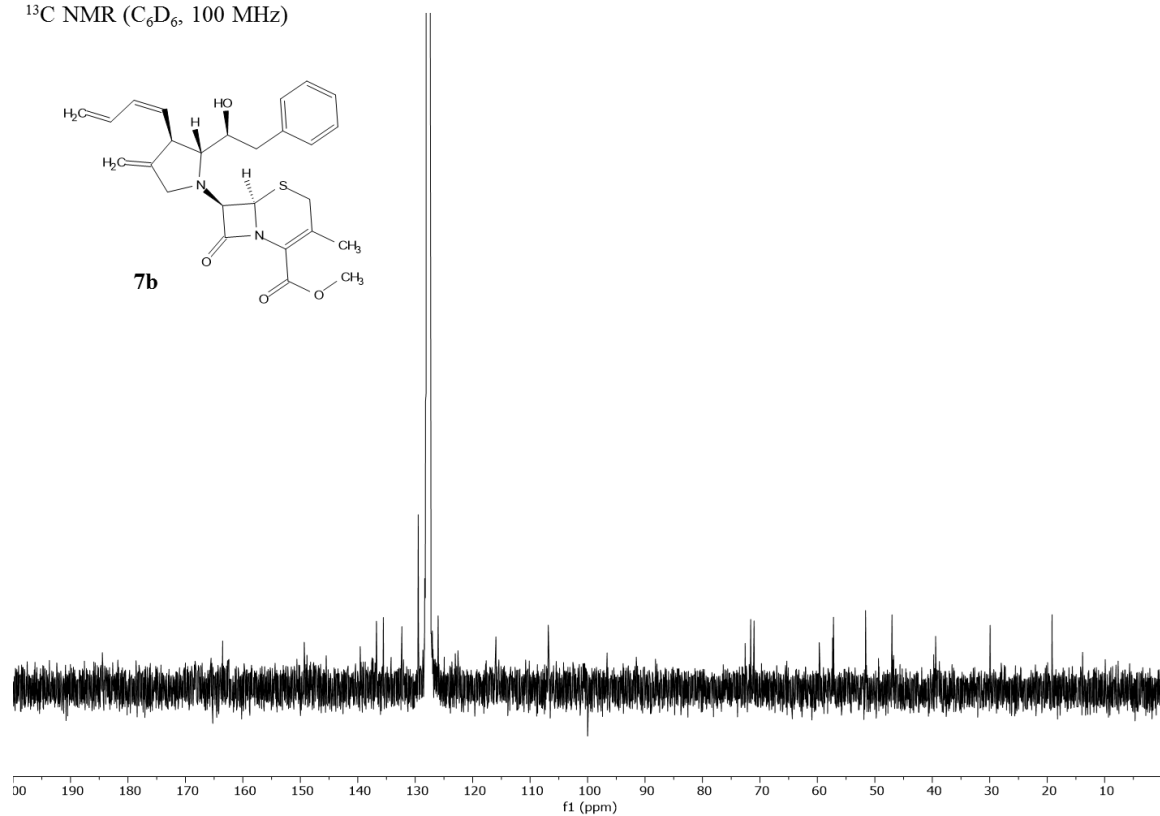


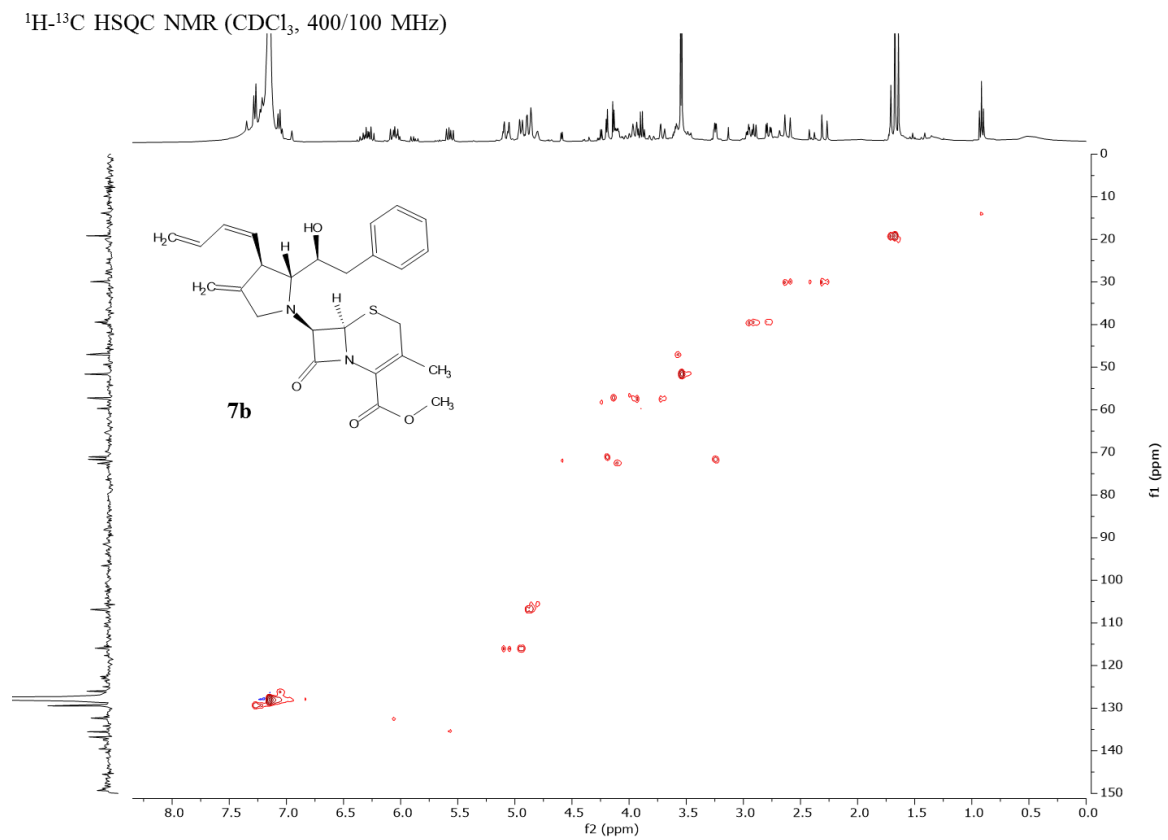
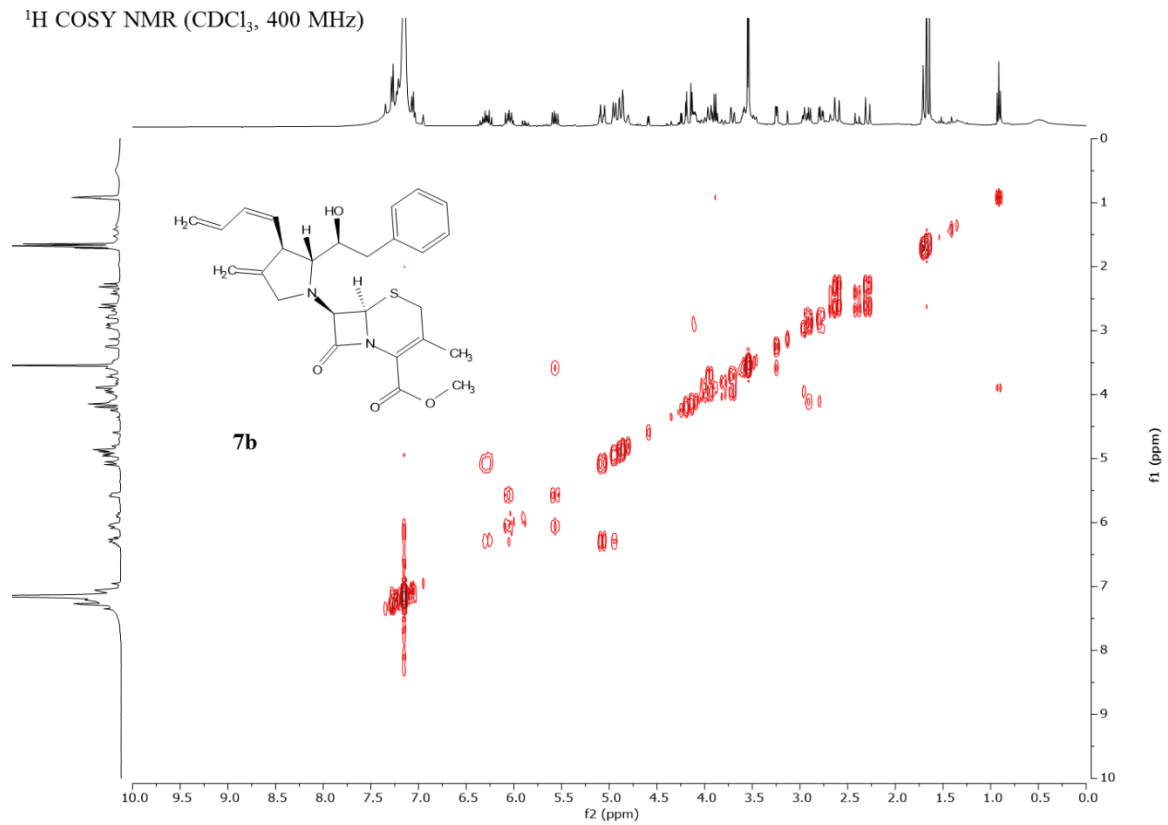
**5b**

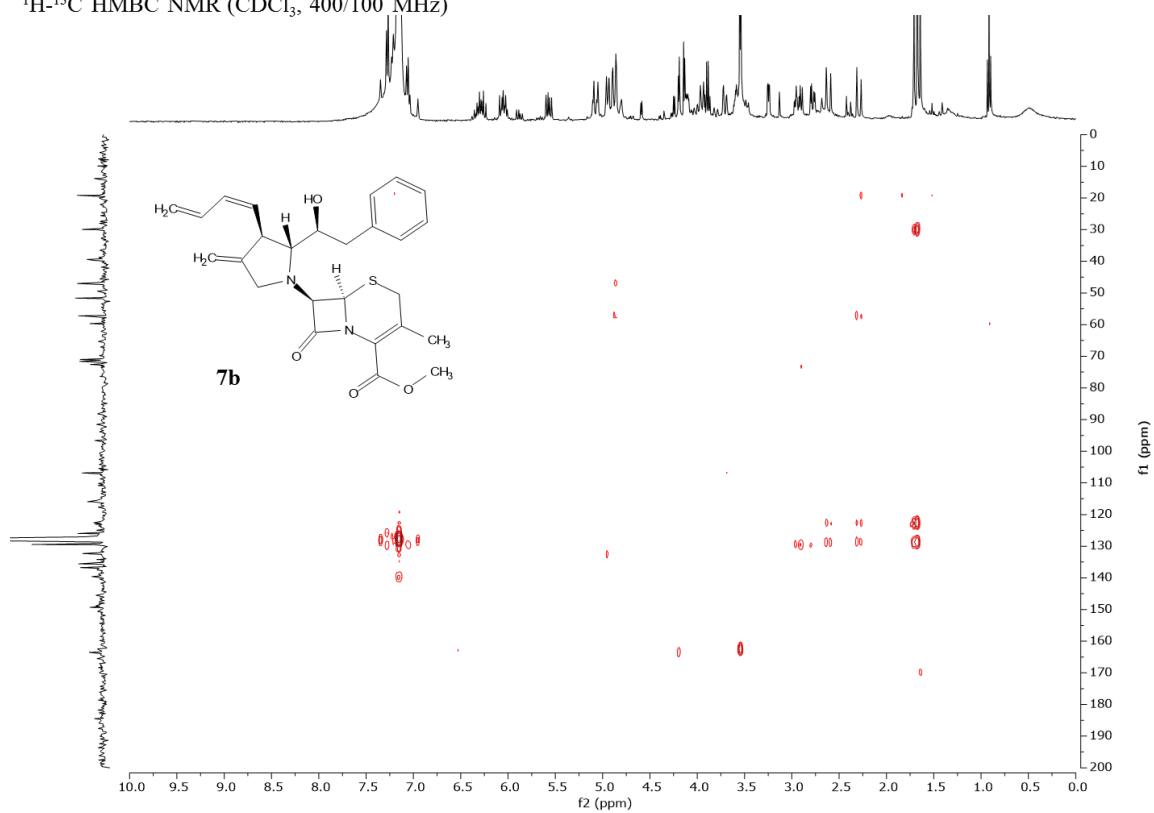


$^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz) $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

$^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

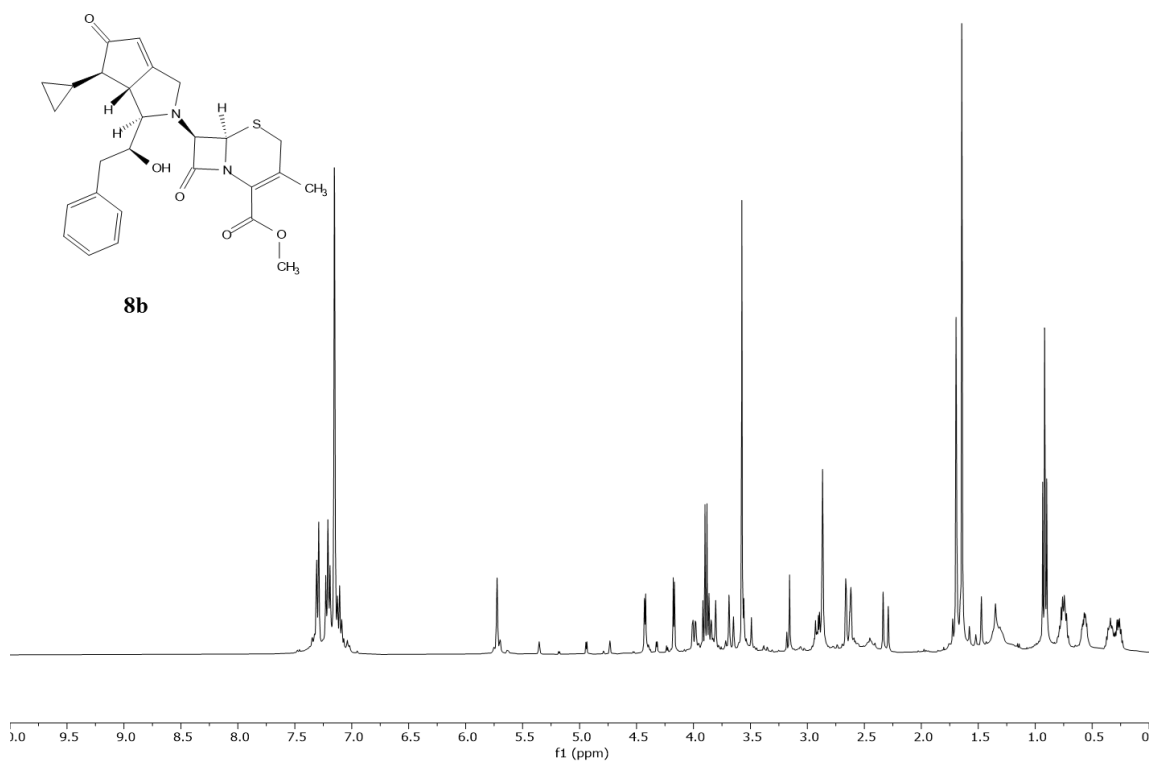
$^1\text{H NMR}$  ( $\text{C}_6\text{D}_6$ , 400 MHz) $^{13}\text{C NMR}$  ( $\text{C}_6\text{D}_6$ , 100 MHz)



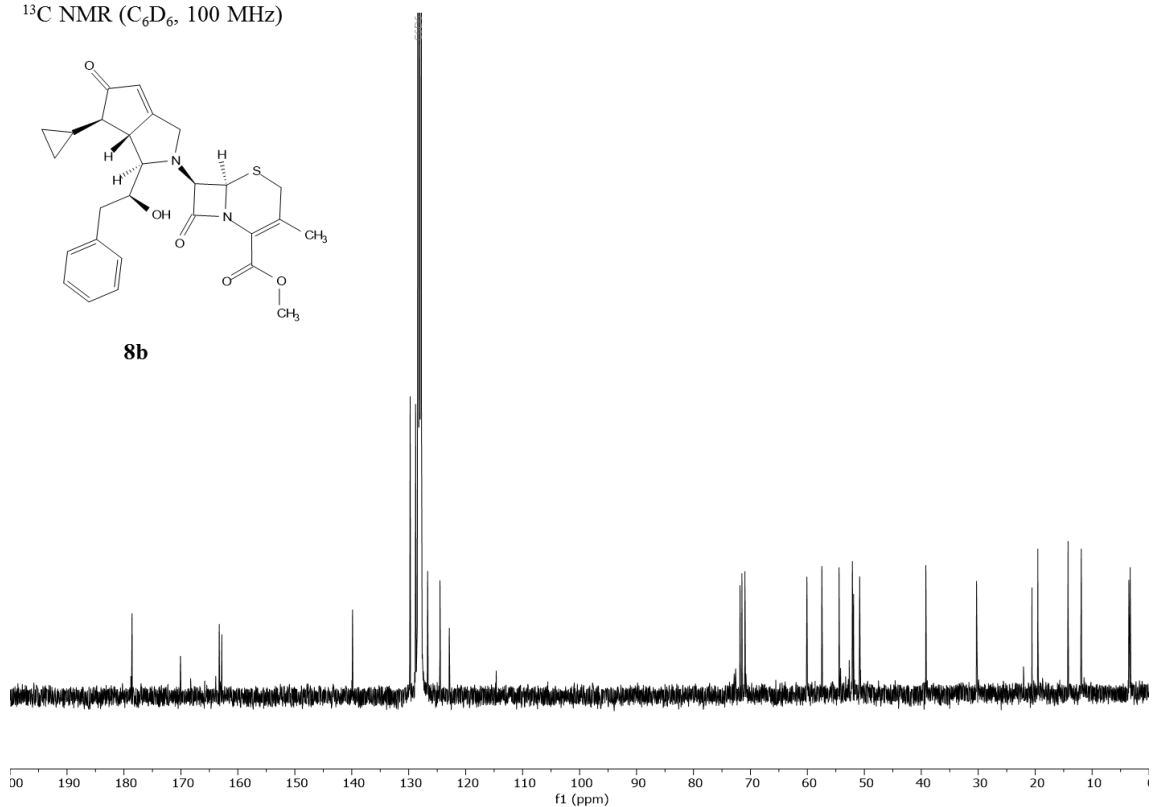
$^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

