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Research review paper

Peptaibiotics: Harnessing the potential of microbial secondary metabolites for mitigation of plant pathogens

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ABSTRACT

Agricultural systems are in need of low-cost, safe antibiotics to protect crops from pests and diseases. Peptaibiotics, a family of linear, membrane-active, amphipathic polypeptides, have been shown to exhibit antibacterial, antifungal, and antiviral activity, and to be inducers of plant resistance against a wide range of phytopathogens. Peptaibiotics belong to the new generation of alternatives to agrochemicals, aligned with the United Nations Sustainable Development Goals and the One Health approach toward ensuring global food security and safety. Despite that, these fungi-derived, non-ribosomal peptides remain surprisingly understudied, especially in agriculture, where only a small number has been tested against a reduced number of phytopathogens. This lack of adoption stems from peptaibiotics' poor water solubility and the difficulty to synthesize and purify them *in vitro*, which compromises their delivery and inclusion in formulations. In this review, we offer a comprehensive analysis of peptaibiotics' classification, biosynthesis, relevance to plant protection, and mode of action against phytopathogens, along with the techniques enabling researchers to extract, purify, and elucidate their structure, and the databases holding such valuable data. It is also discussed how chemical synthesis and ionic liquids could increase their solubility, how genetic engineering and epigenetics could boost *in vitro* production, and how omics can reduce screenings' workload through *in silico* selection of the best candidates. These strategies could turn peptaibiotics into effective, ultra-specific, biodegradable tools for phytopathogen control.

1. Introduction

Agricultural systems are challenged by a plethora of pests and diseases, costing over 290 billion dollars to the global economy each year (IPPC Secretariat, 2021). Within the next decades, climate change is expected to boost biotic and abiotic stress events worldwide (Raza et al., 2019) and the severe weather pattern changes may lead to more favorable conditions for the proliferation of crop pests and diseases (Panno et al., 2021; Pureswaran et al., 2018). These challenges call for innovative measures to protect crops and ensure food security and safety worldwide.

Agricultural systems heavily rely on cultural practices (e.g., sanitary pruning) and the use of agrochemicals (e.g., antibiotics) to control plant pathogens (Brauer et al., 2019; Sundin and Wang, 2018). While cultural practices are viewed as inefficient, agrochemicals, although effective, have numerous drawbacks (Brauer et al., 2019). Additionally, reports of resistance development by the pathogens are becoming increasingly frequent, rendering those chemicals ineffective (Brauer et al., 2019; Massi et al., 2021; Sundin and Wang, 2018). This trend has been observed for an alarming number of active substances, such as benzimidazole, strobilurin (Ma and Michailides, 2005), and copper sulphate ($CuSO_4$) (Cameron and Sarojini, 2014; Lamichhane et al., 2018). Moreover, consumers are becoming increasingly aware of these problems and are demanding a healthier and environmentally friendlier agriculture (Rodríguez-Bermúdez et al., 2020). These paradigms have prompted public organizations to fund innovative, sustainable

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measures, whilst imposing severe limitations on the use of agrochemicals, aligned with the United Nations Development Goals and One Health approach, the European Green Deal, and the Farm to Fork strategy (European Commission, 2020).

In this regard, the European Commission has set targets of having 25% of the European Union's (EU) agricultural land under organic farming and a 50% reduction in the overall use of chemical pesticides by 2030 (European Commission, 2020). Thus, novel bio-based techniques will play a fundamental role in achieving these goals. In that regard, biocontrol and integrated pest management have been rendered as viable alternatives to agrochemicals for the control of phytopathogens (Rahman et al., 2018). Among those methods, we can find microbial organisms acting as pathogen antagonists (Thambugala et al., 2020), plant host symbionts (White et al., 2018), plant growth promoters (Fusco et al., 2022), nanotechnology (Ali et al., 2021), and the use of natural compounds, like essential oils (Menossi et al., 2021). Unfortunately, other promising technologies, such as antimicrobial peptides (AMPs), seem to have a long way to go legislation-wise (Rosa et al., 2022). Indeed, peptides are currently out of the EU's list of approved substances for plant protection (Regulation EC 1107/2009 Annex I 540/2011) and the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA), which regulate pesticides and biocides, have not issued any opinion on the use of those products. Still, due to the unavoidable need for more sustainable agri-food systems, AMPs have a huge potential to rise as a new generation of alternatives to current agrochemicals, even though that will likely be accompanied by high registration costs and strict regulations.

AMPs are promising for the development of new, sustainable, and effective treatments against a wide range of plant pathogens (Rosa et al., 2022). Thousands of naturally-occurring AMPs have been identified as part of the innate immune system of mammals, amphibians, insects, plants, and microorganisms (e.g., bacteria and fungi) (Huan et al., 2020; Zhang et al., 2023). In plants, for instance, thionins, defensins, snakins, and cyclotides have been described as AMPs or, in a broader sense, as host defense peptides (HDPs). Numerous studies have been carried out to interrogate the antimicrobial activity of these molecules to ultimately develop low-cost, safe, bio-based antibiotics (Huan et al., 2020; Zhang et al., 2023). Unlike conventional agrochemicals, AMPs are extremely specific and effective at low doses, while being biodegradable, short-term products (Rosa et al., 2022). However, bacterial mechanisms of resistance are being reported (Abdi et al., 2019).

Peptaibiotics emerge among the most promising AMPs for phytopathogen control (Rosa et al., 2022). They have been described to exhibit antibacterial, antifungal, and antiviral properties (Zhao et al., 2019) and to induce plant resistance (Li et al., 2014; Luo et al., 2010; Viterbo et al., 2007). Moreover, peptaibiotics are remarkably stable to degradation by proteases (De Zotti et al., 2020). Thus, microbial resistance is less likely to occur (Dam et al., 2018; Lorito et al., 1996). Hence, the application of peptaibiotics in agricultural systems could help reducing the need for agrochemicals. Despite that, the first peptaibiotics-inspired formula for plant protection is yet to be developed. In the meantime, several patents have been submitted toward that goal (De Zotti et al., 2022; De Zotti et al., 2021; Olivier, 2001; Pesaresi et al., 2019).

Herein we provide a comprehensive analysis of developments in the last 30 years, concerning the peptaibiotics' relevance towards crop protection. To that end, a search was carried out in SCOPUS and PubMed using "peptaibols", "lipopeptaibols", "lipoaminopeptaibols", "non-ribosomal peptides" as keywords in combination with complementary terms such as "agriculture", "antimicrobial activity", "bioactivity", "channel-forming ability", "phytopathogens" and "crop protection". We focused on works testing peptaibiotics directly against phytopathogens *in vitro* and/or *in planta*. In the cases where an indirect method was used (e.g., dual culture), only studies in which the antimicrobial properties were proven to be a result of peptaibiotics' action were considered. Forty-five publications met these criteria.