Annex 5

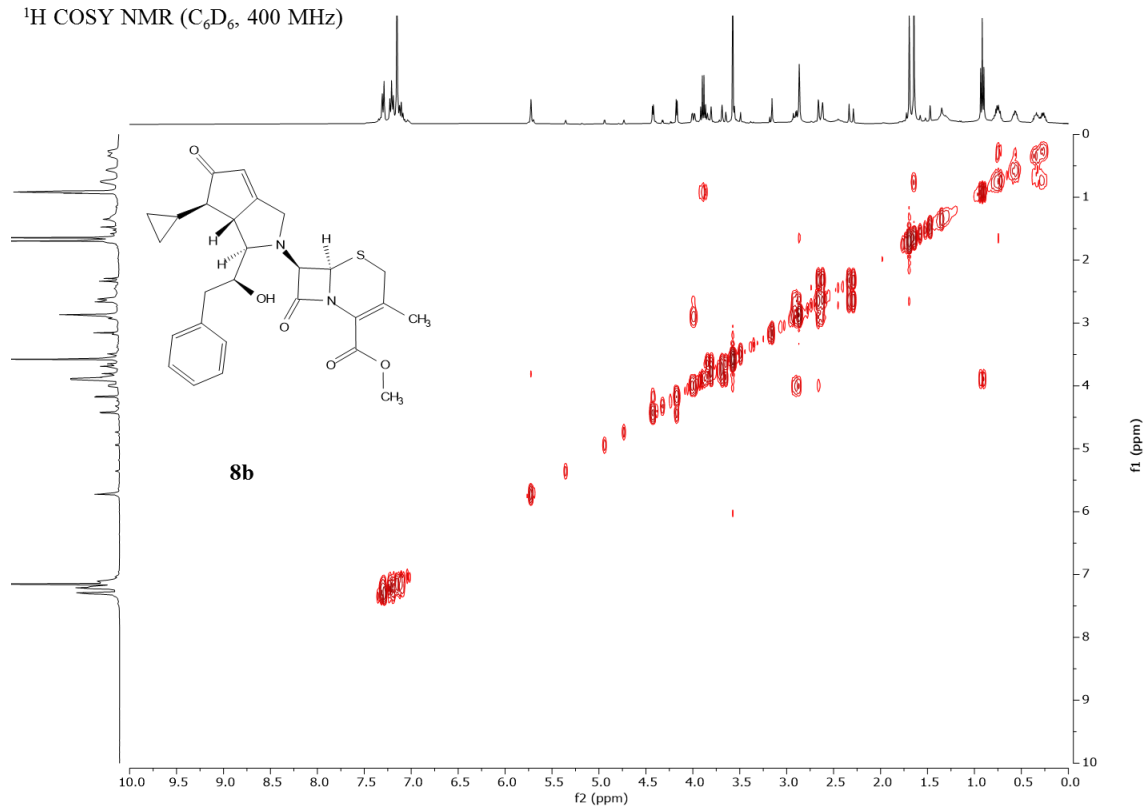
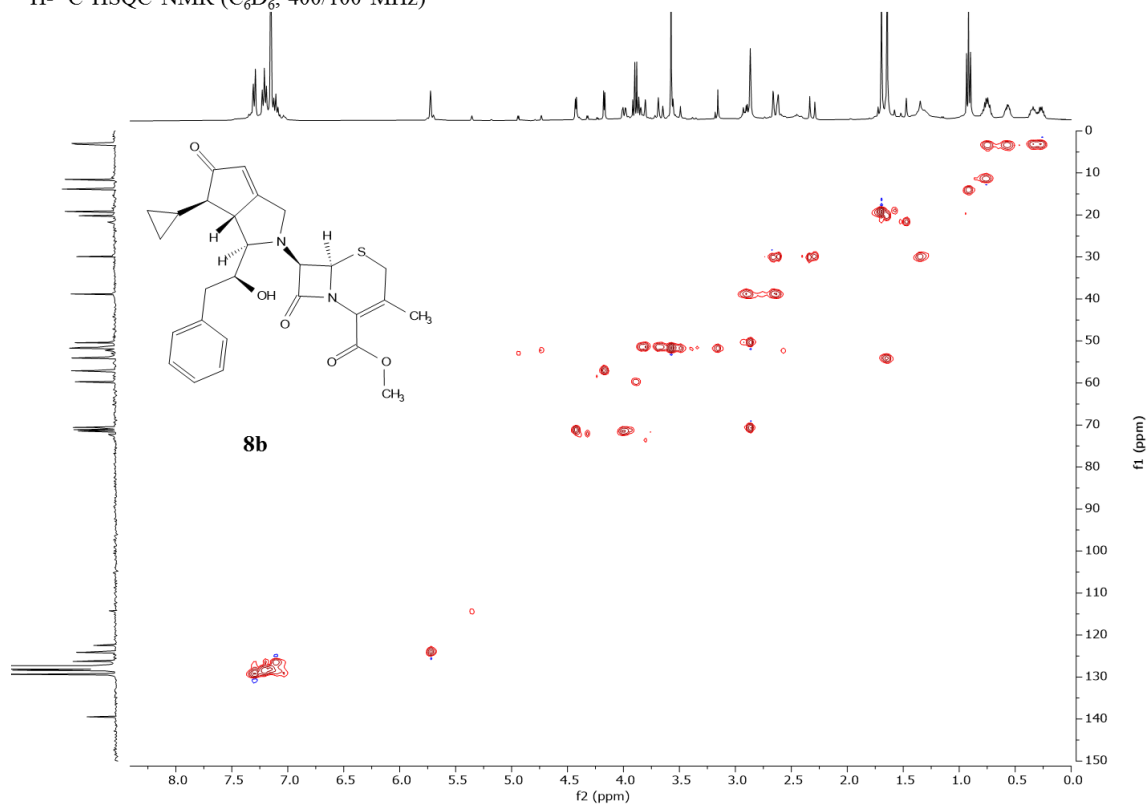
$^1\text{H}$  NMR ( $\text{C}_6\text{D}_6$ , 400 MHz)

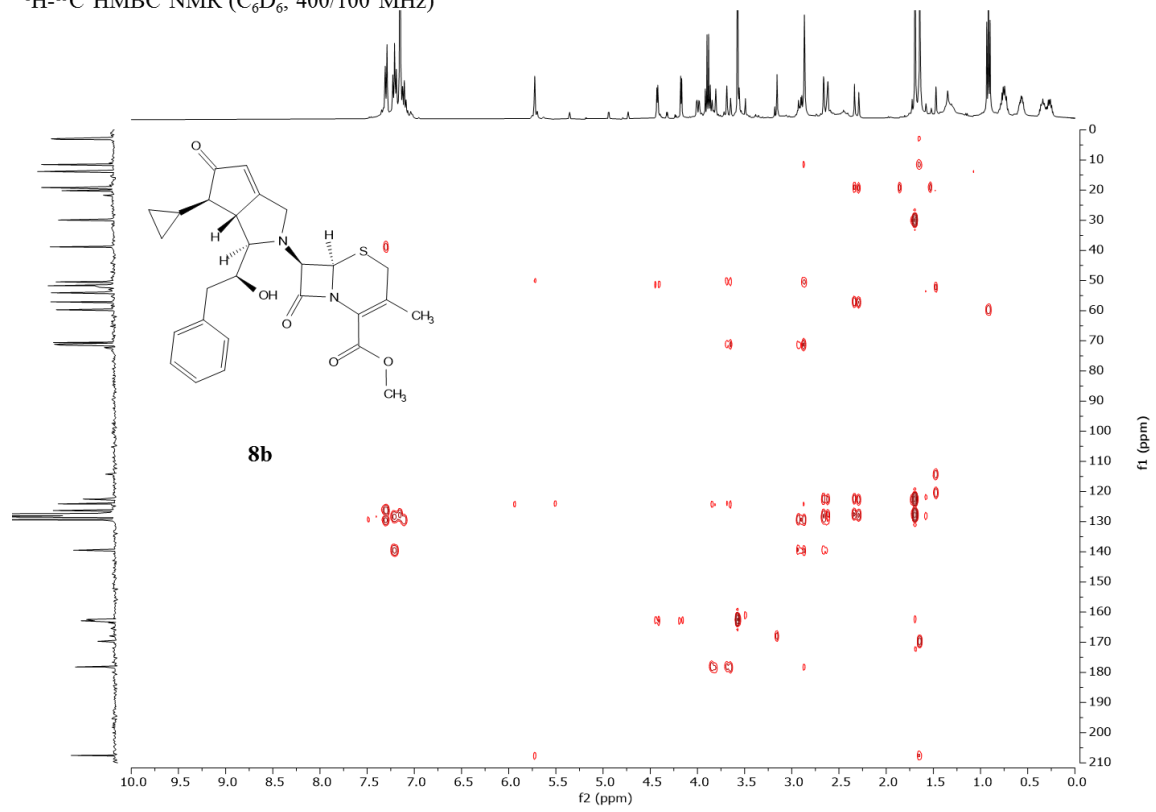


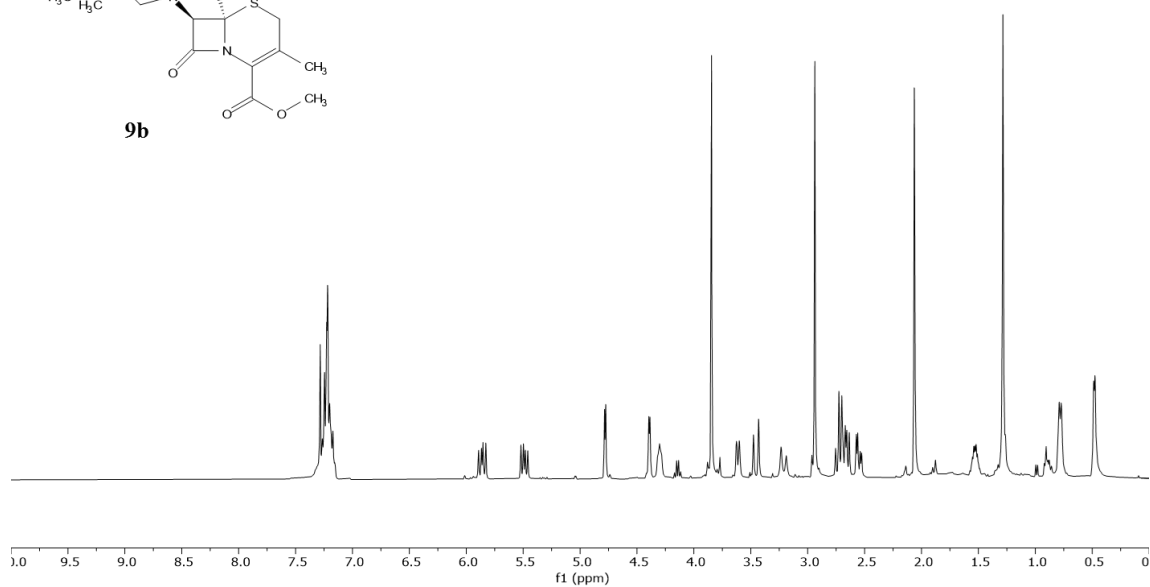
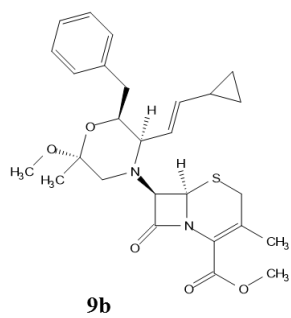
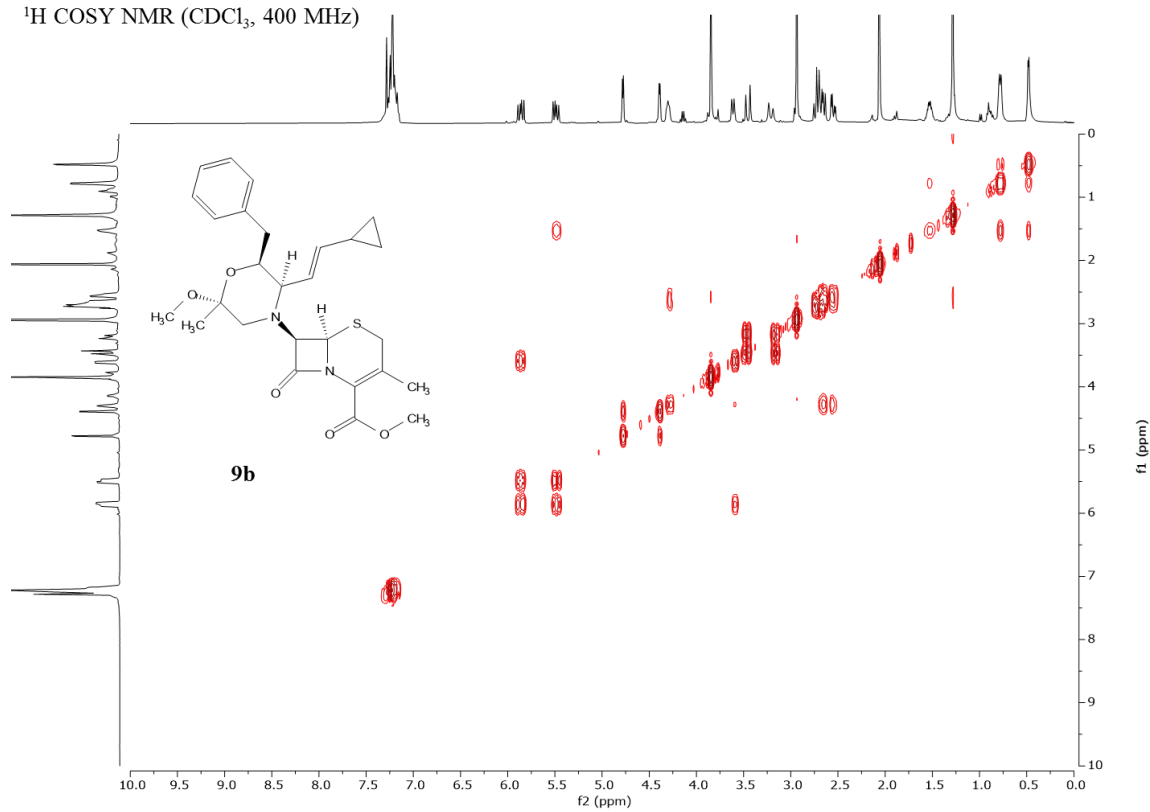
$^{13}\text{C}$  NMR ( $\text{C}_6\text{D}_6$ , 100 MHz)

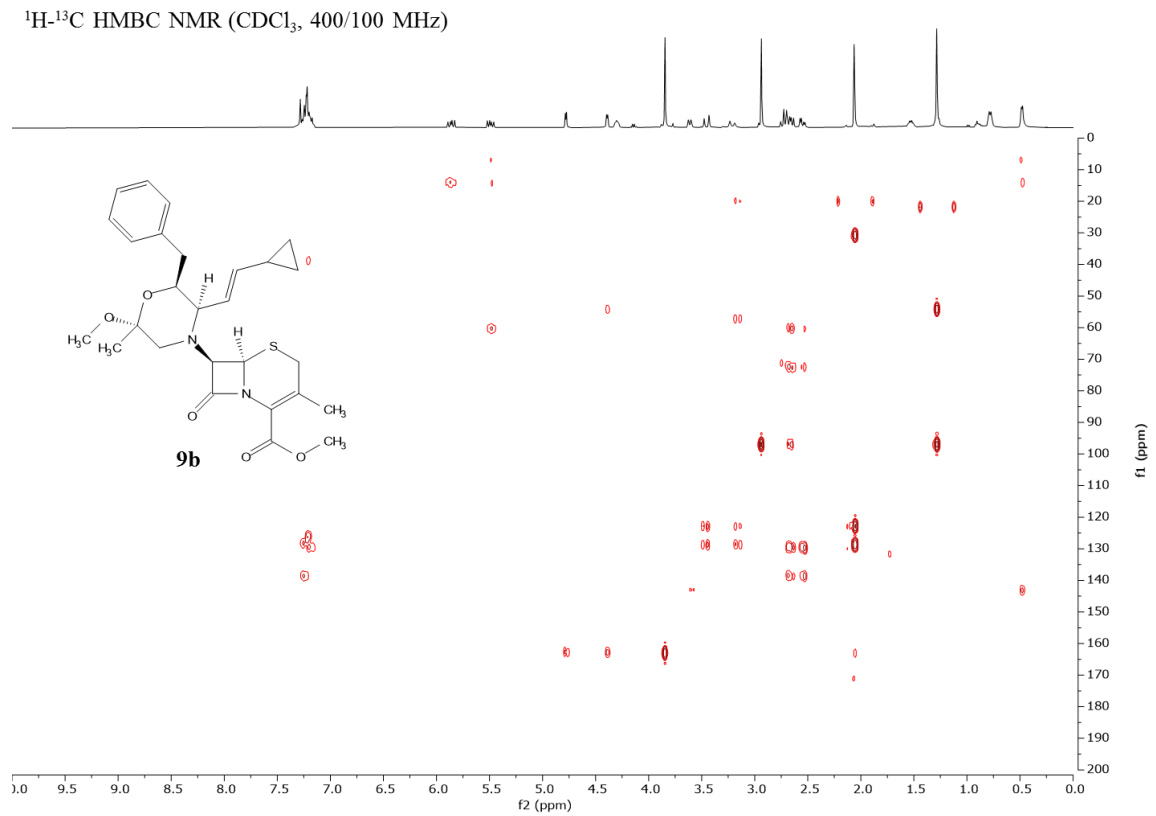
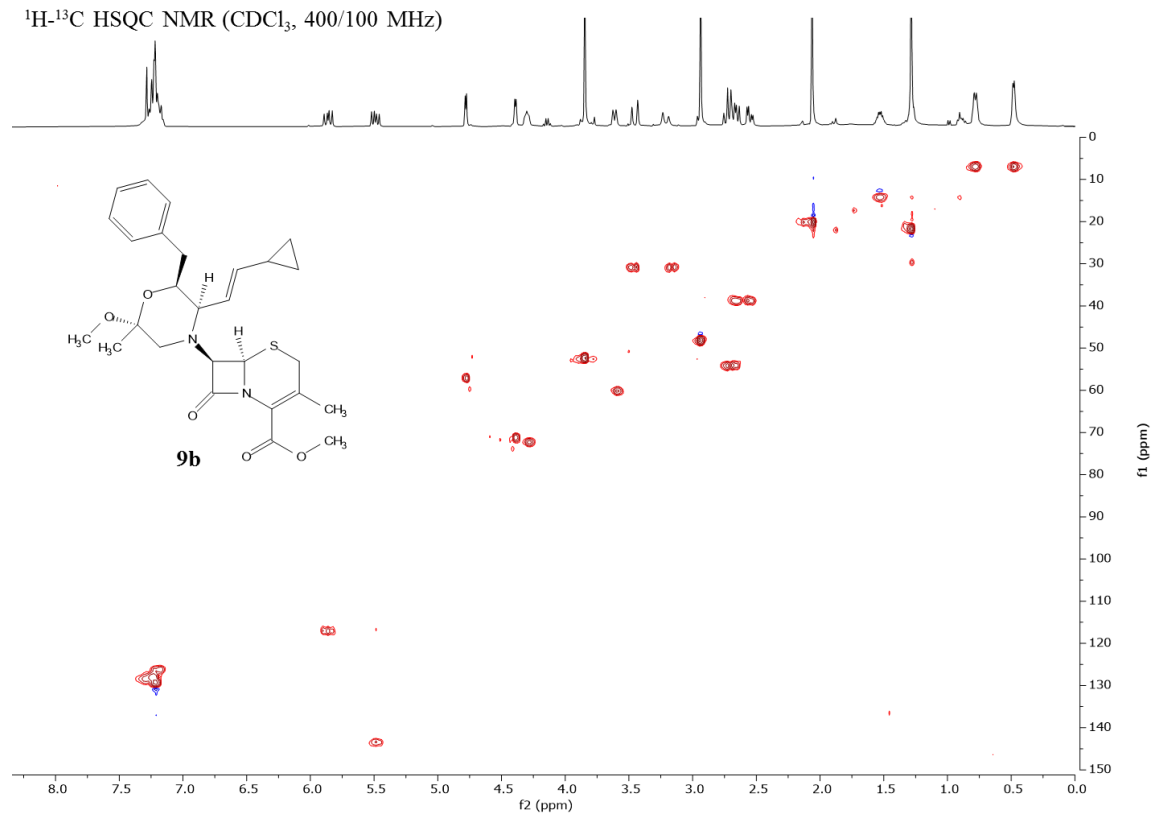


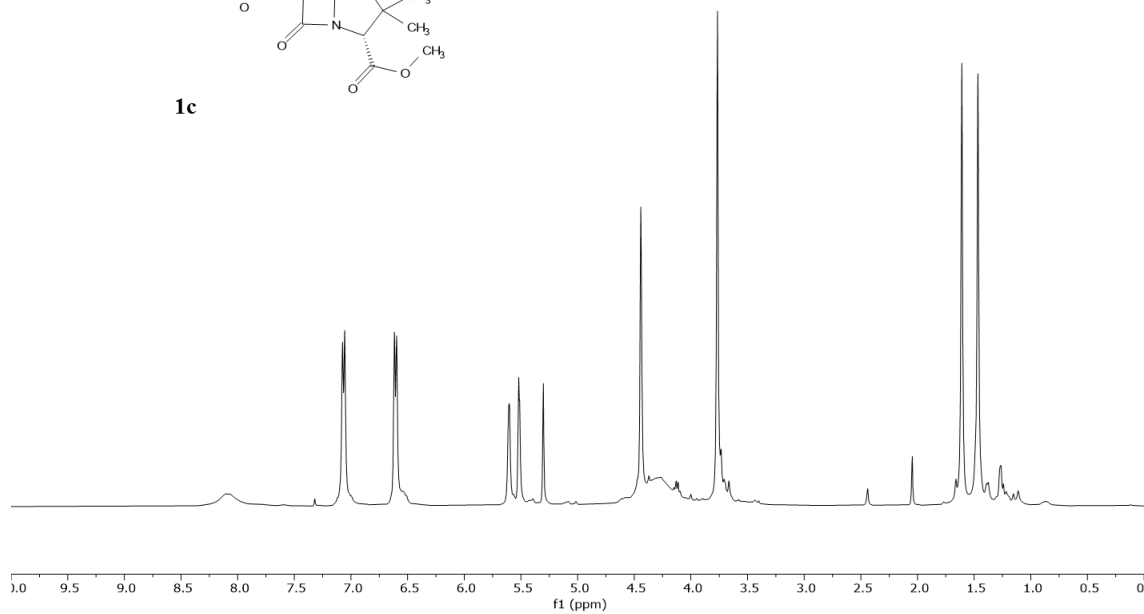
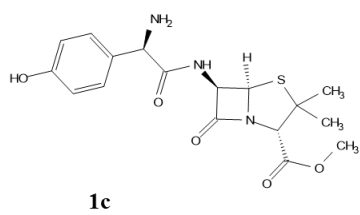


$^1\text{H}$  COSY NMR ( $\text{C}_6\text{D}_6$ , 400 MHz) $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{C}_6\text{D}_6$ , 400/100 MHz)

$^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{C}_6\text{D}_6$ , 400/100 MHz)

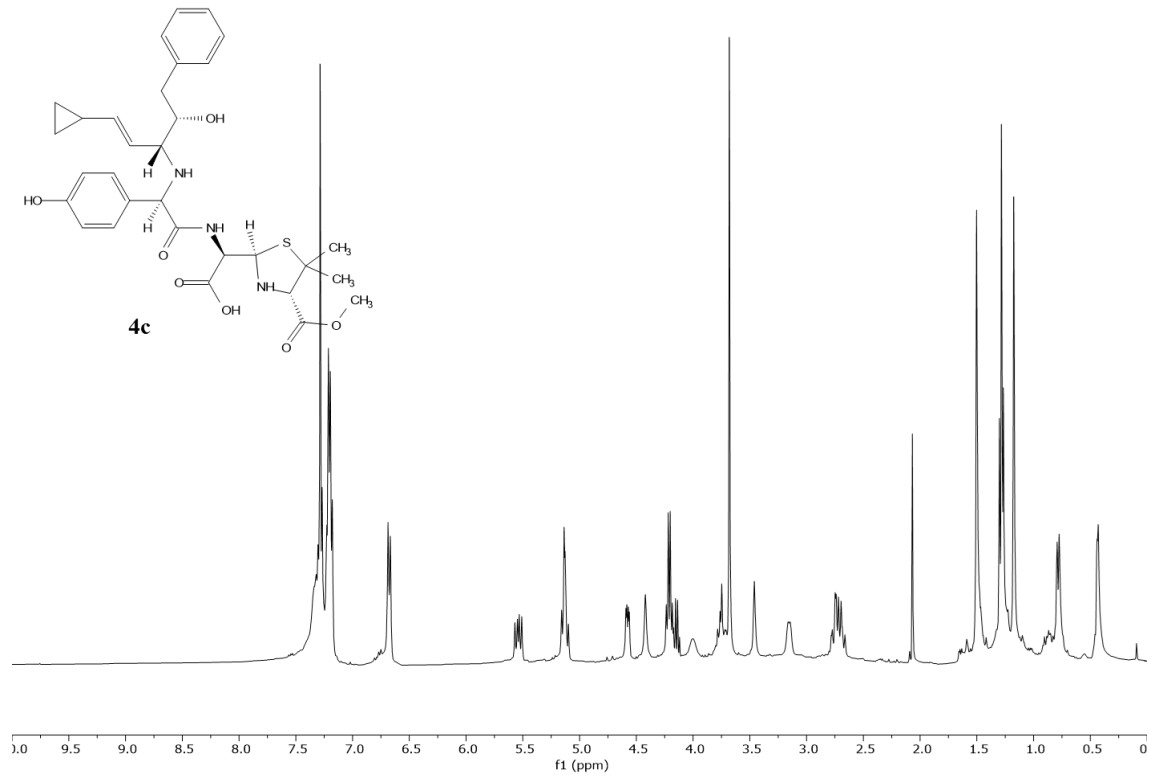
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) $^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz)



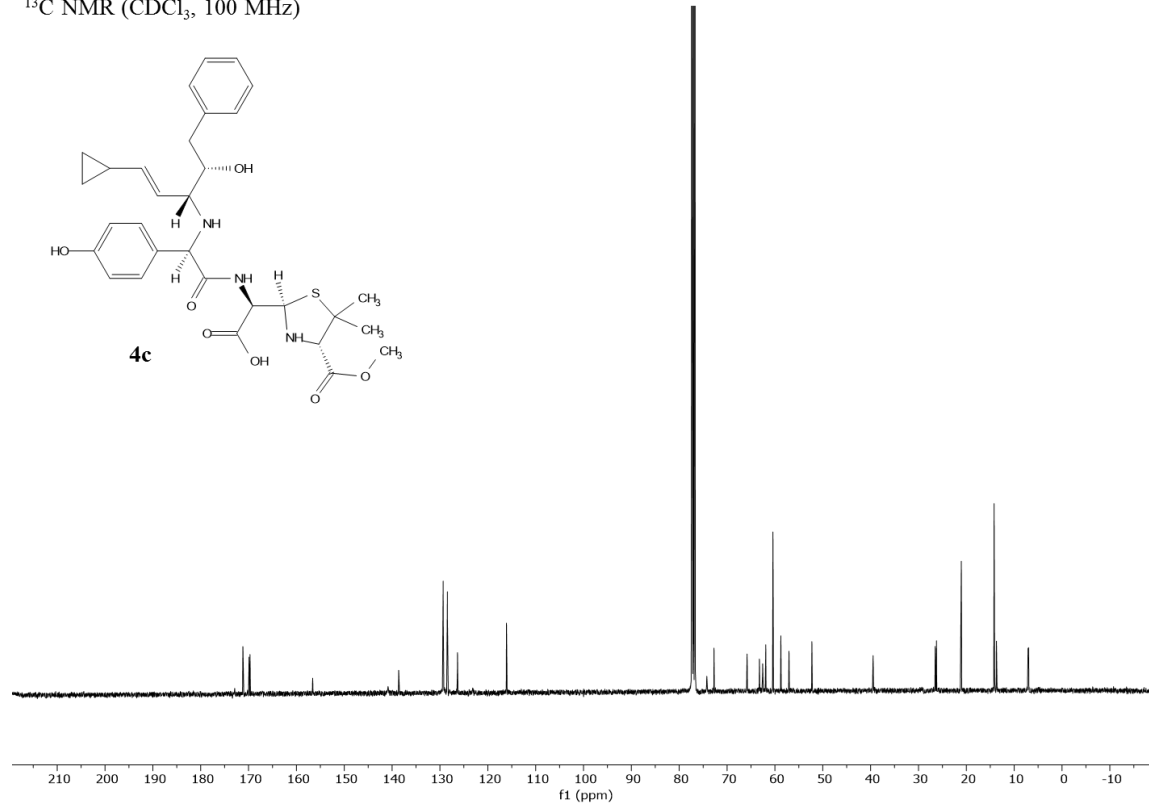
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

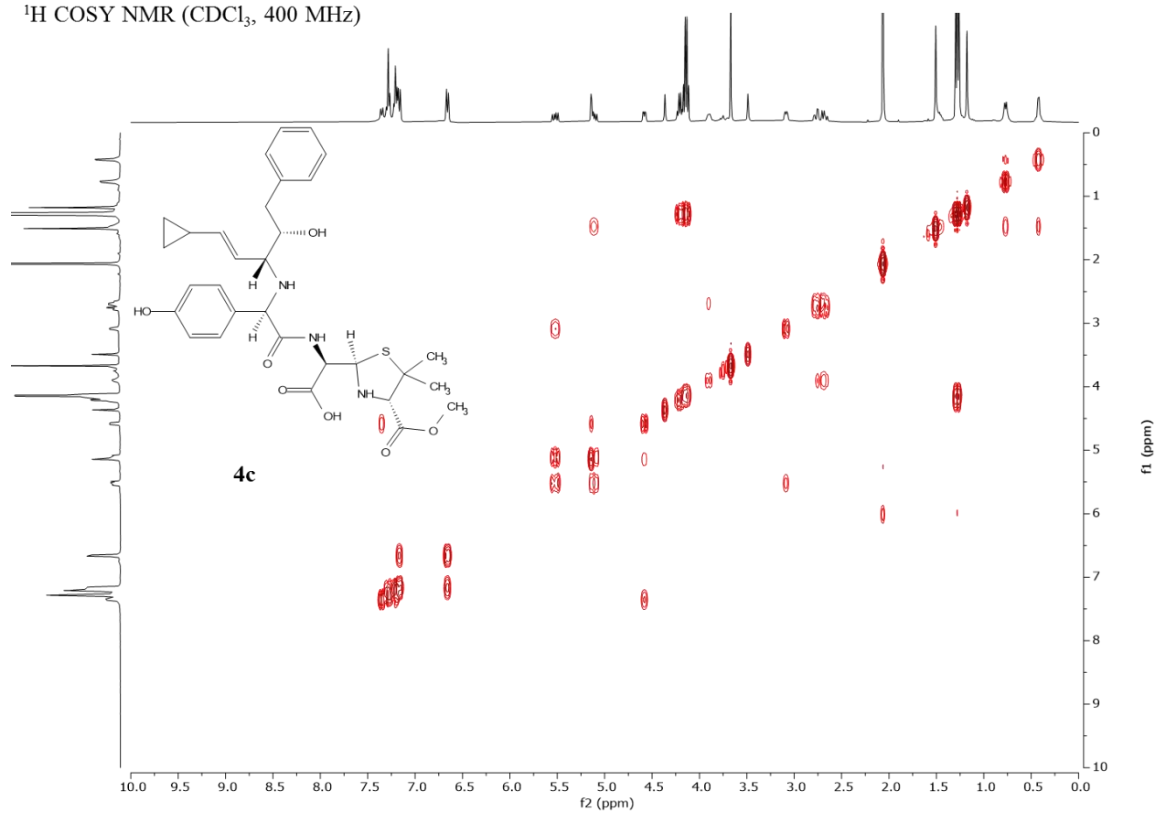
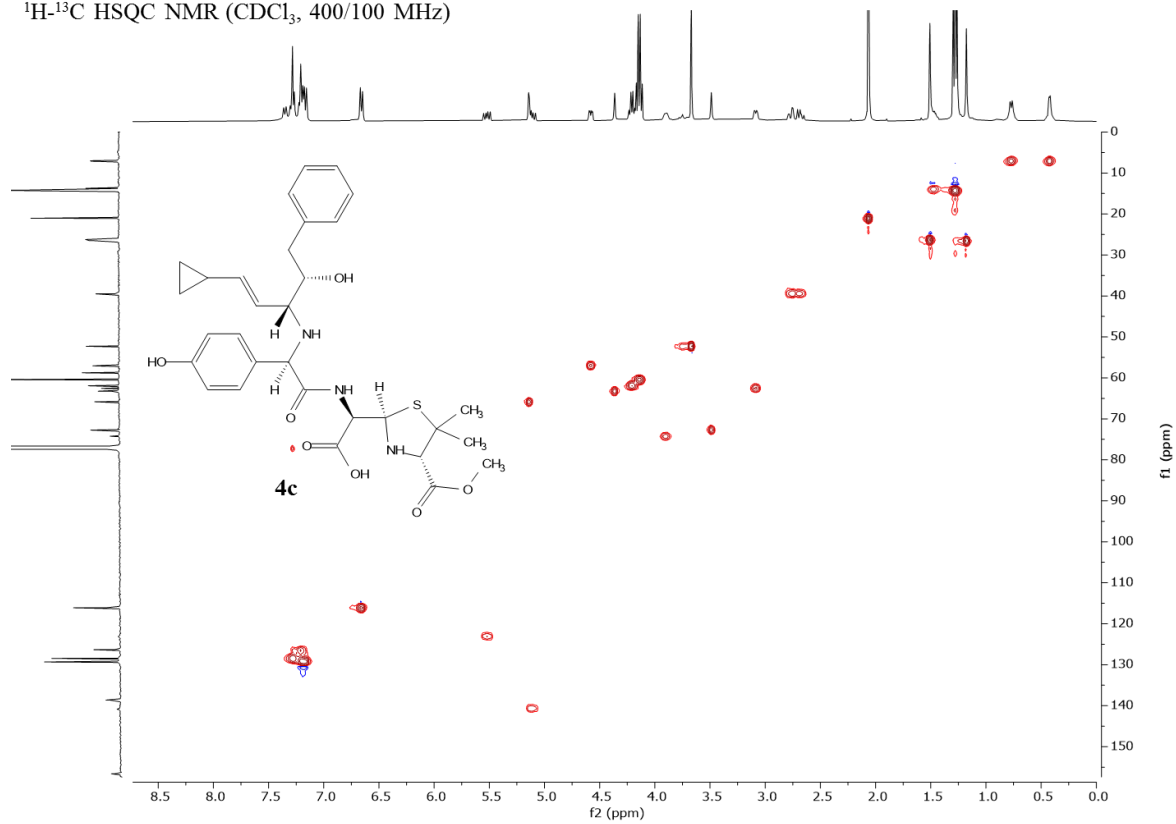
Annex 5