Because this is a hot topic (Rosa et al., 2022; Zhang et al., 2023; Zhao et al., 2019), other reviews have focused on peptaibiotics, although with different scopes. The first review was dedicated to peptaibols from *Trichoderma* (Szekeres et al., 2005). Not long after, Leitgeb et al. (2007) offered an in-depth description of alamethicin's biosynthesis, biological activity and structure. More recently, Gavryushina et al. (2021) and Hou et al. (2022) have compiled the discoveries regarding the structural characterization, biosynthetic pathway, and mode of action of peptaibols. Despite anti-phytopathogenic activity being mentioned in these works, none was dedicated to their application in agriculture or discussed their handicaps. Moreover, the mechanisms of action and methods for producing peptaibols are often left out. Hence, the biosynthesis, action mechanisms, isolation and identification methods, and databases focused on peptaibiotics, are discussed in the next sections. In addition, reasons why peptaibiotics lag behind other green technologies in agriculture are discussed, alongside the factors that, in our opinion, might boost their wide adoption.

2. Peptaibiotics classification: status and challenges

Peptaibiotics are a vast family of linear, amphipathic polypeptides of 5 to 21 amino acid residues derived from the fungal non-ribosomal peptide synthetase (NRPS) pathway, with a molecular mass ranging from 500 to 2200 Da (Zeilinger et al., 2016). Peptaibols are the largest and most studied group of peptaibiotics, which also includes lipopeptaibols and lipoaminopeptaibols or aminolipopeptaibols (Neumann et al., 2015). Peptaibiotics sequences, characteristically, present high proportion of non-coded residues, like Iva and Aib (Fig. 1), the latter being particularly abundant (De Zotti et al., 2012; Degenkolb et al., 2003). This peculiar composition is behind the peptaibols helical 3D structure (e.g., α-helix and 3_{10}-helix) (Ségalas et al., 1999). Deviations to these conformations may occur when Aib–Pro motifs are present, forming a subtype of the 3_{10}-helix, the (Xaa–Yaa–Aib–Pro)-β-bend ribbon spiral (Christoffersen et al., 2015; Ségalas et al., 1999). These traits underly the remarkable properties of peptaibols in regard to both antimicrobial activity and stability to proteolysis (De Zotti et al., 2020; De Zotti et al., 2012). The *N*-terminal residue is usually acetylated in peptaibols, whereas their *C*-terminal group is often an alcohol instead of a carboxyl, i.e., the *C*-terminal residue is an α-amino alcohol such as leucinol, phenylalaninol, or valinol, among others (Fig. 2). Conversely, in lipopeptaibols, the *N*-terminal amino acid is acylated with a short fatty acid (e.g., octanoic, decanoic, or *cis*-dec-4-enoic acids), whereas the *N*-terminal amino acid in lipoaminopeptaibols is typically acylated by long-chain, α-methyl-branched fatty acids (Fig. 2) (Degenkolb et al., 2003; Neumann et al., 2015).

Peptaibiotics are classified according to their sequence length. Hence, long-sequence peptaibiotics consist of 17 to 21 residues (e.g., alamethicins, trichosporins, trichorzins, chrysospermins), medium peptaibiotics of 12 to 16 residues (e.g., emerimicins, harzianins, tylopeptins), and short peptaibiotics of 4 to 11 amino acid residues (e.g., trichogin, trichodecenin) (Fig. 3) (Bortolus et al., 2016; Hou et al., 2022). Long-sequence molecules often present central Pro and Gln residues near both termini, while medium-sequence ones often show an *N*-terminal Ac-Aib-Gln- segment or its analogue where Gln is replaced by an Asn. Both often present Aib-Pro motives. On the other hand, short peptaibiotics usually have a high Gly content (Gavryushina et al., 2021; Szekeres et al., 2005).

3. Peptaibiotics' main source organisms, biosynthesis, and chemical synthesis

3.1. Main source organisms of peptaibiotics

Peptaibiotics are produced as secondary metabolites by fungi of an array of genera, although the main source has been, by far, *Trichoderma* spp. (Röhrich et al., 2014; Rush et al., 2021; Zhao et al., 2019). *T.*

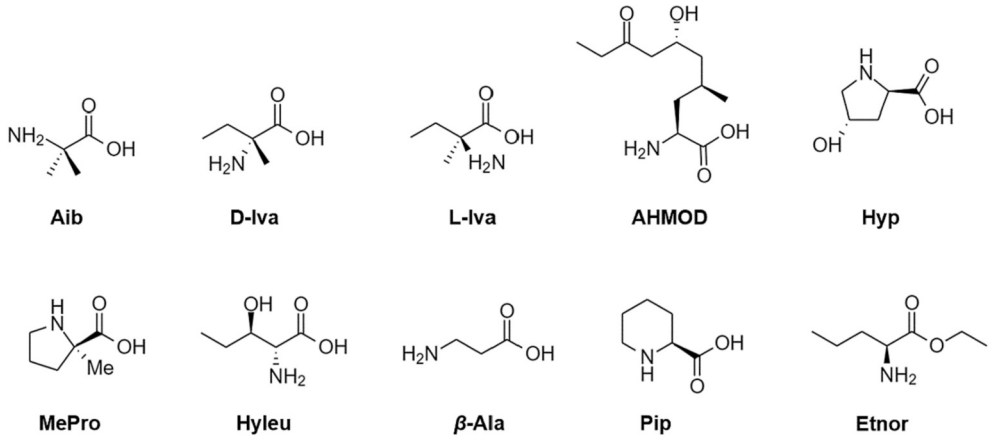

Fig. 1. Structure of some non-proteinogenic α-amino acids commonly present in peptaibiotics: Aib (α-aminoisobutyrate), D-Iva and L-Iva (D and L isomers of isovaline), AHMOD [(2*S*)-amino-(6*R*)-hydroxy-(4*S*)-methyl-8-oxo-decanoic acid], Hyp (hydroxyproline), MePro (L-methylproline), Hyleu (hydroxy-L-leucine), β-Ala (β-alanine), Pip (L-pipecolic acid), and Etnor (α-ethyl-norvaline).