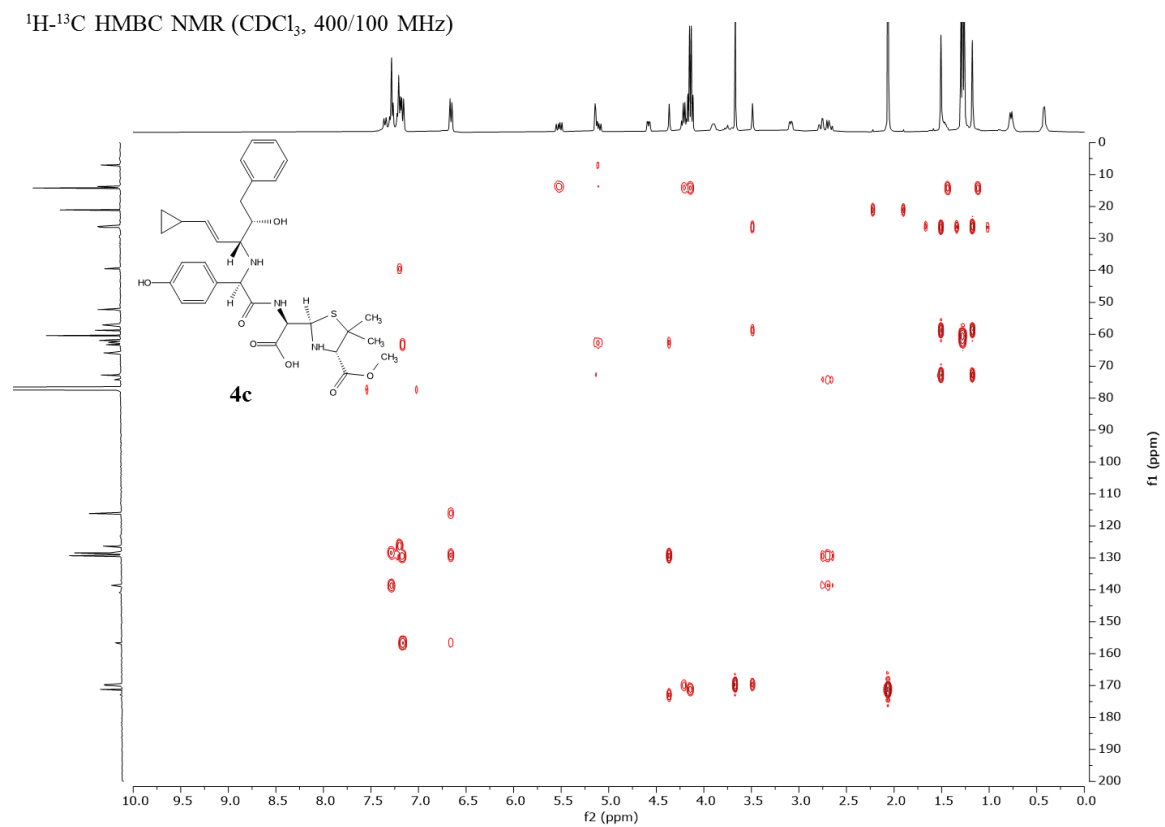
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



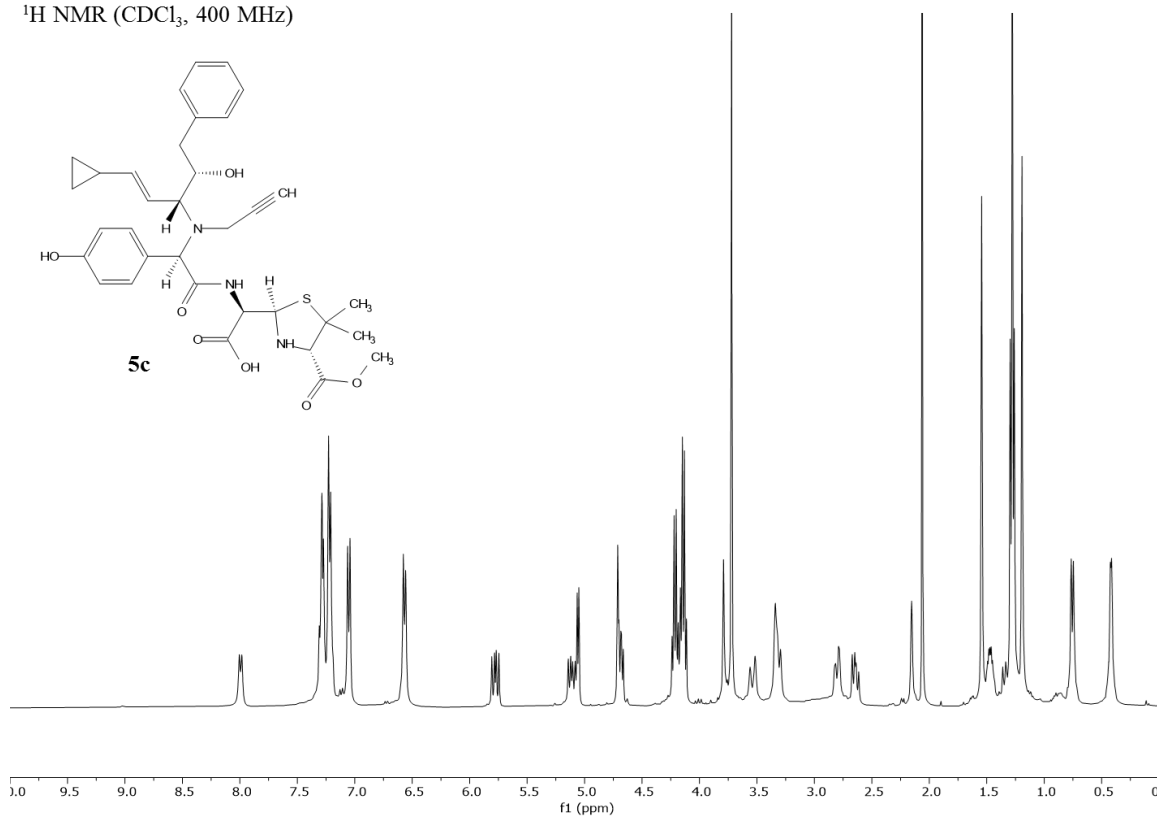
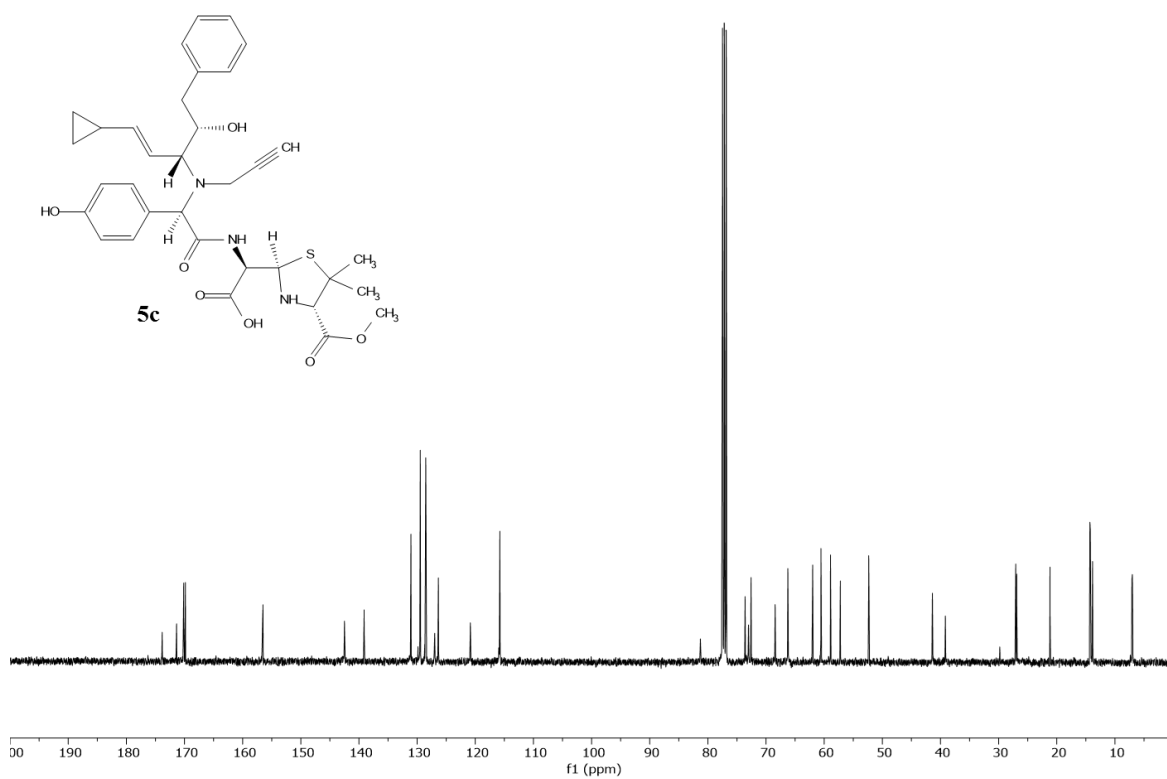
$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)

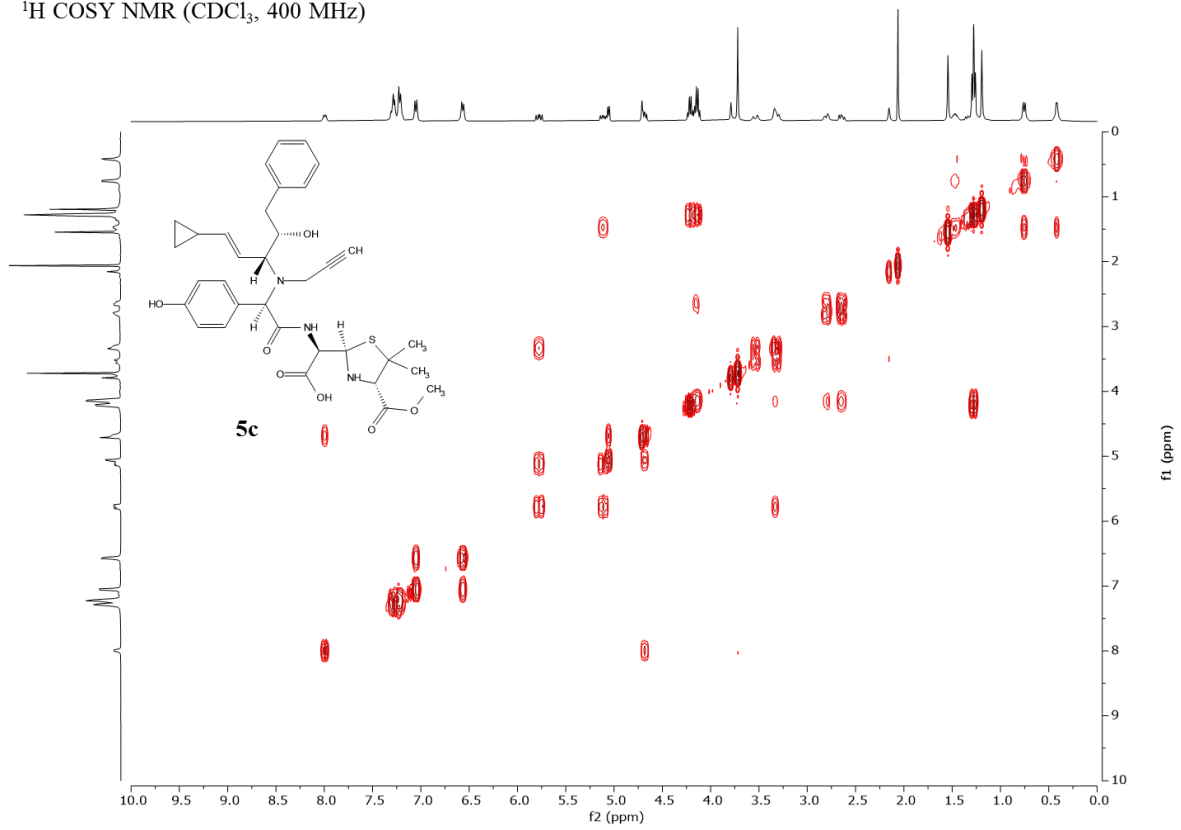
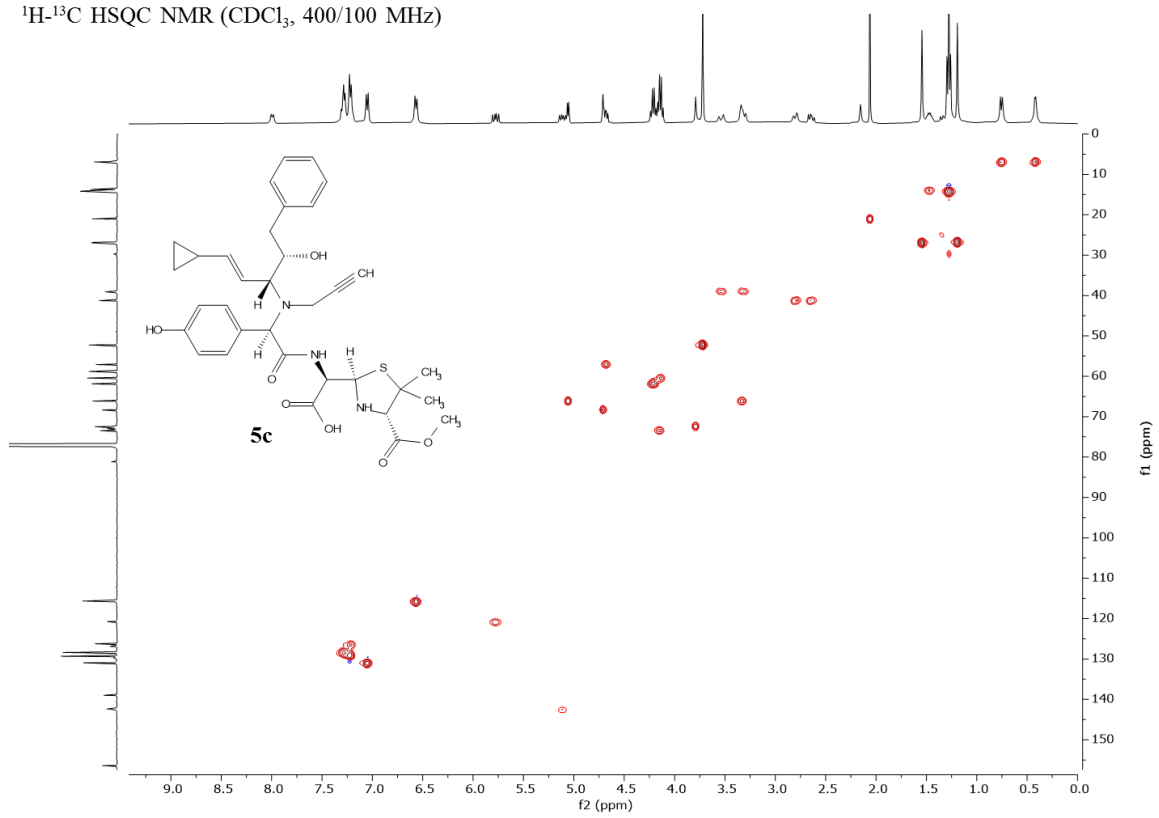


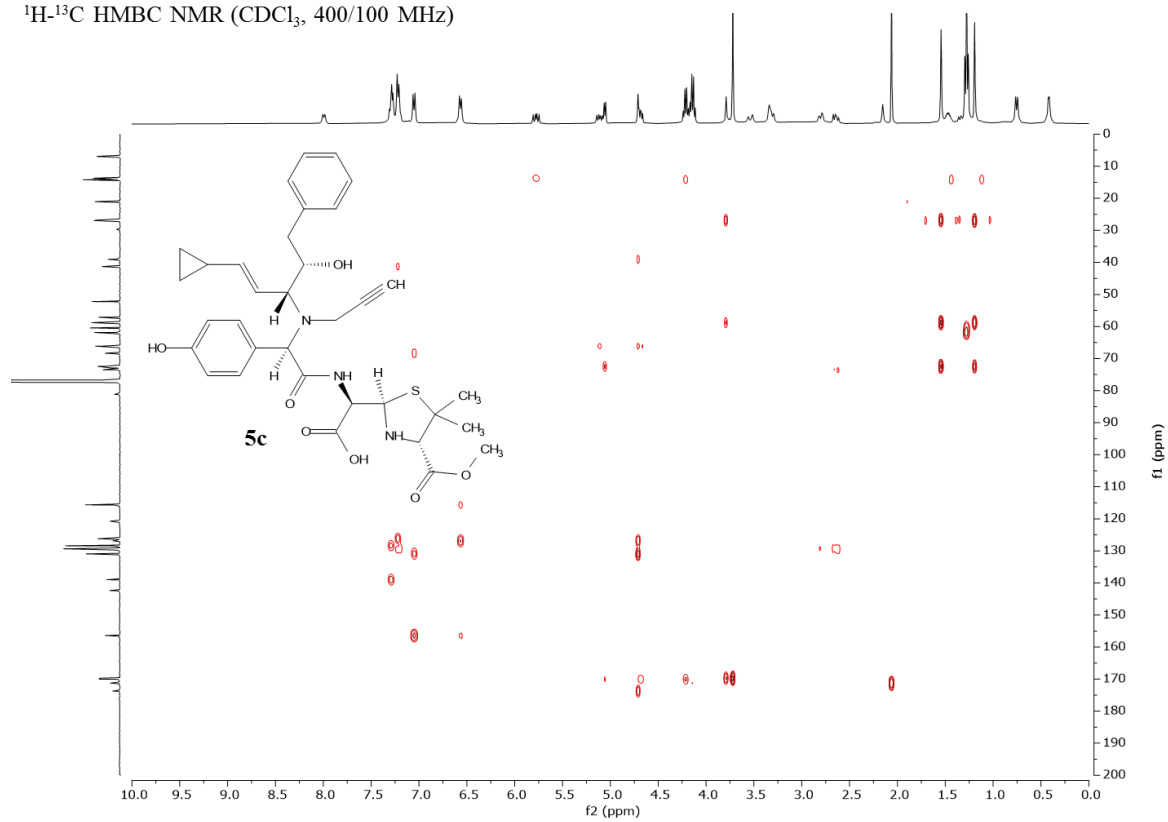
$^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz) $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{CDCl}_3$ , 400/100 MHz)