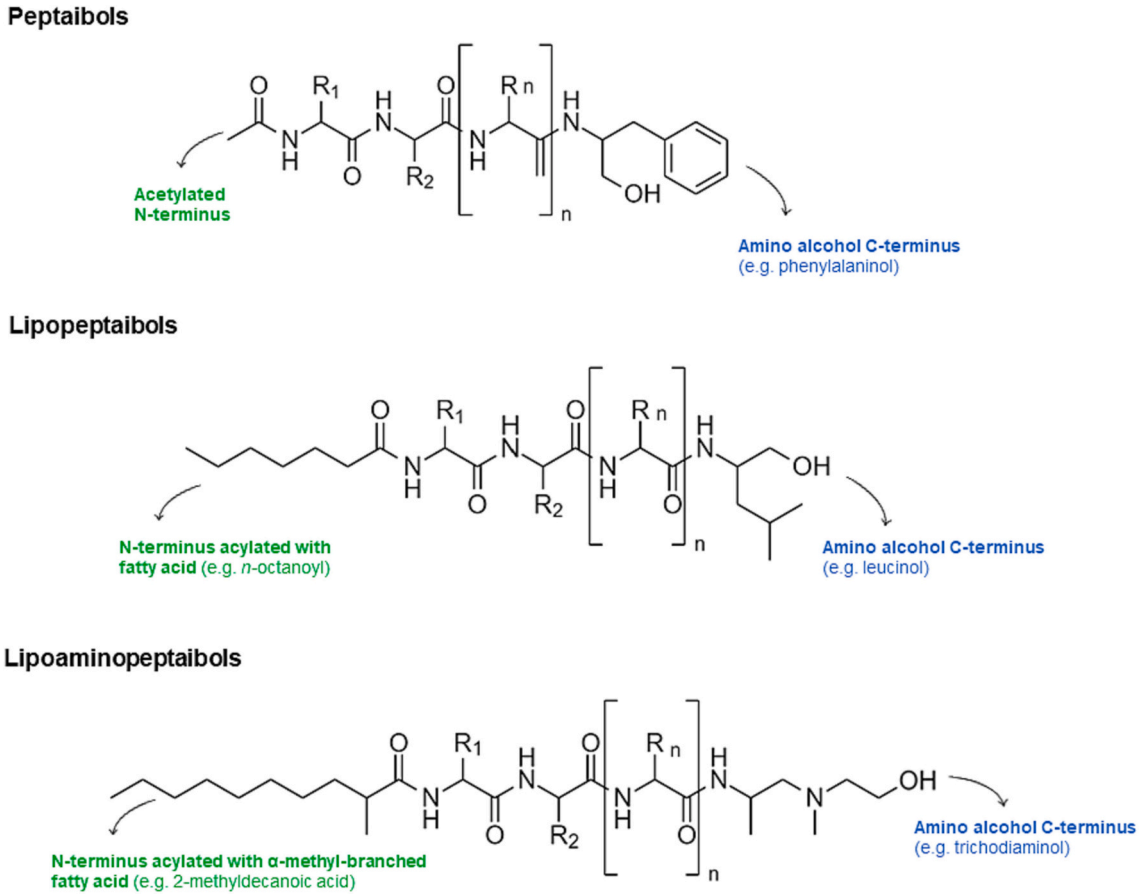

Fig. 2. General scheme of the different classes of peptaibiotics: peptaibols, lipopeptaibols, and lipoaminopeptaibols. $R_{1,\ 2,\ n}$ represent the amino acids' side-chains, usually containing a high proportion of Aib and other non-proteinogenic amino acid residues.

harzianum is the most relevant regarding agricultural applications (Degenkolb et al., 2015; Fraceto et al., 2018; Rush et al., 2021). Its peptaibiotics profile has been tested in five works, against four different fungal phytopathogens (Goulard et al., 1995; Lorito et al., 1996; Rebuffat et al., 1995; Schirmbock et al., 1994) and one plant virus (Kai et al., 2018) (Table 1). *Trichoderma asperellum* (Alfaro-Vargas et al., 2022; Tamandegani et al., 2020), *T. atroviride* (Oh et al., 2002), *T. cerinum* (Khare et al., 2018), *T. citronoviride* (Maddau et al., 2009), *T. effusum* (Balázs et al., 2023), *T. gamsii* (Marik et al., 2018), *T. koningiopsis* (Marik et al., 2018), *T. longibrachiatum* (Balázs et al., 2023; Tamandegani et al., 2020), *T. longibrachiatum* f. *bissettii* (Balázs et al., 2023), *T. pseudokoningii* (Li et al., 2014; Luo et al., 2010; Shi et al., 2012; Song et al., 2006; Zhao et al., 2018), *T. Reesei* (Balázs et al., 2023; Marik et al., 2019), *T. saturnisporum* (Balázs et al., 2023), and *T. virens* (Viterbo et al., 2007) are the other species prospected in this field (Table 1). Outside of that genus, *Apiocrea* spp. (Dornberger et al., 1995; Grigoriev et al., 2003; Kim et al., 2000; Yeo et al., 2002) and *Sepedonium* spp. (Grigoriev et al., 2003; Kronen et al., 2001; Lam et al., 2021; Otto

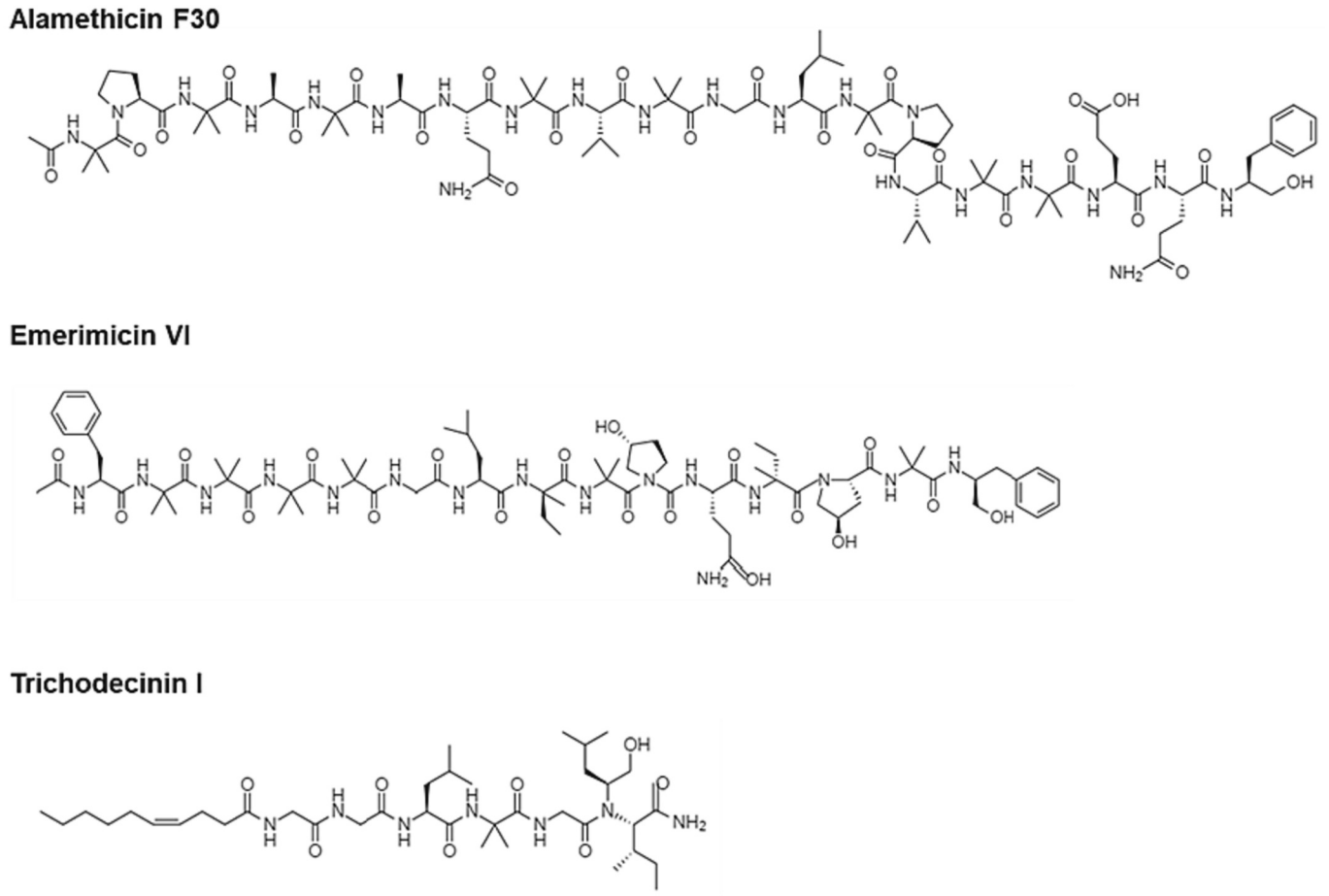

Fig. 3. Structures of representative peptaibiotics with different lengths: alamethicin F30, emerimicin VI and trichodecinin I. They are examples of long-, medium- and short-sequenced peptaibiotics, respectively.

et al., 2016a, 2016b; Ritzau et al., 1997) have been the source organisms in four and six different works, respectively, whereas *Purpureocillium* spp., has been in two (Liu et al., 2020; Wang et al., 2016), and *Acremonium* spp. (Grigoriev et al., 2003), *Boletus* spp. (Lee et al., 1999a), *Clonostachys* spp. (Rodríguez et al., 2011), *Emericellopsis* spp. (Grigoriev et al., 2003), *Gliocladium* spp. (Otto et al., 2015), *Moszia* spp. (Grigoriev et al., 2003), *Mycogone* spp. (Gräfe et al., 1995), and *Tylopilus* spp. (Lee et al., 1999b) have only been tested against phytopathogens in a single work (Table 1).

The exploration of new habitats will translate into new molecules to be exploited. This has been demonstrated for deep sea *M. sediminis*-derived microbacterins (Liu et al., 2015), for emerimicin extracted from *E. minima* from marine sediments (Inostroza et al., 2017), and alkalophilic *E. alkaline* (Grigoriev et al., 2003; Kuvarina et al., 2021). Many more species are known for their ability to biosynthesize peptaibiotics (Gavryushina et al., 2021; Hou et al., 2022). However, their potential effects remain undisclosed. Due to the high number of untested compounds and strains it is likely that some of these substances will become the new generation of bio-based antibiotics.

3.2. Biosynthetic pathway to peptaibiotics

The biosynthesis of peptaibiotics is completely independent of mRNA and ribosomes. Their assembly is mediated by non-ribosomal peptide synthases (NRPSs), which are large multi-module protein complexes, where each module is composed by different catalytic domains with specific functions (Zhang et al., 2016). NRPSs consist of three types of modules: one initiation module, one termination module, and one or more elongation modules. Each module is a semiautonomous unit that recognizes, activates, loads and modifies a single monomer of the final peptide (Reimer et al., 2018; Üssmuth and Mainz, 2017). The synthesis proceeds in the *N*- to *C*-terminal direction (Martínez-Núñez and López, 2016). In the last synthesis step, the peptide chain is released from the enzymatic complex, either by hydrolysis (linear peptide) or cyclization. Alternatively, the NRPS can release the final peptide by reducing the thioester bond to a terminal aldehyde or alcohol (Reimer et al., 2018; Zhang et al., 2016).

In addition to the variability in the number of modules that compose the NRPSs, five other factors account for the tremendous diversity among NRPs structures and biological activities: i) NRPS modules can load over 500 different substrates; ii) NRPS modules usually present optional domains which can modify the loaded residue in many different ways; iii) some modules have been found to act twice, thus incorporating an extra amino acid to the sequence (Komon-Zelazowska et al., 2007); iv) module-skipping modules during synthesis lead to shorter peptaibiotics (Degenkolb et al., 2012); v) after being released, the peptide can undergo additional chemical modifications, a.k.a 'editing', eventually generating the final molecule (Reimer et al., 2018; Üssmuth and Mainz, 2017).

The publication of the first genomes of *Trichoderma* (Kubicek et al., 2011; Martinez et al., 2008) proved the presence of genes belonging to a large cluster, the NRPS (Bansal and Mukherjee, 2016; Mukherjee et al., 2012). Surveys of those genomes revealed the presence of 7-, 14- and 18-20 module peptaibiotics synthetases (Degenkolb et al., 2012). The first peptaibol synthetase genes to be cloned were *tex1* (Wiest et al., 2002) and *tex2* genes of *T. virens*, *T. reesei*, and *T. atroviridae* (Degenkolb et al.,

Table 1
List of peptaibiotics screened for antimicrobial activity against phytopathogenic bacteria, fungi, and viruses. This includes synthetic peptaibiotics, carrying different amino acidic modifications compared to their natural counterparts, as well as fungal micro-heterogeneous extracts, composed of several undisclosed peptaibiotics molecules. For each tested compound, or group of compounds, information is provided regarding its length (when applicable), source organism, target phytopathogens, effective dosage, mode of action (when reported by the authors), and the original reference. This table is a shortened version of Table S1 included in the Supplementary Data, where source organism isolates, MIC, type of test performed, and inhibition degree are included.