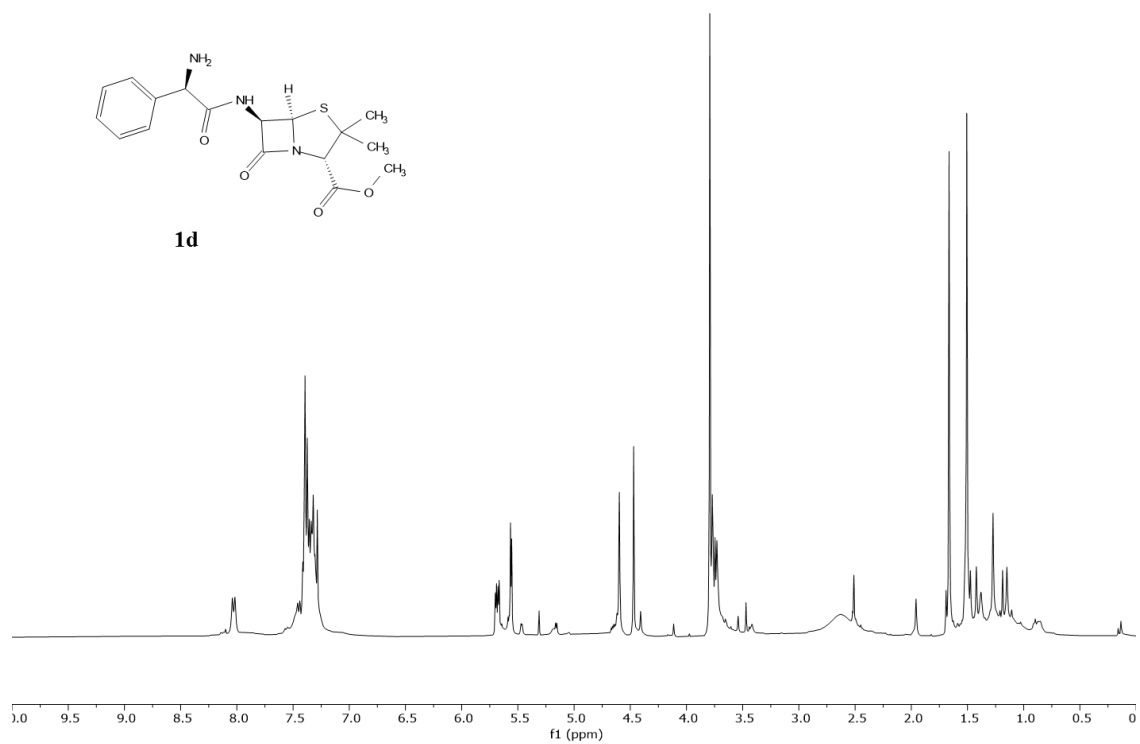
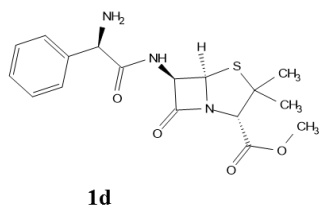
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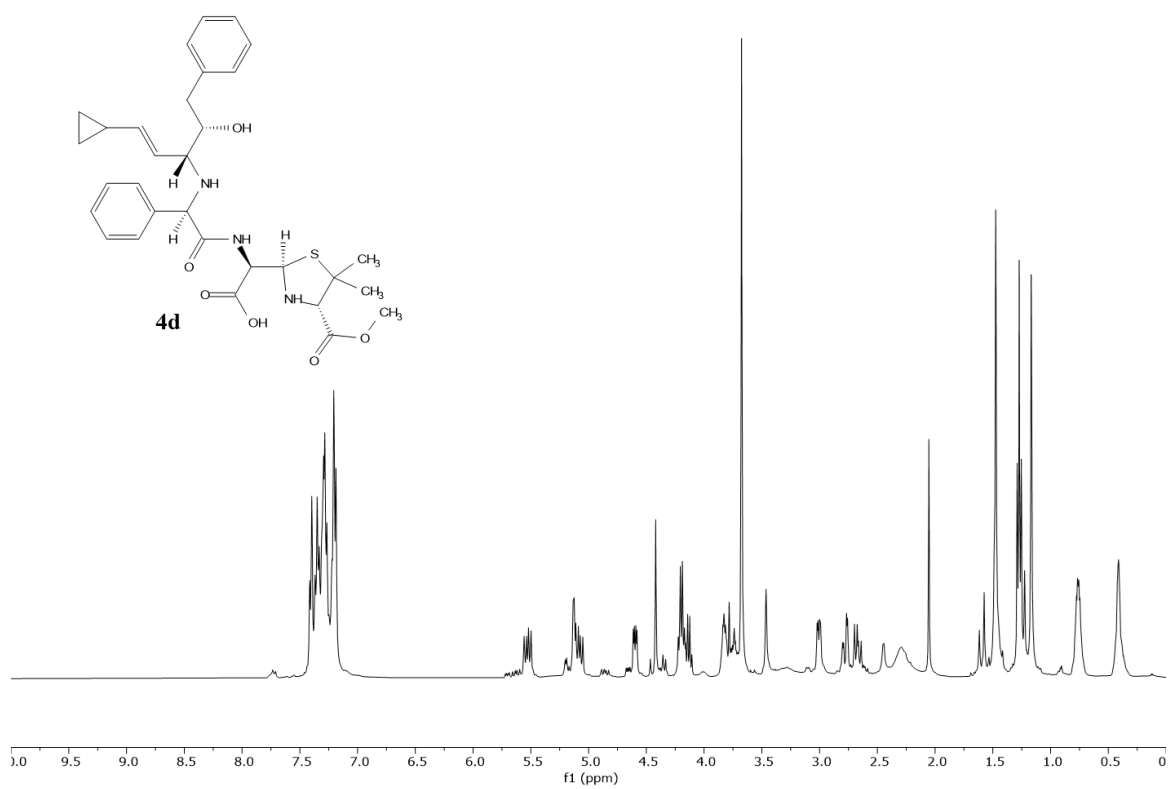
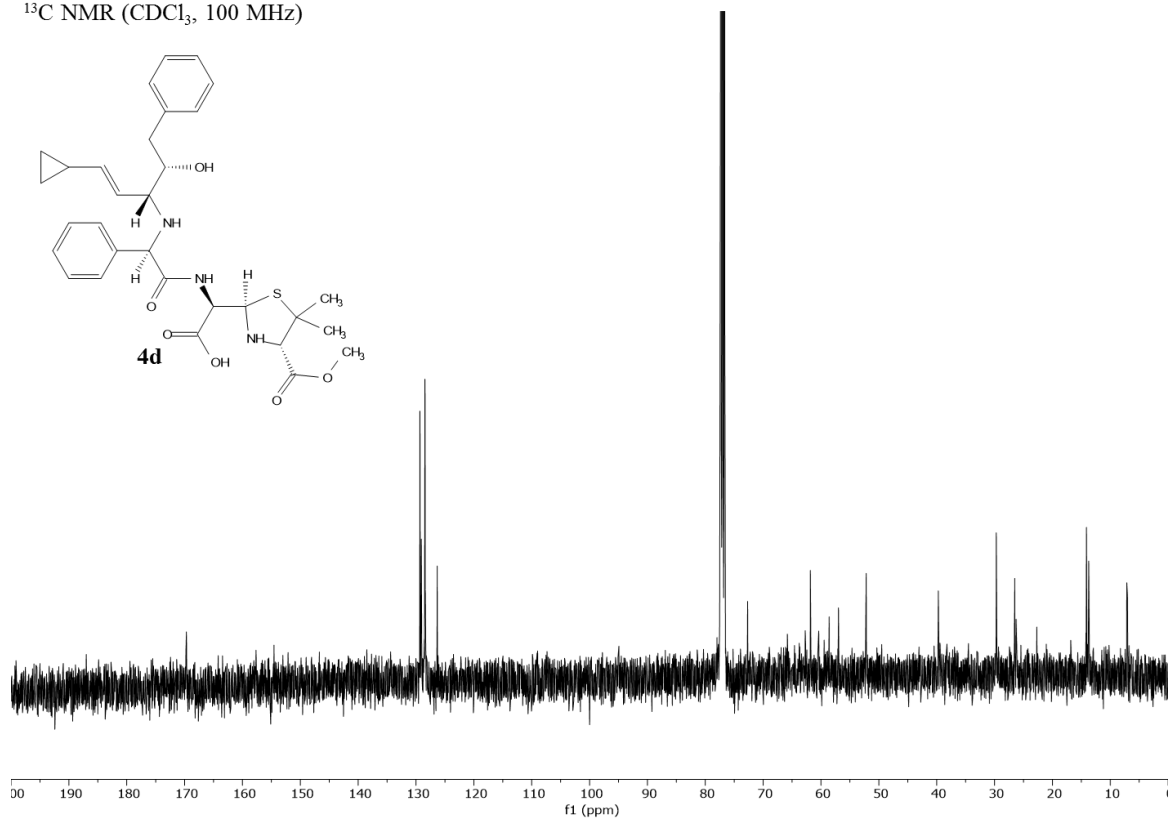
$^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz) $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

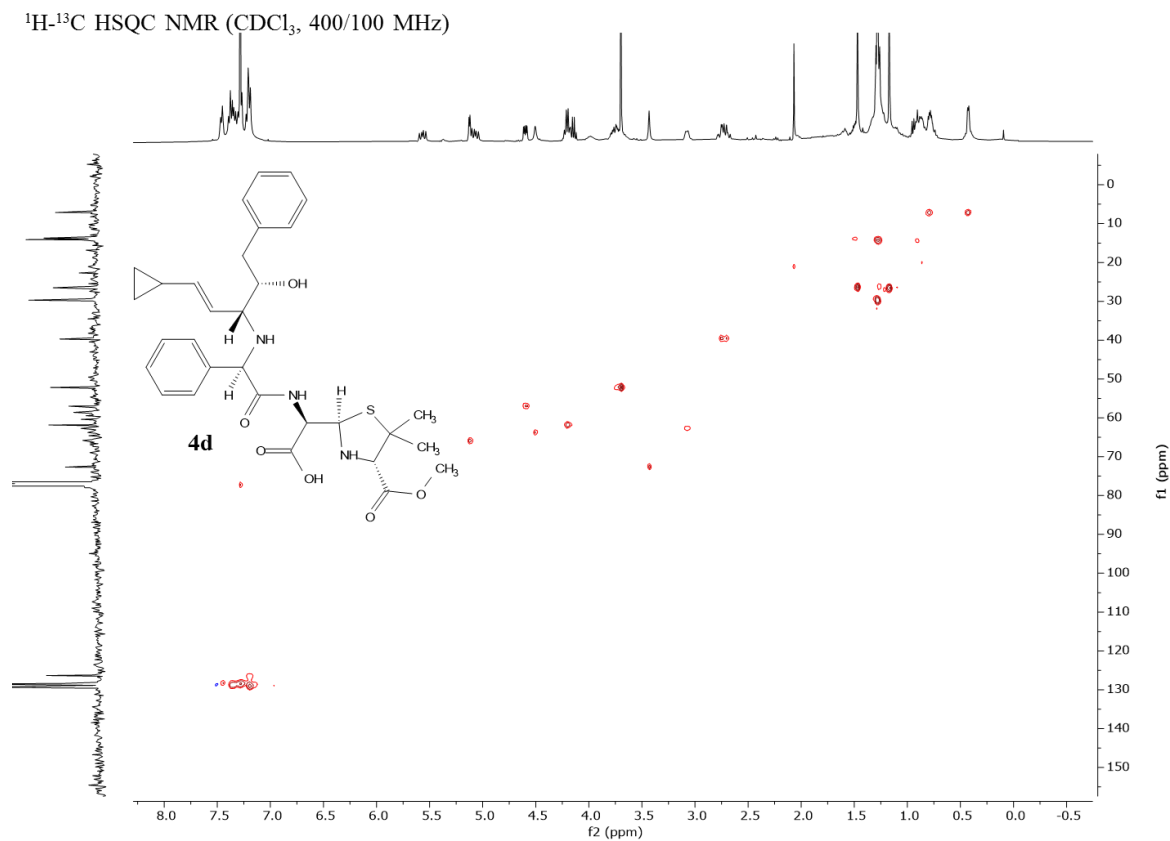
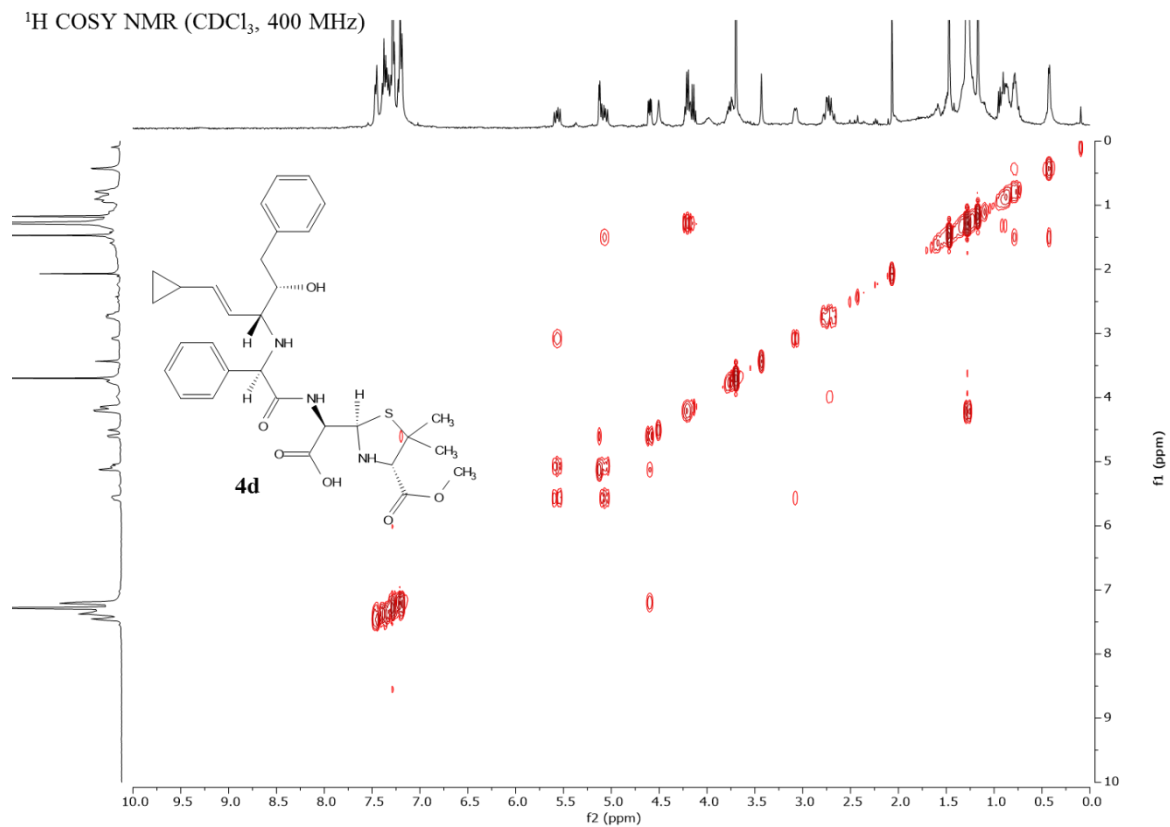
$^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

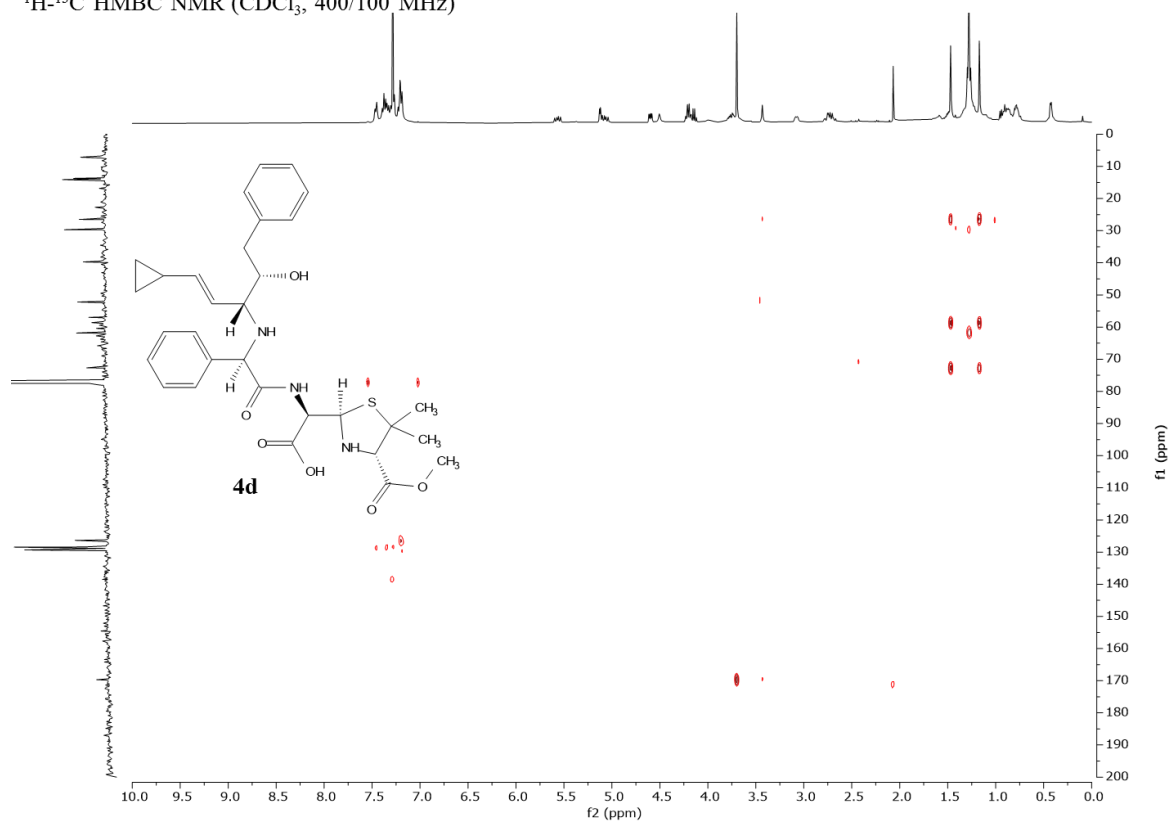
Annex 5

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)