Peptaibiotics *[1, 2, 3, n]	Source organisms	Target pathogens ** []	Mode of action	References
Trialed against bacteria				
Boletusin[1]	*Boletus* spp.	*Corynebacterium lilium* [50 μg/paper disc]	Not specified	(Lee et al., 1999a)
Chrysospermins B-D[1]	*Apiocrea* sp.	*Corynebacterium lilium* [30 μg/paper disc]	Not specified	(Kim et al., 2000)
Peptaivirins A-B[1]	*Apiocrea* sp.	*Corynebacterium lilium* [30 μg/paper disc]	Not specified	(Yeo et al., 2002)
TvB I-II[1]	*Trichoderma virens*	*Pseudomonas syringae* pv. *lachrymans* [9.6 nmol]	Induction plant defenses	(Viterbo et al., 2007)
Tylopeptins A-B[2]	*Tylopilus neofelleus*	*Corynebacterium lilium* [50 μg/paper disc]	Not specified	(Lee et al., 1999b)
Trichokonins VI-VIII[1]	*Trichoderma pseudokoningii; Trichoderma longibrachiatum*	*Clavibacter michiganensis* ssp. *michiganensis* [100 μg/mL]; *Erwinia carotovora; Pectobacterium carotovorum* subsp. *carotovorum* [0.3 μg/mL]; *Ralstonia solanacearum; Xanthomonas oryzae* pv. *oryzae* [27-100 μg/mL]	Induction plant defenses; Cell membrane rupture	(Li et al., 2014; Song et al., 2006; Zhang et al., 2022)
Trichogin GA IV (analogues)[n]	*Synthetic*	*Agrobacterium tumefaciens* [15 μM]; *Bacillus subtilis* [3 μM]; *Erwinia carotovora* subsp. *carotovora* [3 μM]; *Pseudomonas corrugata* [3-15 μM]; *Pseudomonas savastanoi* pv. *savastanoi* [3-15 μM]; *Ralstonia solanacearum* [3 μM]; *Xanthomonas arboricola* [15 μM]; *Xanthomonas campestris* pv. *campestris* [1-15μM]	Not specified	(Caracciolo et al., 2023)
Micro-heterogeneous extracts [n]	*Trichoderma longibrachiatum* f. *bissettii; Trichoderma longibrachiatum; Trichoderma reesei; Trichoderma saturnisporum; Trichoderma effusum*	*Clavibacter michiganensis* [156-1250 μg/mL]; *Rhodococcus fascians* [312-5000 μg/mL]; *Rhizobium radiobacter* [1250-10000 μg/mL]; *Xanthomonas campestris* [10000 μg/mL]; *Erwinia amylovora* [10000 μg/mL]; *Pantoea ananatis* [10000 μg/mL]	Not specified	(Balázs et al., 2023)
Trialed against fungi				
Alamethicin F30[1]	*Trichoderma* sp.	*Phoma destructiva* [2000 μg/mL]	Cell membrane rupture	(Grigoriev et al., 2003)
Albupeptins A-D[2]	*Gliocladium album*	*Botrytis cinerea* [25-50 μM]; *Phytophthora infestans* [84-97 μM]; *Septoria tritici*	Not specified	(Otto et al., 2015)
Ampullosporins A-F[2]	*Sepedonium ampullosporum*	*Botrytis cinerea* [4.6-11.4 μM]; *Phytophthora infestans* [14.7-19.5 μM]; *Phoma destructiva* [50 μg/agar well-2000 μg/mL]; *Septoria tritici*	Not specified	(Grigoriev et al., 2003; Kronen et al., 2001; Lam et al., 2021; Ritzau et al., 1997)
Atroviridins A-C[1]	*Trichoderma atroviride*	*Cladosporium* spp. [50 μg/paper disc]; *Colletotrichum dematium* [50 μg/paper disc]; *Curvularia inaequalis* [50 μg/paper disc]; *Fusarium oxysporum* [50 μg/paper disc]; *Phytophthora infestans* [50 μg/paper disc]; *Verticillium dahliae* [50 μg/paper disc]	Cell membrane rupture	(Oh et al., 2002)
Bergofungins A-B[2]	*Emericellopsis donezkii*	*Phoma destructiva* [2000 μg/mL]	Cell membrane rupture	(Grigoriev et al., 2003)
Boletusin[1]	*Boletus* spp.	*Alternaria mali; Colletotrichum lagenarium; Fusarium solani; Magnaporthe gricea*	Not specified	(Lee et al., 1999a)
Cephaibols B-E[2]	*Acremonium tubaki*	*Phoma destructiva* [2000 μg/mL]	Cell membrane rupture	(Grigoriev et al., 2003)
Chilenopeptins A-B[2]	*Sepedonium* aff. *chalcipori*	*Botrytis cinerea* [5.3-11 μM]; *Phytophthora infestans* [10.1-17.8 μM]; *Septoria tritici*	Not specified	(Otto et al., 2016b)
Chrysospermins A-D[1]	*Apiocrea* sp.; *Apiocrea chrysosperma*	*Alternaria mali; Colletotrichum lagenarium; Fusarium culmorum* [250-500 μg/mL]; *Fusarium solani; Magnaporthe gricea; Phoma destructiva* [250-2000 μg/mL]	Cell membrane rupture	(Dornberger et al., 1995; Grigoriev et al., 2003; Kim et al., 2000)
Harzianins HC[2]	*Trichoderma harzianum*	*Sclerotium cepivorum* [100 μg/mL]	Cell membrane rupture	(Rebuffat et al., 1995)
Helioferins A-B[3]	*Mycogone rosea*	*Fusarium culmorum* [6.25 μg/mL]	Not specified	(Gräfe et al., 1995)
Leucinostatins A-B, Z[3]	*Purpureocillium lilacinum*	*Phytophthora capsici* [60 μg/agar well]; *Phytophthora infestans* [60 μg/agar well]; *Botrytis cinerea* [5000 μg/mL]	Not specified	(Liu et al., 2020; Wang et al., 2016)

(*continued on next page*)

Table 1 (*continued*)

Peptaibiotics *[1, 2, 3, n]	Source organisms	Target pathogens ** []	Mode of action	References
Neoatroviridins A-D[1]	*Trichoderma atroviride*	*Cladosporium* spp. [50 μg/paper disc]; *Colletotrichum dematium* [50 μg/paper disc]; *Curvularia inaequalis* [50 μg/paper disc]; *Fusarium oxysporum* [50 μg/paper disc]; *Phytophthora infestans* [50 μg/paper disc]; *Verticilium dahliae* [50 μg/paper disc]	Cell membrane rupture	(Oh et al., 2002)
Paracelsin A[1]	*Trichoderma* sp.	*Phoma destructiva* [2000 μg/mL]	Cell membrane rupture	(Grigoriev et al., 2003)
Peptaivirins A-B[1]	*Apiocrea* sp.	*Alternaria mali; Colletotrichum lagenarium; Fusarium solani; Magnaporthe gricea*	Not specified	(Yeo et al., 2002)
Texenomycin A[1]	*Moszia lindtneri*	*Phoma destructiva* [2000 μg/mL]	Cell membrane rupture	(Grigoriev et al., 2003)
Trichofumins A-D[2]	*Trichoderma* sp.	*Phoma destructiva* [1000-2000 μg/mL]	Cell membrane rupture	(Berg et al., 2003; Grigoriev et al., 2003)
Trichogin GA IV[2]	*Trichoderma longibrachiatum*	*Rhizoctonia solani; Fusarium oxysporum*	Not specified	(De Zotti et al., 2009)
Trichokonins VI-VIII[1]	*Trichoderma pseudokoningii; Trichoderma longibrachiatum*	*Ascochyta citrullina* [25 μM]; *Bipolaris sorokiniana* [320 AU/mL]; *Botrytis cinerea* [25 μM and 80-200 μg/mL]; *Colletotrichum lagenarium* [320 AU/mL]; *Curvularia lunata* [320 AU/mL]; *Fusarium oxysporum* [25 μM]; *Fusarium oxysporum* f.sp. *niveum* [320 AU/mL]; *Fusarium oxysporum* f.sp. *phaseoli* [320 AU/mL]; *Fusarium oxysporum* f.sp. *vasinfectum* [320 AU/mL]; *Phytophthora parasitica* [25 μM]; *Rhizoctonia solani* [320 AU/mL]; *Valsa mali* [320 AU/mL]; *Verticilium dahliae* [50 μM]	Cell membrane rupture; Induced programmed cell death	(Shi et al., 2012; Song et al., 2006; Zhao et al., 2018)
Trichorzianins A-B[1]	*Trichoderma harzianum*	*Botrytis cinerea* [200-800 μg/mL]; *Fusarium oxysporum* f.sp. *phaseoli* [200 μg/mL]	Synergy with lytic enzymes; Inhibition of glucan synthase	(Lorito et al., 1996; Schirmbock et al., 1994)
Trichorzins[1]	*Trichoderma harzianum*	*Sclerotium cepivorum* [100 μg/mL] *Alternaria alternata* [800 μg/mL];	Cell membrane rupture	(Goulard et al., 1995)
Trichotoxins[1]	*Trichoderma asperellum*	*Botrytis cinerea* [800 μg/mL]; *Colletotrichum gloeosporioides* [800 μg/mL]; *Fusarium oxysporum* [800 μg/mL]	Cell membrane rupture	(Alfaro-Vargas et al., 2022)
Trilongins B I-IV[1]	*Trichoderma* spp.	*Colletotrichum gloeosporioides* [40-320 μM]	Not specified	(Grigoletto et al., 2020)
Tulasporins A-D[1]	*Sepedonium tulasneanum*	*Botrytis cinerea* [3.7-10 μM]; *Phytophthora infestans* [4-22 μM]; *Septoria tritici*	Not specified	(Otto et al., 2016a)
Tylopeptins A-B[2]	*Sepedonium* aff. *chalcipori*	*Botrytis cinerea* [5-11 μM]; *Phytophthora infestans* [10.1-17.5 μM]; *Septoria tritici*	Not specified	(Otto et al., 2016b)
Desacetyltryptophanyl-Ampullosporin A[2]	*Synthetic*	*Phoma destructiva* [2000 μg/mL]	Cell membrane rupture	(Grigoriev et al., 2003)
Hexamethyl Ampullosporin A[2]	*Synthetic*	*Phoma destructiva* [2000 μg/mL]	Cell membrane rupture	(Grigoriev et al., 2003)
TvB I-II[1]	*Synthetic*	*Rhizoctonia solani* [200 μg/mL]	Not specified	(Viterbo et al., 2007)
Trichogin GA IV (analogues)[n]	*Synthetic*	*B. sorokiniana* [15 μg/mL]; *Botrytis cinerea* [5-50 μM]; *Fusarium oxysporum; Fusarium graminearum* [5-15 μM]; *Penicillium expansum* [5-15 μM]; *Pyricularia oryzae* [50 μM]; *Plasmopara viticola* [30-50 μM]; *Rhizoctonia solani Alternaria alternata* [200-6300 μg/mL]; *Alternaria solani* [100-200 μg/mL]; *Apiognomonia quercina* [50-100 μg/mL]; *Aspergillus fumigatus* [200 μg/mL]; *Biscogniauxia mediterranea* [50-100 μg/mL]; *Botryosphaeria corticola* [50-100 μg/mL]; *Botryosphaeria obtuse* [50-100 μg/mL]; *Botryosphaeria parva* [50-100 μg/mL]; *Colletotrichum truncatum* [not specified]; *Colletotrichum gloeosporioides* [320 μM]; *Diplodia pinea* [50-100 μg/mL]; *Diplodia scrobiculata* [50-100 μg/mL]; *Fusarium culmorum* [100-200 μg/mL]; *Fusarium graminearum* [100-200 μg/mL]; *Fusarium moniliforme* [100-200 μg/mL]; *Fusarium oxysporum* f.sp. *ciceris* [not specified]; *Fusarium falciforme* [200 μg/mL]; *Fusarium keratoplasticum* [200 μg/mL]; *Fusarium solani* [200-100000 μg/mL]; *Macrophomina phaseolina* [not specified]; *Phoma cucurbitacearum* [200-	Cell membrane rupture	(Baccelli et al., 2022; Bolzonello et al., 2023; De Zotti et al., 2020, De Zotti et al., 2009; Sella et al., 2021)
Micro-heterogeneous extracts [n]	*Clonostachys rosea; Trichoderma asperellum; Trichoderma cerinum; Trichoderma citrinoviride; Trichoderma gamsii; Trichoderma koningiopsis; Trichoderma longibrachiatum; Trichoderma reesei*		Synergy with mycoparasitism; Synergy with lytic enzymes; PGP activity of the fungus	(Grigoletto et al., 2020; Khare et al., 2018; Maddau et al., 2009; Marik et al., 2019, Marik et al., 2018; Rodríguez et al., 2011; Tamandegani et al., 2020)

(*continued on next page*)

Table 1 (*continued*)

Peptaibiotics *[1, 2, 3, n]	Source organisms	Target pathogens ** []	Mode of action	References
		25000 μg/mL]; *Rhizoctonia solani* [6300-25000 μg/mL]; *Sclerotinia sclerotiorum* [not specified]; *Trichoderma gamsii* [6300 μg/mL]; *Trichoderma koningiopsis* [6300 μg/mL]; *Trichoderma aggressivum* f. *europaeum* [25000-100000 μg/mL]; *Trichoderma pleuroti* [25000-100000 μg/mL]		
Trialed against viruses				
Chrysospermins B-D[1]	*Apiocrea* sp.	Tobacco Mosaic Virus [100 μg/mL]	Not specified	(Kim et al., 2000)
Peptaivirins A-B[1]	*Apiocrea* sp.	Tobacco Mosaic Virus [10-100 μg/mL]	Not specified	(Yeo et al., 2002; Yun et al., 2000)
Trichokonins[1]	*Trichoderma pseudokoningii*	Tobacco Mosaic Virus [0.2 μg/mL]	Induction plant defenses	(Luo et al., 2010)
Trichorzins	*Trichoderma harzianum*	Cucumber Mosaic Virus [10 μM]	Not specified	(Kai et al., 2018)

*[1, 2, 3 and n] indicate the sequence length of peptaibiotics, where [1] means long-sequence (17-21 residues), [2] means medium-sequence (12-16 residues), [3] means short sequences (4-11 residues), and [n] indicates variable sequence length; notably, Trichogin GA IV synthetic analogues usually fall into the short sequence category. ** Values between [] refer to the minimum concentration (or range, when different molecules show different activities) needed for inhibition for that particular peptaibiotic and pathogen. No value indicates no activity detected.

2012).

3.3. Isolation and structural elucidation of natural peptaibiotics

The extraction of peptaibiotics is usually performed by applying various organic solvents, like ethyl acetate (Maddau et al., 2009; Grigoletto et al., 2020; Liu et al., 2020), methanol (Marik et al., 2013), *n*-butanol (Goulard et al., 1995; Rebuffat et al., 1995; Yeo et al., 2002), among others, to the broth or mycelium of cultured strains. For instance, Katoch et al. (2019) and Viterbo et al. (2007) used acetonitrile:methanol:water (1:1:1) to extract both the polar and non-polar fractions from fungal myceliae, whereas Marik et al. (2018) used a chloroform:methanol 2:1 solution.

After obtaining the crude extract, peptaibiotics' fractions can be further isolated from impurities and purified through a series of chromatographic techniques like flash chromatography, employing polymeric adsorbents of organic substances (e.g., Amberlite XAD-16, Diaion HP 20) (Song et al., 2006; Rodríguez et al., 2011; Lam et al., 2021; Balázs et al., 2023), and or HPLC (Otto et al., 2015, 2016b; Otto et al., 2016a). The major chromatographic techniques employed for separation and purification of peptide-based molecules are size-exclusion chromatography (SEC) and reverse-phase chromatography (RP-HPLC) (Mant et al., 2007). Otto et al. (2015, 2016b), for instance, used SEC to purify chilenopeptins and albupeptins from *S.* aff. *chalcipori* and *G. album*, respectively, whereas Song et al. (2006) used RP-HPLC to isolate trichokonins before employing preparative-HPLC to separate trichokonin VI from the trichokonins microheterogenous mixture.