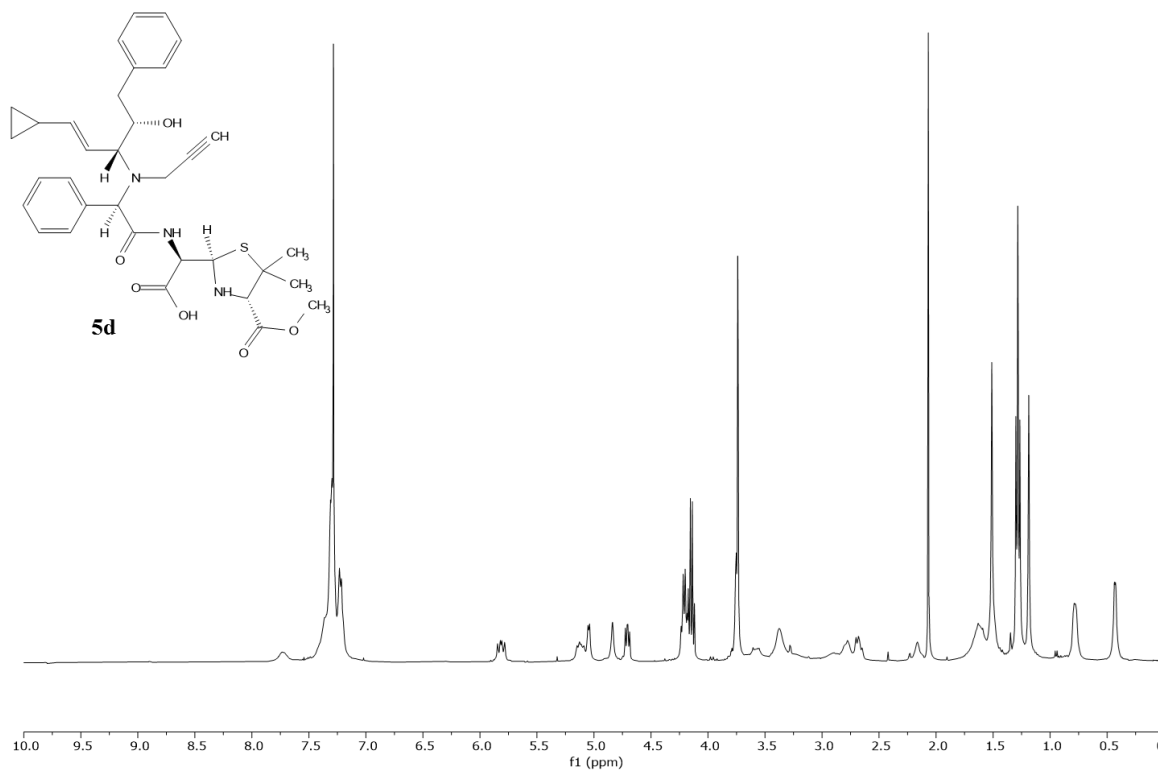
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)



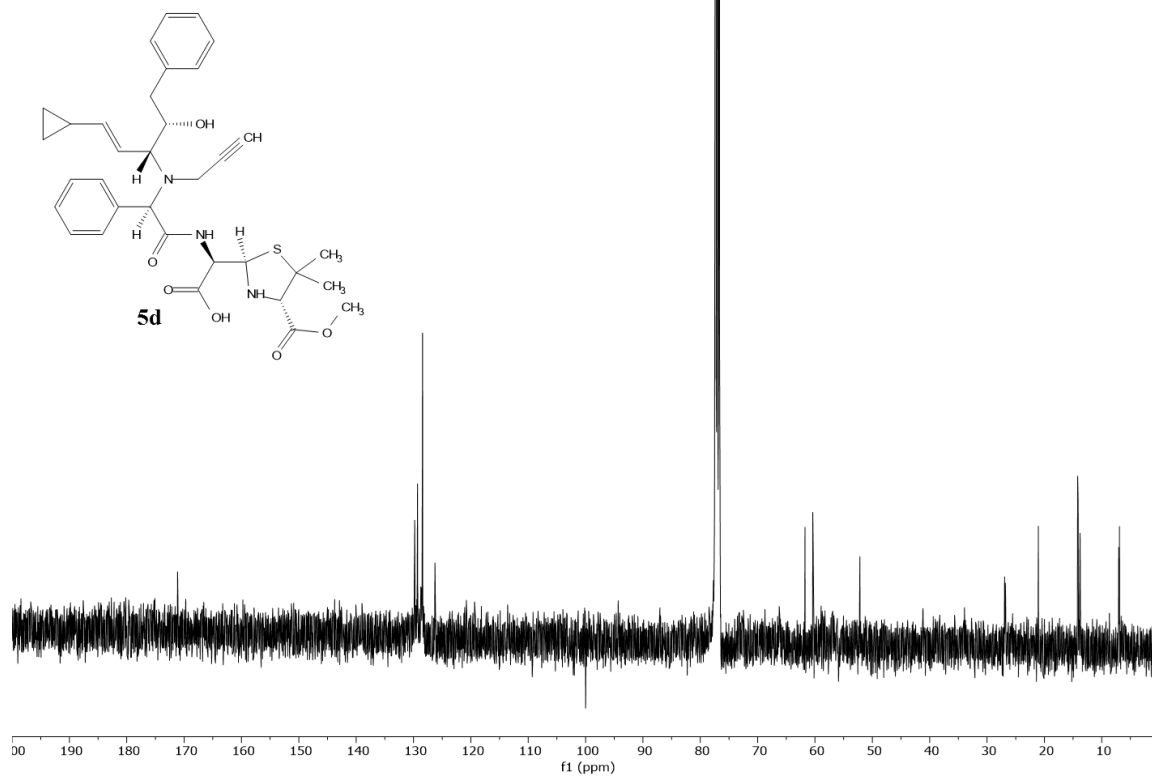
$^1\text{H}$ - $^{13}\text{C}$  HMBC NMR ( $\text{CDCl}_3$ , 400/100 MHz)

Annex 5

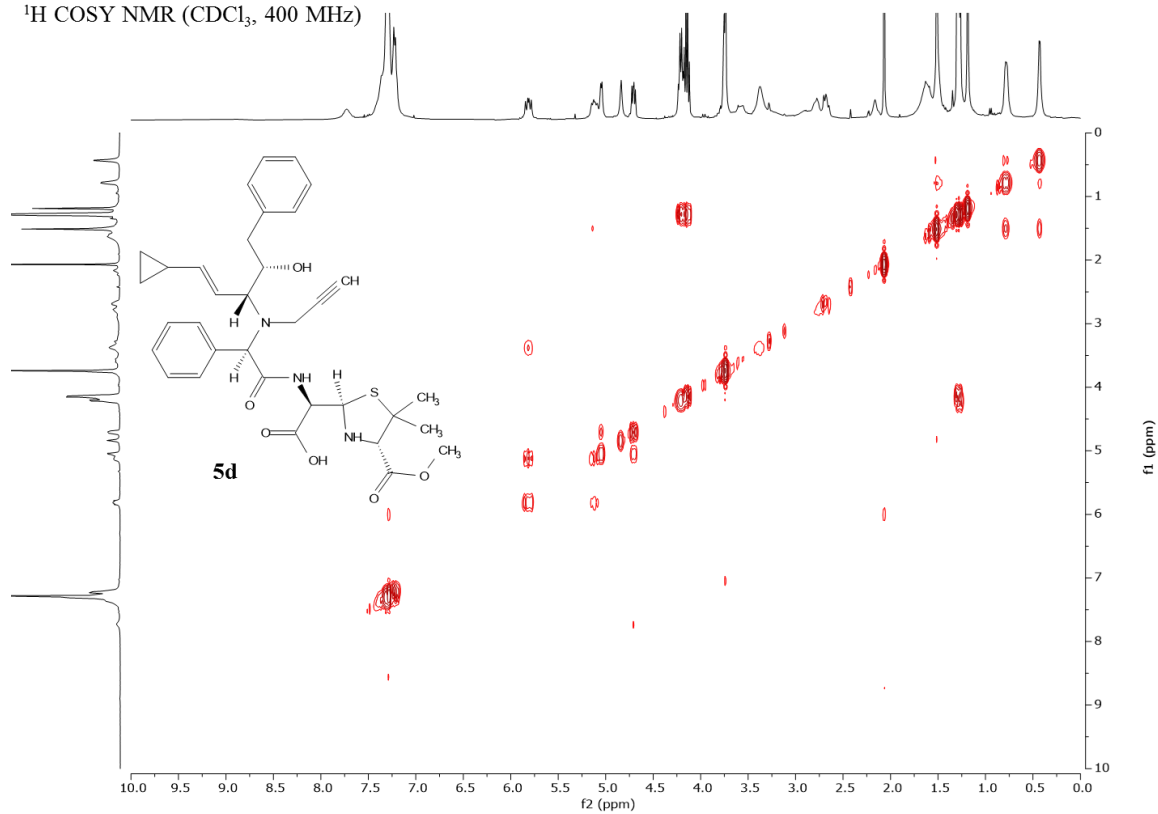
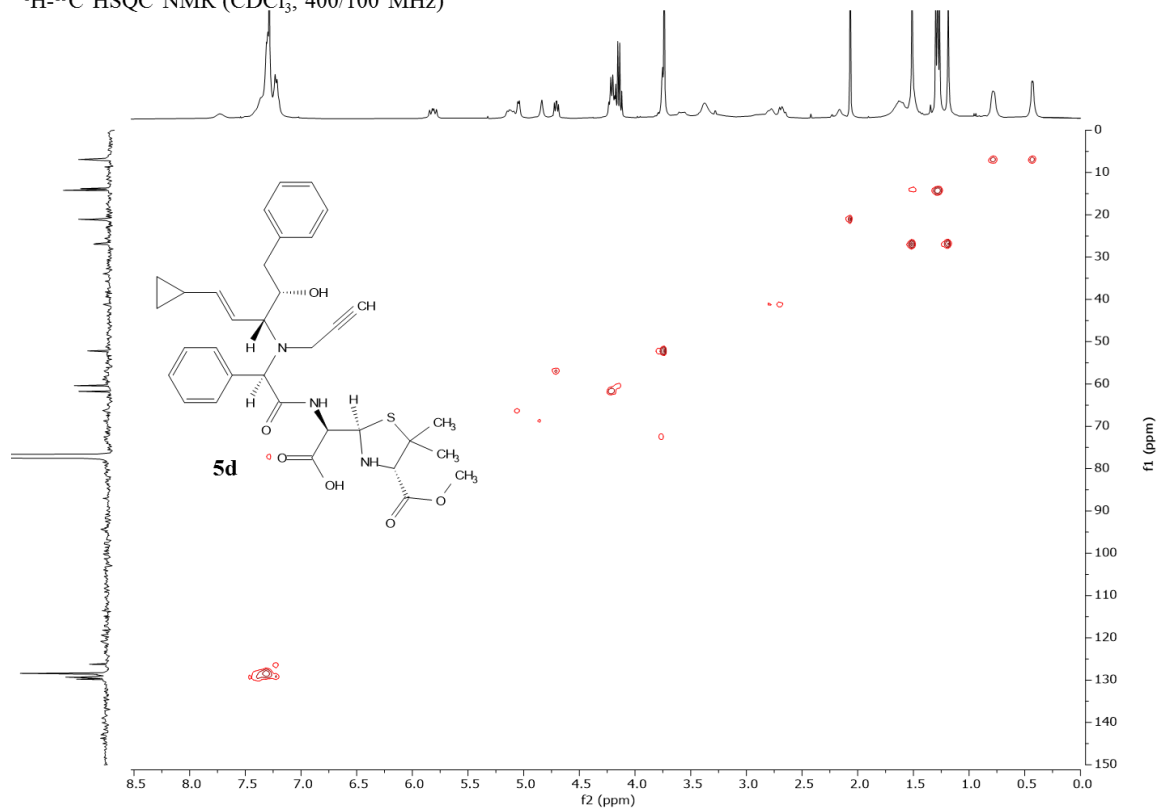
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

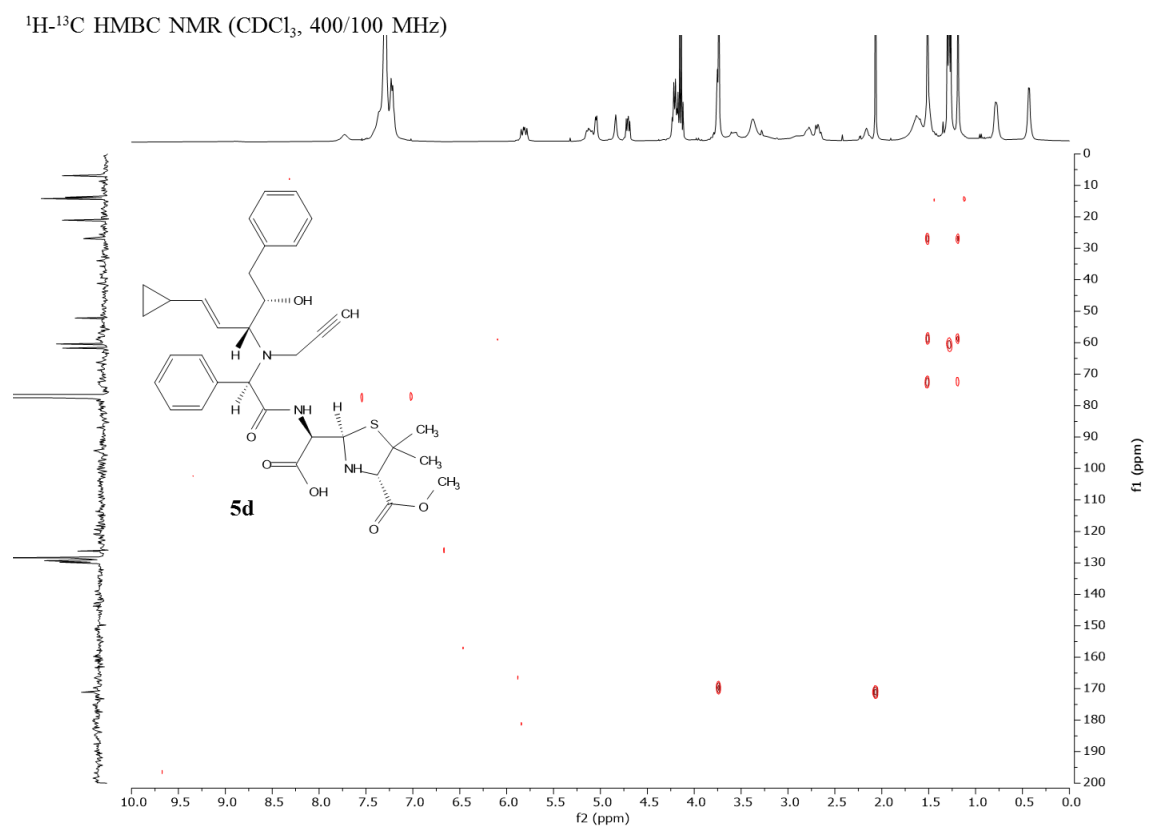


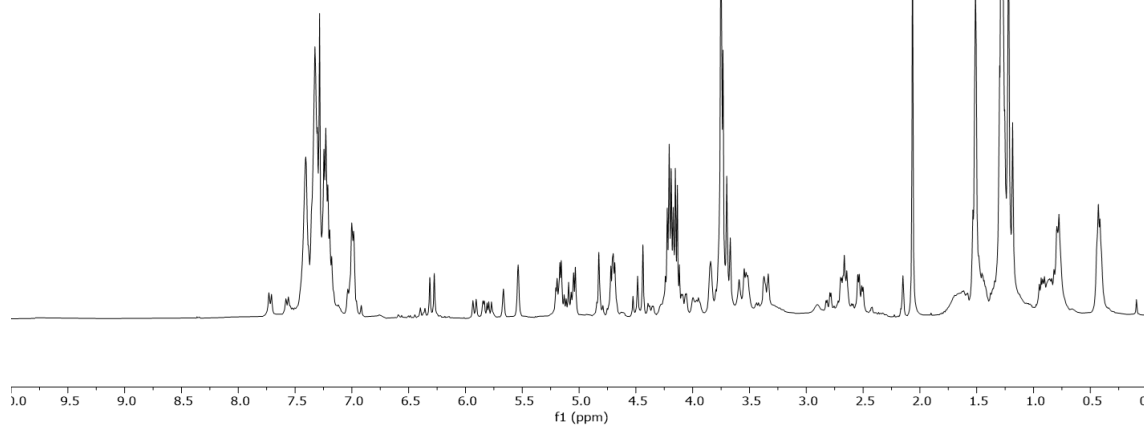
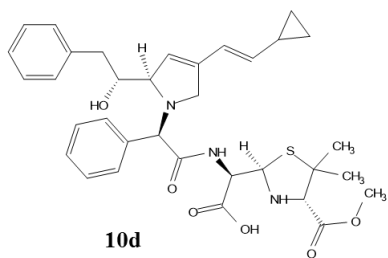
$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)





$^1\text{H}$  COSY NMR ( $\text{CDCl}_3$ , 400 MHz) $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR ( $\text{CDCl}_3$ , 400/100 MHz)



$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)

**Table 6.5.S1.** Optimization conditions for the Petasis reaction

<b>Entry</b>	<b>Solvent</b>	<b>Conditions</b>	<b>Molarity (M)</b>	<b>Equivalents 2:1a-f:3</b>	<b>Yield (%)</b>
<b>1</b>	EtOH/HFIP 9:1	40 °C, 72 h	0,20	1:1:1	30
<b>2</b>	EtOH/HFIP 9:1	0 °C, 72 h	0,20	1:1:1	-
<b>3</b>	EtOH	rt, 72 h	0,25	1:1:1	-
<b>4</b>	DCM	rt, 72 h	0,25	1:1:1	-
<b>5</b>	DCM	rt, 24 h, 3Å molecular sieves	0,20	1:1:1	-
<b>6</b>	EtOH/HFIP 9:1	40 °C, 72 h, 3Å molecular sieves	0,20	1:1.5:1.5	53
<b>7</b>	EtOH/HFIP 9:1	40 °C, 72 h, 3Å molecular sieves, glycerol	0,20	1:1.5:1.5	23
<b>8</b>	EtOH/HFIP 9:1	rt, 24 h, (E)-2-Cyclopropylvinylboronic acid pinacol ester	0,20	1:1:1	-
<b>9</b>	EtOH/HFIP 9:1	40 °C, 72 h, 3Å molecular sieves, (E)-2-Cyclopropylvinylboronic acid pinacol ester	0,20	1:1.5:1.5	9

#### 4. Immunoassay

##### *Antigen preparation*

All synthesized compounds (7.0  $\mu\text{mol}$ , 1,000 and 240 equiv. for HSA and H1, respectively), dissolved in potassium carbonate 0.5 M, pH 11.0 in 10 % DMF, were mixed with the carrier protein (0.5 mg) and stirred overnight at room temperature. Reference antigens for PG, AMX, CFT, AZT and MRP were also prepared following the major configuration. The corresponding BLC, as commercially supplied from Sigma (240 equiv), dissolved in potassium carbonate 0.5 M, pH 11.0, was reacted with the carrier protein (2.0 mg) and stirred overnight at room temperature. Prepared antigens were purified by gel filtration chromatography on Amicon Ultra 0.5 pre-concentrated 10 K filters using PBS 1X, pH 7.4, as elution buffer. Carrier proteins used were HSA and H1.

New Zealand white rabbits were immunized to raise specific IgG for PG, CFT, AZT, and MRP. To prepare the immunogenic antigens, the same protocol as for the preparation of the reference antigens was followed, but KLH (1,000 equiv.) was used as the immunogenic protein. Immunogens were purified by size exclusion chromatography on dextran desalting columns using PBS 1X, pH 7.4, as the elution buffer.

All antigens were diluted to 1.0 mg/mL and stored at  $-20\text{ }^{\circ}\text{C}$  until use. Antigen concentrations were determined by the Bradford protein assay<sup>[1]</sup> and the protein-hapten molar ratio was established by MS-MALDI-TOF.<sup>[2]</sup>

##### *Multiplexed DVD in vitro assay protocol*

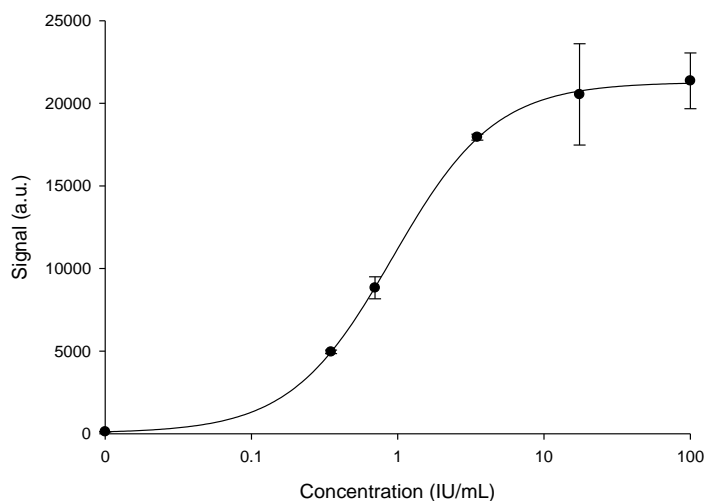
Reagents printed were (I) the prepared and reference antigens and (II) controls. HSA and rabbit IgG (or human IgE) were used as negative and positive controls of the assay, respectively. Reference antigens were used to test the specificity of each serum. Antigens were prepared in sodium carbonate/bicarbonate buffer 0.1 M, pH 9.6, and controls in PBS 1X, pH 7.4.

Reagents (25 nL, 40  $\mu\text{g}/\text{mL}$ ) were spotted in microarray format (20 arrays per disk of  $5 \times 4$  spots), using a noncontact printing device (AD 1500 BioDot, Inc., Irvine, CA) and DVDs (CD Rohling-up GmbH, Saarbrücken, Germany). After printing, the DVDs were incubated overnight at  $37\text{ }^{\circ}\text{C}$ .

To detect specific IgG against PG, CFT, MRP, and AZT, different dilutions (1/250-1/16,000) of rabbit sera and control (PBS-T) (25  $\mu$ L per sample) were added to each array and incubated for 15 min. Then, the DVD is washed with PBS-T and water. 25  $\mu$ L of goat anti-rabbit antibody labelled with horseradish peroxidase (Abcam, Cambridge, UK) in PBS-T (dilution 1/400) was added and incubated for 15 min followed by the final washing step.

To detect specific IgE from allergic patients and controls, 25  $\mu$ L of sample was added to each array and incubated for 30 min. Then, the DVD was washed and 25  $\mu$ L of  $\alpha$ -human IgE monoclonal antibody (Ingenasa, Madrid, Spain) in PBS-T (1  $\mu$ g/mL) was added and incubated for 15 min. After washing as before, 25  $\mu$ L of a 1/100 dilution of goat anti-mouse antibody labelled with horseradish peroxidase (Abcam, Cambridge, UK) was added and incubated for 15 min.

The immunoreaction was developed by homogeneously dispensing 1.0 mL of TMB (SDT GmbH, Baesweiler, Germany) along the whole disc surface. The reaction was stopped by washing the disk with water after 8 min. Signals were read by a modified DVD drive and data analyzed as previously described.<sup>[3]</sup> All experiments were repeated 3 times in parallel.



Calibrators used: 0, 0.35, 0.70, 3.50, 17.5 and 100 IU/mL. The standard data points (signal vs. semi-log concentration) were the mean of 5 curves performed in different days and on different disks. A four-parameter logistic (4PL) curve was fitted through the points, using SigmaPlot 11 software. Sensitivity ( $EC_{50} = 0.92 \pm 0.03$  IU/mL), slope= 1.24,  $r^2 = 0.9998$ . Limit of quantification ( $\geq 0.06$  IU/mL) is equal to the signal of the blank plus 10 times the SD (mean + 10xSD), with a relative standard deviation (RSD) ranging from 3 to 15 %.

**Figure 6.5.S1.** Calibration curve for IgE using IgE human serum (3<sup>rd</sup> WHO International Standard) and the capture antibody Omalizumab

## 5. Samples from allergic patients and controls

All samples from allergic patients and controls were provided by the Hospital Universitari i Politènic La Fe (Valencia, Spain) and informed consent for the diagnostic procedures was obtained from all patients. All participants were enrolled after giving written informed consent according to protocols approved by the ethics review board at La Fe University Hospital (registry no. COBIOPHAD). The procedures followed were in accordance with the Helsinki Declaration of 1975 as revised in 2008. The patients were diagnosed following the procedure described in the European Network of Drug Allergy (ENDA) protocol based on skin testing, *in vitro* tests or drug provocation test, whenever necessary.

**Table 6.5.S2.** Clinical characteristics of the cohort of allergic patients

Patient Number	Sex <sup>[a]</sup>	Age (years)	Culprit drug	Clinical manifestation	Route	Time
1	M	68	Augmentin	Anaphylaxis	Parenteral	Immediate
2	F	36	Amoxicillin	Anaphylaxis	Oral	Immediate
3	F	42	Augmentin	Anaphylaxis	Oral	Immediate
4	M	49	Augmentin	Cutaneous	Oral	Delayed
5	M	80	Augmentin	Anaphylaxis	Parenteral	Immediate
6	M	44	Augmentin	Cutaneous	Oral	Delayed
7	F	32	Augmentin	Anaphylaxis	Oral	Immediate
8	F	47	Amoxicillin	Anaphylaxis	Oral	Immediate
9	M	56	Augmentin	Cutaneous	Oral	Immediate
10	F	32	Augmentin	Cutaneous	Oral	Delayed
11	M	56	Amoxicillin	Cutaneous	Oral	Immediate
12	F	73	Augmentin	Anaphylaxis	Oral	Immediate
13	F	75	Cefuroxime	Cutaneous	Oral	Immediate
14	F	44	Cefuroxime	Cutaneous	Oral	Immediate
15	M	76	Amoxicillin	Cutaneous	Oral	Immediate
16	F	76	Augmentin	Cutaneous	Oral	Delayed
17	M	30	Amoxicillin	Anaphylaxis	Oral	Immediate
18	F	53	Augmentin	Anaphylaxis	Oral	Immediate
19	F	40	Augmentin	Cutaneous	Oral	Immediate

<sup>[a]</sup>Sex: F=Female, M=Male.

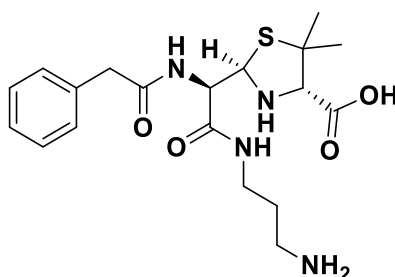
## REFERENCES

- [1] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.
- [2] Y. Wang, C. H. He, H. Zheng, H. Bin Zhang, *Int. J. Mol. Sci.* **2012**, *13*, 84–96.
- [3] P. Dobosz, S. Morais, E. Bonet, R. Puchades, Á. Maquieira, *Anal. Chem.* **2015**, *87*, 9817–9824.
- [4] S. Wang, S. Liu, Y. Zhang, J. He, D. H. Coy, L. Sun, *Heal. Sci. J.* **2020**, *14*, 1–8.



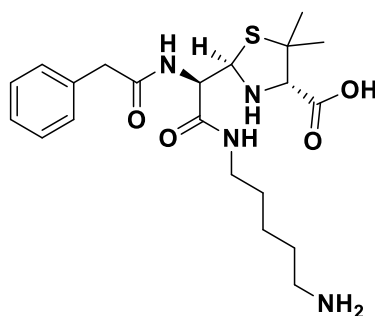
## 6.6. Annex 6

### Summary of all the haptens described in this dissertation



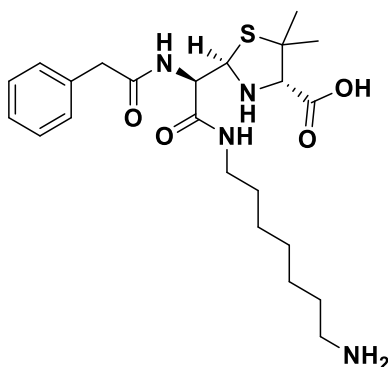
*(2R,4S)-2-((R)-2-((3-aminopropyl)amino)-2-oxo-1-(2-phenylacetamido)ethyl)-5,5-dimethylthiazolidine-4-carboxylic acid*

Described in Chapter 1.1 and labeled as Hapten **1**. Synthetic procedure and data characterization included in Annex 1 (pp. 210 and 217).



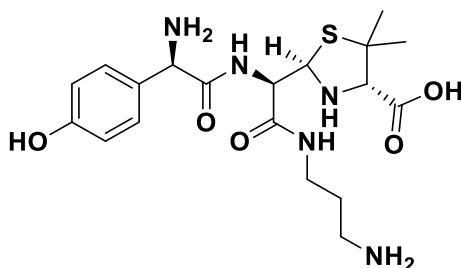
*(2R,4S)-2-((R)-2-((5-aminopentyl)amino)-2-oxo-1-(2-phenylacetamido)ethyl)-5,5-dimethylthiazolidine-4-carboxylic acid*

Described in Chapter 1.1 and labeled as Hapten **2**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 210 and 218).



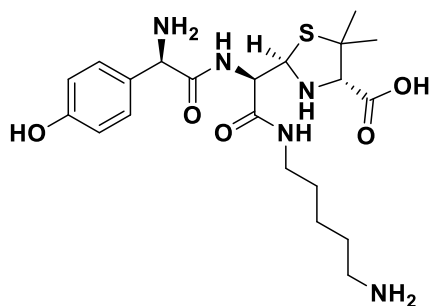
*(2R,4S)-2-((R)-2-((7-aminoheptyl)amino)-2-oxo-1-(2-phenylacetamido)ethyl)-5,5-dimethylthiazolidine-4-carboxylic acid*

Described in Chapter 1.1 and labeled as Hapten **3**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 211 and 219).



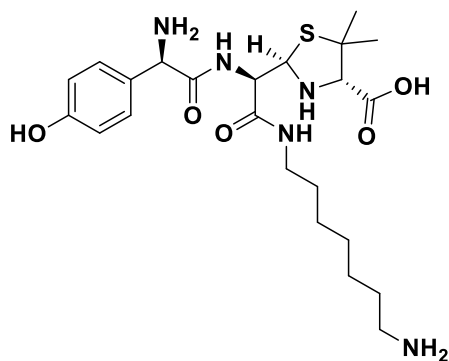
*(2R,4S)-2-((R)-1-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-2-((3-aminopropyl)amino)-2-oxoethyl)-5,5-dimethylthiazolidine-4-carboxylic acid*

Described in Chapter 1.1 and labeled as Hapten **4**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 211 and 220).



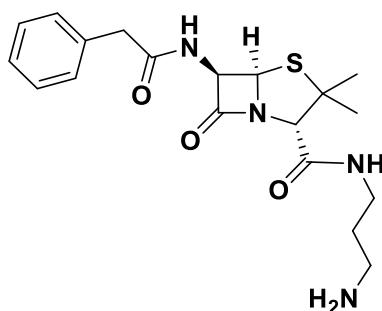
*(2R,4S)-2-((R)-1-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-2-((5-aminopentyl)amino)-2-oxoethyl)-5,5-dimethylthiazolidine-4-carboxylic acid*

Described in Chapter 1.1 and labeled as Hapten **5**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 211 and 221).



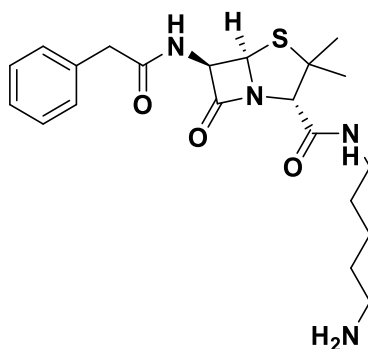
*(2R,4S)-2-((R)-1-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-2-((7-aminoheptyl)amino)-2-oxoethyl)-5,5-dimethylthiazolidine-4-carboxylic acid*

Described in Chapter 1.1 and labeled as Hapten **6**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 212 and 222).



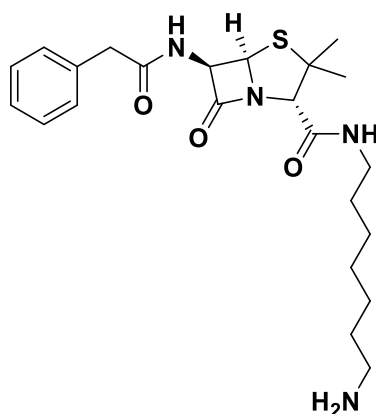
*(2S,5R,6R)-N-(3-aminopropyl)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxamide*

Described in Chapter 1.1 and labeled as Hapten **7**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 212 and 223).



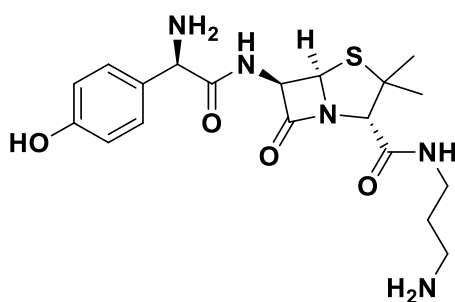
*(2S,5R,6R)-N-(5-aminopentyl)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxamide*

Described in Chapter 1.1 and labeled as Hapten **8**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 213 and 224).



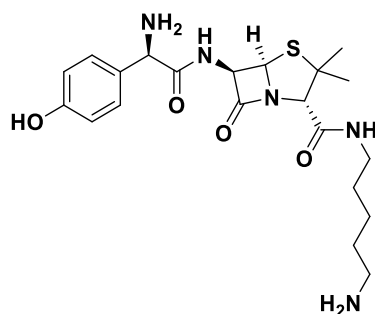
*(2S,5R,6R)-N-(7-aminoheptyl)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxamide*

Described in Chapter 1.1 and labeled as Hapten **9**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 213 and 225).



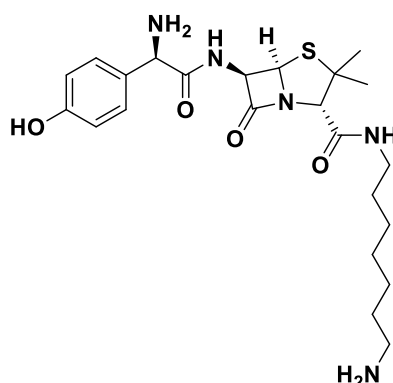
*(2S,5R,6R)-6-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-N-(3-aminopropyl)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxamide*

Described in Chapter 1.1 and labeled as Hapten **10**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 213 and 226).



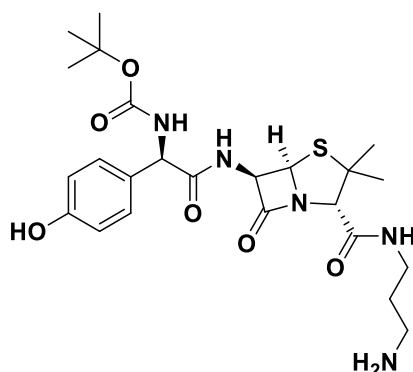
*(2S,5R,6R)-6-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-N-(5-aminopentyl)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxamide*

Described in Chapter 1.1 and labeled as Hapten **11**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 214 and 227).



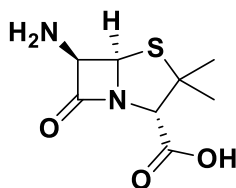
*(2S,5R,6R)-6-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-N-(7-aminoheptyl)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxamide*

Described in Chapter 1.1 and labeled as Hapten **12**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 214 and 228).



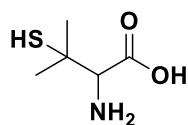
*Tert-butyl ((R)-2-(((2S,5R,6R)-2-((3-aminopropyl)carbamoyl)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptan-6-yl)amino)-1-(4-hydroxyphenyl)-2-oxoethyl)carbamate*

Described in Chapter 1.1 and labeled as Hapten **13**. Synthetic procedure and NMR spectrum included in Annex 1 (pp. 214 and 229).



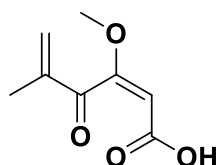
*6-aminopenicillanic acid*

Described in Chapter 1.2 and labeled as Hapten **6-APA**. Used as commercially supplied.



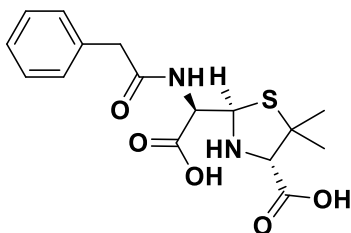
*DL-penicillamine*

Described in Chapter 1.2 and labeled as Hapten **PA**. Used as commercially supplied.



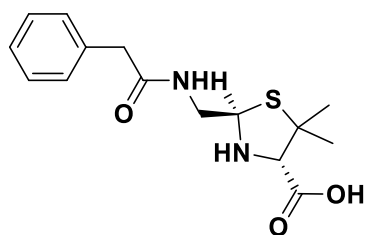
*Penicillic acid*

Described in Chapter 1.2 and labeled as Hapten **PLA**. Used as commercially supplied.



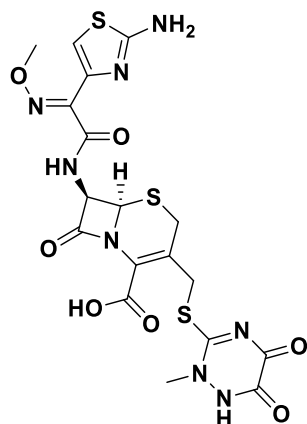
*Benzylpenicilloic acid*

Described in Chapter 1.2 and labeled as Hapten **PGoyl**. Synthetic procedure and data characterization included in Chapter 1.2 (p. 104). NMR spectrum included in Annex 2 (p. 246).