After separation and elution, fractions containing the molecule of interest can be collected for structural elucidation (Fig. 6). Peptaibiotics' high molecular weight and intricate amino acidic composition make it difficult to accurately determine their structure (Hou et al., 2022). Mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) techniques are the most frequently used to accurately elucidate the molecular structure of peptaibols. X-ray crystallography has also been employed in some studies.

Soft-ionization techniques, namely, electrospray ionization (ESI) and matrix-assisted laser desorption-ionization (MALDI), have become the golden standard for peptaibiotics analysis, either in stand-alone or HPLC-coupled modes (Neuhof et al., 2007; Van Bohemen et al., 2016). In ESI, a liquid solution of the analyte(s) is firstly exposed to a strong electric field and then sprayed, resulting in highly charged droplets of both the sample and mobile phase (Gaskell, 1997). The resulting ions are then directed to the detector/mass analyzer (Glish and Vachet, 2003; Van Bohemen et al., 2016; Marik et al., 2018; Balázs et al., 2023), in many cases a quadrupole ion trap (QIT) or an Orbitrap (Marik et al., 2018, 2019; Alfaro-Vargas et al., 2022). In MALDI, samples are co-crystallized with an energy-absorbent matrix and exposed to pulse-laser irradiation. This technique is used to trace a mass fingerprint of each peptide (Jurinke et al., 2004; Singhal et al., 2015). MALDI-TOF has been one of the most reliable tools for the analysis of peptaibiotics since recent improvements in resolution, sensitivity, and versatility made this analytical method highly competitive (Neuhof et al., 2007; Maddau et al., 2009; Katoch et al., 2019; Liu et al., 2020). Neuhof et al. (2007) investigated the peptaibol production by intact-cell MALDI-TOF-MS of 28 *Trichoderma* species, using microgram amounts of mycelia, and showed that peptaibol spectra can readily be obtained. Likewise, Liu et al. (2020) used MALDI-TOF-IMS as a fast identification method of a leucinostatin Z from *P. lilacinum* from the inhibition zone of a dual culture assay with *B. cinerea*.

In addition to a proper ionization and detection of the analyte(s), the controlled additional fragmentation of relevant ions is of chief importance for structural elucidation. Tandem MS (MS/MS, MS^2, and MS^3) is widely used to this end (Song et al., 2006; Otto et al., 2016b; Marik et al., 2018, 2019; Katoch et al., 2019; Alfaro-Vargas et al., 2022). Based on those fragments, a putative peptaibiotics amino acidic sequences can be hypothesized (Hou et al., 2022). These techniques have been used to characterize chilenopeptins from *S.* aff. *chalcipori* (Otto et al., 2016b), peptaibiotics extracted from *Trichoderma* (Berg et al., 2003; Katoch et al., 2019; Alfaro-Vargas et al., 2022), ampullosporins from *S. ampullosporum* (Lam et al., 2021), and lipopeptaibol leucinostatin from *P. lilacinum* (Liu et al., 2020).

Structural analysis of peptides by NMR mostly involves 2D techniques, such as total correlation spectroscopy (TOCSY) and correlated spectroscopy via long-range couplings (COLOC). These have been used to elucidate the structure of peptaibiotics such as ampullosporin A (Kronen et al., 2001) and peptaivirins (Yun et al., 2000). Other NMR techniques, like homonuclear Hartmann-Hahn spectroscopy (HOHAHA), have been used in the structural analysis of tylopeptins (Lee et al., 1999b). Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectroscopies have also been employed in the study of ampullosporin (Ritzau et al., 1997). Relevantly, due to the complex nature of peptaibiotics' structure, and particularly due to the tandem mass spectrometry sequencing difficulty in distinguishing between isobaric amino acids (e.g., leucine and isoleucine), these techniques are often in used in combination with ESI and MALDI to produce accurate predictions (Degenkolb et al., 2003; Otto et al., 2015, 2016b; Otto et al., 2016a; Lam et al., 2021).

X-ray crystallography is one of the most widely used methods for crystal structure determination of proteins, however, due to their chemical structures and physical properties only a few peptaibiotics

have been determined using this technique (Hou et al., 2022). This technique is based on the principle that when electromagnetic radiation interacts with the electrons of crystalized materials, the electromagnetic wave bends. The X-ray detector registers the waves and generates a diffraction map that can then be modelled into an electron density map that is used to build a structural model of the protein (Gawas et al., 2019). Kronen et al. (2003) used this technique to elucidate the structure of ampullosporin A and De Zotti et al. (2012) to investigate the role of Aib residues on the 3D-structure and bioactivity of trichogin GA IV.

3.4. Chemical synthesis of peptaibiotics

The solid-phase peptide synthesis (SPPS) method (Merrifield, 1963) has been a breakthrough in both peptide science and synthetic organic chemistry. SPPS is a fast, straightforward, controlled, and customizable way to produce peptides and small proteins in high yield and purity (Gutte and Merrifield, 1969; Marglin and Merrifield, 1966). The major advantage of SPPS is the possibility to produce not only "standard" peptides, but also NRPs as well as peptide analogues and mimetics with no match in nature, by enabling tailored insertion of non-coded/artificial amino acids in the peptide backbone and/or *N*-/*C*-terminal/side-chain modifications other than natural post-translational ones. The versatility and impact of SPPS explains why it remains a hot research topic (Yan and Chen, 2022), encompassing from development of new automated microflow techniques (Masui and Fuse, 2022) to much-needed greener methods (Varnava and Sarojini, 2019) to face the sustainability challenges posed by chemical synthesis and purification of peptides at both the research and industrial scales (Isidro-Llobet et al., 2019).

While SPPS conveys a virtually endless number of possible sequences that can include non-canonical amino acids and modifications, these are often quite demanding from the chemical synthesis perspective, requiring a substantial optimization effort. For instance, SPPS works generally well for production of linear hydrophilic peptides of up to a few dozen residues but can be quite challenging to produce the so-called "difficult peptides", which mostly comprise highly hydrophobic sequences (Mueller et al., 2020; Paradís-Bas et al., 2016). Therefore, given their typically high hydrophobicity and the presence of sterically hindered α,α-dialkyl α-amino acid residues in their backbone, peptaibiotics are demanding target-molecules for chemical synthesis, requiring adaptation of not only SPPS but also earlier classical solution-phase approaches.