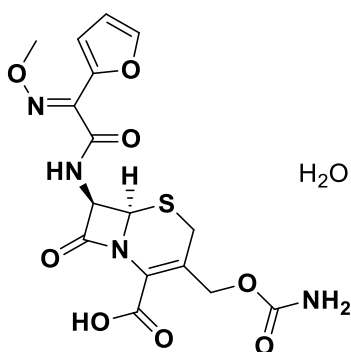
*Benzylpenilloic acid*

Described in Chapter 1.2 and labeled as Hapten **BPG**. Synthetic procedure and data characterization included in Chapter 1.2 (p. 104). NMR spectrum included in Annex 2 (p. 247).



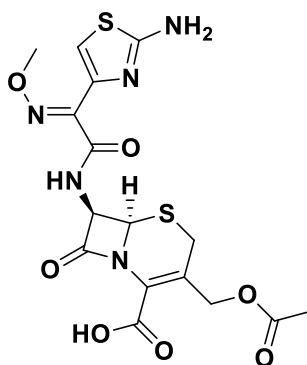
*4-((E)-2-(((6R,7R)-2-carboxy-3-(((2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl)thio)methyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-7-yl)amino)-1-(methoxyimino)-2-oxoethyl)thiazol-2-aminium*

Described in Chapter 2.1 and labeled as Hapten **CFT**. Synthetic procedure, data characterization and NMR spectrum included in Annex 3 (pp. 250 and 253).

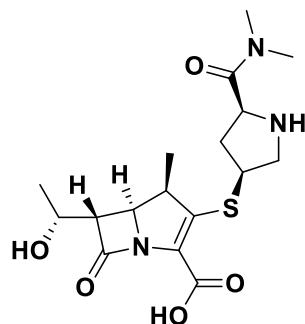


*(6R,7R)-3-((carbamoyloxy)methyl)-7-((E)-2-(furan-2-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate*

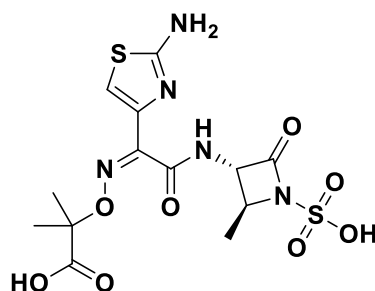
Described in Chapter 2.1 and labeled as Hapten **CFR**. Synthetic procedure, data characterization and NMR spectrum included in Annex 3 (pp. 251 and 254).



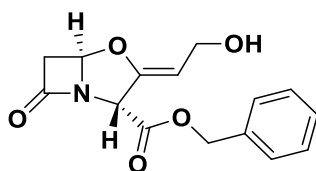
(6*R*,7*R*)-3-(acetoxymethyl)-7-((*E*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid  
 Described in Chapter 2.1 and labeled as Hapten **CFO**. Synthetic procedure, data characterization and NMR spectrum included in Annex 3 (pp. 252 and 255).



(4*R*,5*S*,6*S*)-3-(((3*S*,5*S*)-5-(dimethylcarbamoyl)pyrrolidin-3-ylthio)-6-((*R*)-1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid  
 Described in Chapter 2.1 and labeled as Hapten **MRP**. Used as commercially supplied.



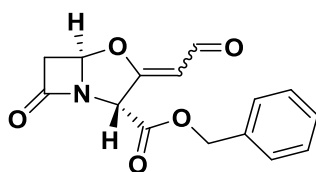
2-(((*Z*)-1-(2-aminothiazol-4-yl)-2-(((2*S*,3*S*)-2-methyl-4-oxo-1-sulfoazetid-3-yl)amino)-2-oxoethylidene)amino)oxy)-2-methylpropanoic acid  
 Described in Chapter 2.1 and labeled as Hapten **AZT**. Used as commercially supplied.



Benzyl (2*R*,5*R*,*Z*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate

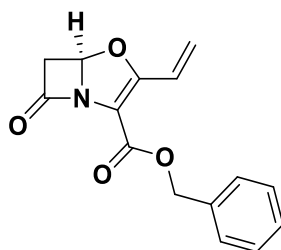
Described in Chapter 2.2 and labeled as Hapten **CVL-1**. Synthetic procedure and data characterization included in Chapter 2.2 (pp. 155 and 156). NMR spectrum included in Annex 4 (p. 269).





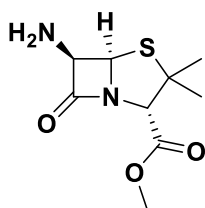
*Benzyl (2R,5R)-7-oxo-3-(2-oxoethylidene)-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate*

Described in Chapter 2.2 and labeled as Hapten **CVL-2**. Synthetic procedure and data characterization included in Chapter 2.2 (p. 156). NMR spectrum included in Annex 4 (pp. 270-272).



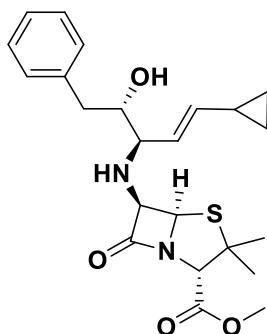
*Benzyl (R)-7-oxo-3-vinyl-4-oxa-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate*

Described in Chapter 2.2 and labeled as Hapten **CVL-3**. Synthetic procedure and data characterization included in Chapter 2.2 (p. 157). NMR spectrum included in Annex 4 (p. 272).



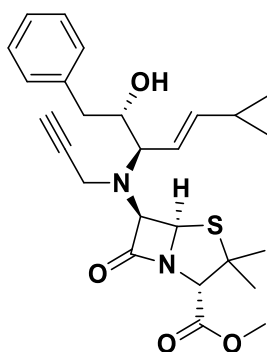
*6-aminopenicillanic acid methyl ester*

Described in Chapter 3 and labeled as Hapten **1a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 280 and 296).



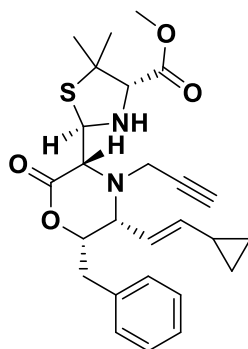
*(2S,3R,E)*-5-Cyclopropyl-2-hydroxy-3-((2S,5R,6R)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)amino-1-phenyl-4-pentene

Described in Chapter 3 and labeled as Hapten **4a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 280 and 297).



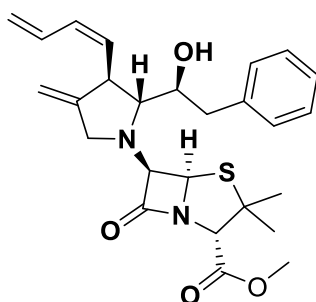
*(2S,3R,E)*-5-Cyclopropyl-2-hydroxy-3-(((2S,5R,6R)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)(2-propynyl)amino)-1-phenyl-4-pentene

Described in Chapter 3 and labeled as Hapten **5a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 281 and 298).



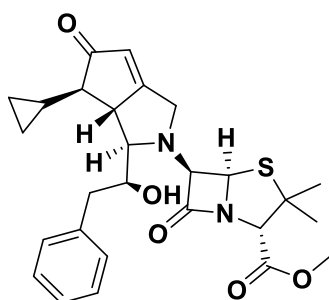
*(3R,5R,6S)*-6-Benzyl-5-((E)-2-cyclopropylvinyl)-3-((2R,4R)-4-methoxycarbonyl-5,5-dimethylthiazolidin-2-yl)-4-propynylmorpholin-2-one

Described in Chapter 3 and labeled as Hapten **6a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 282 and 299).



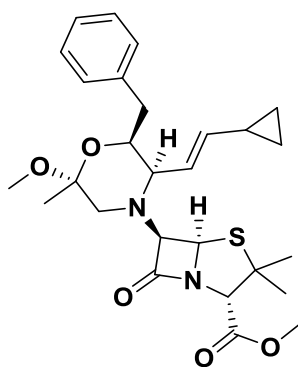
*(2R,3R)-3-((1E)-Buta-1,3-dienyl)-2-((S)-1-hydroxy-2-phenylethyl)-N-((2S,5R,6R)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)-4-methylenepyrrolidine*

Described in Chapter 3 and labeled as Hapten **7a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 282 and 300-301).



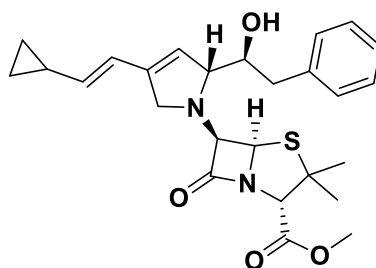
*(1R,2R,5E,8R)-8-Cyclopropyl-2-((S)-1-hydroxy-2-phenylethyl)-3-((2S,5R,6R)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)-3-azabicyclo[3.3.0]oct-5-en-6-one*

Described in Chapter 3 and labeled as Hapten **8a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 283 and 302).



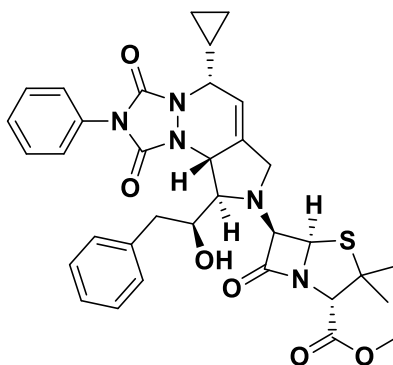
*(2R,5R,6S)-6-Benzyl-5-((E)-2-cyclopropylvinyl)-2-methoxy-4-((2S,5R,6R)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)-2-methylmorpholine*

Described in Chapter 3 and labeled as Hapten **9a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 284 and 303).



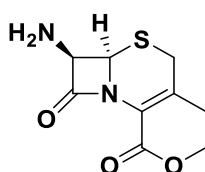
*(2R)-4-((E)-2-Cyclopropylvinyl)-2-((S)-1-hydroxy-2-phenylethyl)-N-((2S,5R,6R)-2-methoxycarbonyl-3,3-dimethyl-1-aza-7-oxo-4-thiabicyclo[3.2.0]heptan-6-yl)-3-pyrroline*

Described in Chapter 3 and labeled as Hapten **10a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 285 and 304-306).



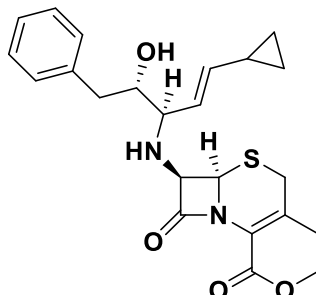
*Methyl (2S,5R)-6-((5R,9aR)-5-cyclopropyl-9-((R)-1-hydroxy-2-phenylethyl)-1,3-dioxo-2-phenyl-2,3,5,7,9,9a-hexahydro-1H,8H-pyrrolo[3,4-c][1,2,4]triazolo[1,2-a]pyridazin-8-yl)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate*

Described in Chapter 3 and labeled as Hapten **11a**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 285 and 307).



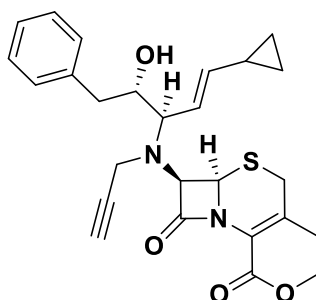
*7-amino-deacetoxycephalosporanic acid (7-ACDA) methyl ester*

Described in Chapter 3 and labeled as Hapten **1b**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 286 and 308).



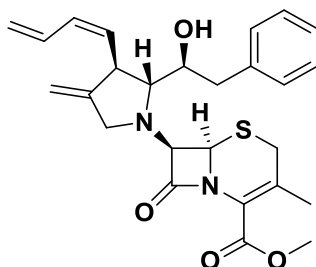
*Methyl (6R,7R)-7-(((4R,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)amino)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate*

Described in Chapter 3 and labeled as Hapten **4b**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 286 and 309-311).



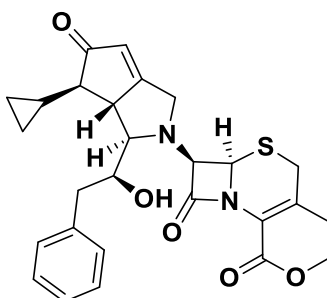
*Methyl (6R,7R)-7-(((4R,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)(prop-2-yn-1-yl)amino)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate*

Described in Chapter 3 and labeled as Hapten **5b**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 287 and 312-314).



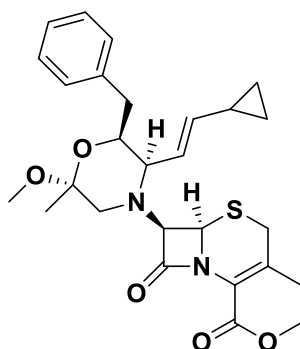
*Methyl (6R,7R)-7-(((2R,3R)-3-((Z)-buta-1,3-dien-1-yl)-2-((S)-1-hydroxy-2-phenylethyl)-4-methylenepyrrolidin-1-yl)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate*

Described in Chapter 3 and labeled as Hapten **7b**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 288 and 315-317).



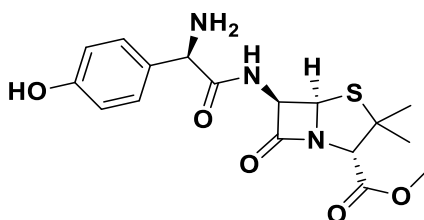
*Methyl (6R,7S)-7-((3S,3aS,4S)-4-cyclopropyl-3-((R)-1-hydroxy-2-phenylethyl)-5-oxo-3,3a,4,5-tetrahydrocyclopenta[c]pyrrol-2(1H)-yl)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate*

Described in Chapter 3 and labeled as Hapten **8b**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 288 and 318-320).



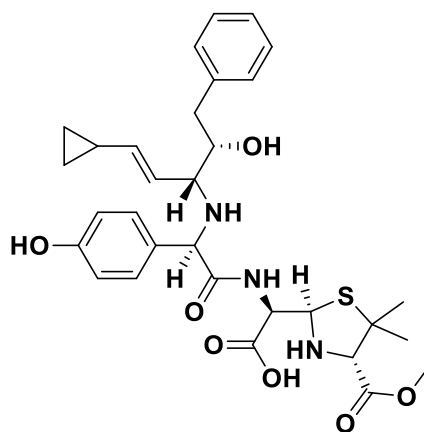
*Methyl (6R,7R)-7-((6R)-6-benzyl-5-((E)-2-cyclopropylvinyl)-2-methoxy-2-methylmorpholino)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate*

Described in Chapter 3 and labeled as Hapten **9b**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 289 and 321-322).



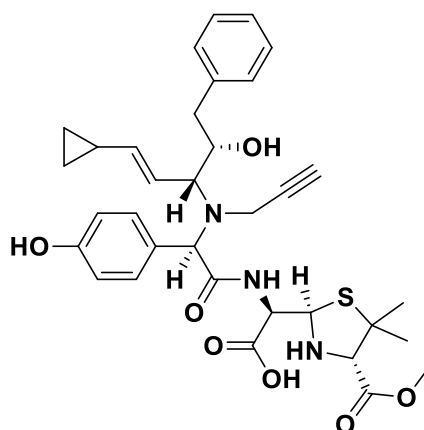
*Amoxicillin methyl ester*

Described in Chapter 3 and labeled as Hapten **1c**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 290 and 323).



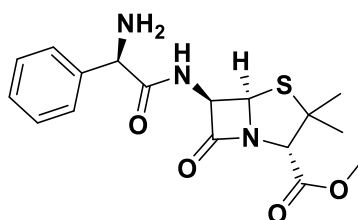
*(R)-2-((R)-2-(((3R,4S,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)amino)-2-(4-hydroxyphenyl)acetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid*

Described in Chapter 3 and labeled as Hapten **4c**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 290 and 324-326).



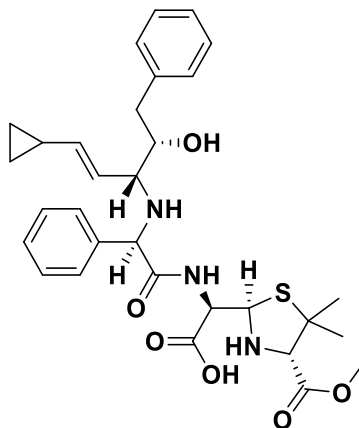
*(R)-2-((R)-2-(((3R,4S,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)(prop-2-yn-1-yl)amino)-2-(4-hydroxyphenyl)acetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid*

Described in Chapter 3 and labeled as Hapten **5c**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 291 and 327-329).



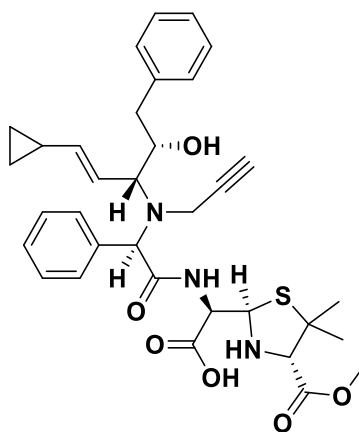
*Ampicillin methyl ester*

Described in Chapter 3 and labeled as Hapten **1d**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 292 and 330).



*(R)-2-((R)-2-(((3R,4S,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)amino)-2-phenylacetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid*

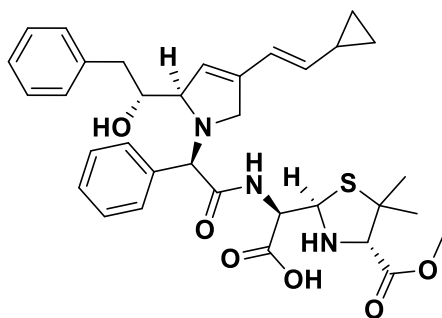
Described in Chapter 3 and labeled as Hapten **4d**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 292 and 331-333).



*(R)-2-((R)-2-(((3R,4S,E)-1-cyclopropyl-4-hydroxy-5-phenylpent-1-en-3-yl)(prop-2-yn-1-yl)amino)-2-phenylacetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid*

Described in Chapter 3 and labeled as Hapten **5d**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 293 and 334-336).





*(2R)-2-((2R)-2-(4-((E)-2-cyclopropylvinyl)-2-((R)-1-hydroxy-2-phenylethyl)-2,5-dihydro-1H-pyrrol-1-yl)-2-phenylacetamido)-2-((2R,4S)-4-(methoxycarbonyl)-5,5-dimethylthiazolidin-2-yl)acetic acid*

Described in Chapter 3 and labeled as Hapten **10d**. Synthetic procedure, data characterization and NMR spectrum included in Annex 5 (pp. 294 and 337).