In their pioneer work, Toniolo and co-workers employed a classical convergent synthesis approach in solution (Benedetti et al., 1983; Benedetti et al., 1982b; Benedetti et al., 1982a; Toniolo et al., 1983). This approach made use of methods reported by Sheppard and co-workers before or concomitantly to the emergence of SPPS, based on pivaloyl chloride-mediated activation of the Aib carboxyl group to form a reactive oxazolone intermediate (Jones et al., 1965; Leplawy et al., 1960). This allowed the successful production of Aib-rich sequences with as much as nine residues (Toniolo et al., 1983) and was the driving force, alongside the growing awareness on the importance of peptaibiotics (Brückner and Toniolo, 2013; Hou et al., 2022), for researchers to endure in the optimization of convergent synthesis in solution, targeting larger peptaibols. One successful example is the aziridine-oxazolone approach developed by Heimgartner and co-workers that enabled the total synthesis of hypomurocins A1, A3 and A5 (Fig. 4), natural undecapeptaibols from the ascomycetous fungus *Hypocrea muroiana* (Pradeille et al., 2012; Pradeille et al., 2005). Still, this is an extremely laborious and time-consuming approach that, despite being advocated as best suited for industrial scale production (Pradeille et al., 2005), does not allow for swift and automated production of large arrays of peptaibols, especially of longer ones. Therefore, SPPS methods tailored for the specificities of peptaibols have been actively pursued since the last decade of the 20th century.

The standard SPPS protocols available until the late 1980s (Atherton and Sheppard, 1989) were unsuited to overcome the peptaibol-associated synthetic hurdles, namely; i) incomplete/slow coupling of α,α-dialkyl α-amino acids and of subsequent ones, with consequent higher racemization levels, and ii) lack of resins suitable to obtain *C*-terminal peptide alcohols *via* cleavage conditions compatible with the acid-labile Aib-Pro linkage. The latter issue was overcome by introduction of the 2-chlorotrityl chloride (2CTC) resins that enable cleavage under very mild conditions (Chatzi et al., 1991) and the direct anchoring

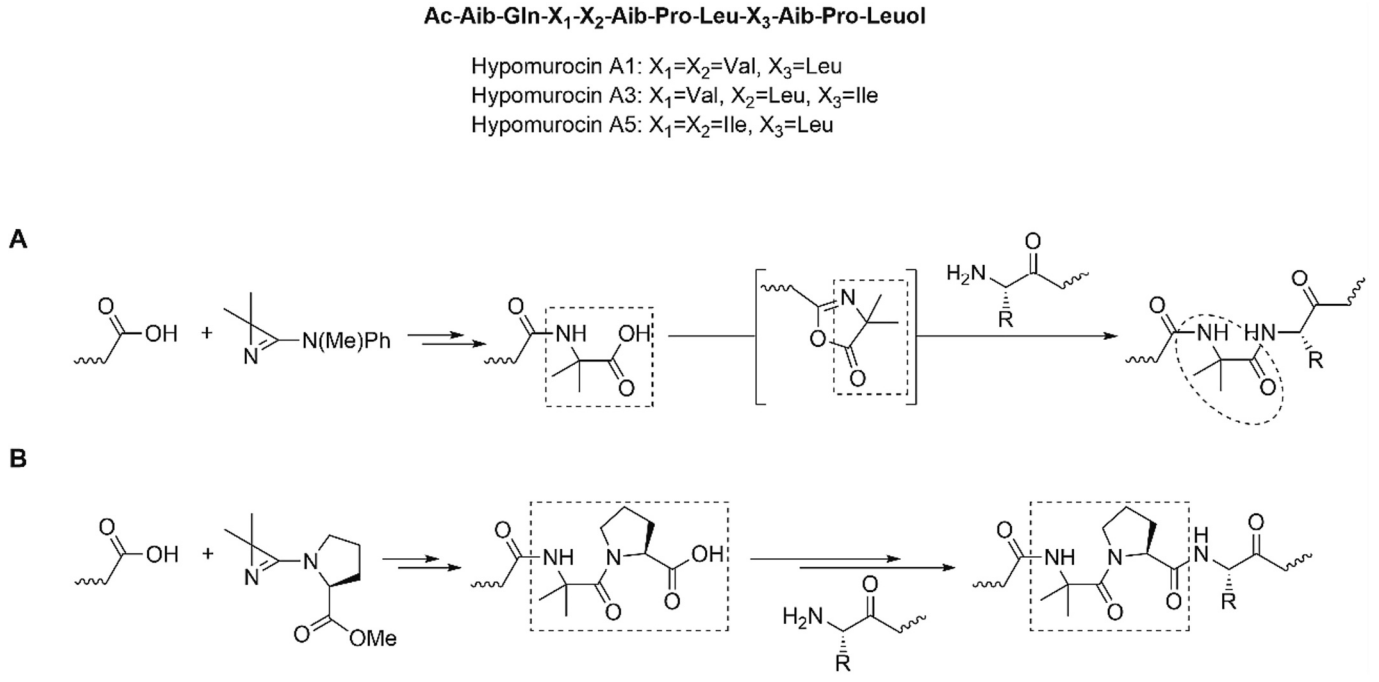

Fig. 4. Amino acid sequences of hypomurocins A1, A2 and A3 whose total synthesis was accomplished by means of a convergent solution-phase approach reported by (Pradeille et al., 2012, Pradeille et al., 2005), based on the aziridine-oxazolone method for incorporation of the critical Aib (A), and Aib-Pro (B) building blocks.

of *C*-terminal α-amino alcohols (Wenschuh et al., 1995) (Fig. 5A). Currently, there are other options for the SPPS of peptide alcohols, but the 2CTC resin remains the preferred one (Ferrer-Gago and Koh, 2020). Sluggish couplings of sterically hindered amino acids, and consequent racemization issues, could not be resolved while carbodiimides (Rebek and Feitler, 1975) remained the golden standard coupling agents. Automated SPPS of natural peptaibols like alamethicins, saturnisporins, and trichotoxins was successfully accomplished in the mid-1990s by combined use of the 2CTC resin and N^{α}-protected amino acid fluorides (Wenschuh et al., 1995) previously prepared using, e.g., cyanuric fluoride (Carpino et al., 1990)(Fig. 5B). Despite these fluorides can be generated *in situ* by use of tetramethylfluoroformamidinium hexafluorophosphate (TFFH) (Bertelsen et al., 2007; Lam et al., 2021), this method has been considered unsuitable for fully automated synthesis (Hjørringgaard et al., 2009).

The introduction of efficient *in situ* peptide coupling agents, namely, phosphonium and aminium salts such as benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), among many others (El-Faham and Albericio, 2011), contributed to the improvement of solid-phase synthesis of peptaibiotics (Lam et al., 2021). The emergence of microwave-assisted SPPS further facilitated the automated solid-phase assembly of peptaibiotics (Hjørringgaard et al., 2009), allowing even the rescue of carbodiimides as a much cheaper alternative to those phosphonium and aminium salts (Ben Haj Salah and Inguimbert, 2014). Therefore, peptaibiotics can now be produced using reagents and methods of the current standard portfolio in SPPS. Recently, De Zotti and colleagues have been employing greener and more cost-efficient SPPS procedures to efficiently produce diverse peptaibols and modified analogues (Baccelli et al., 2022; Caracciolo et al., 2023; De Zotti et al., 2020; Sella et al., 2021), inspired in the ReGreen SPPS concept (Pawlas and Rasmussen, 2019). In summary, robust methods are now available for the swift production of diverse peptaibiotics and optimized surrogates, based on SPPS procedures of increasing simplicity and sustainability.

4. Bioactivity of peptaibiotics against phytopathogens

Peptaibiotics have a wide spectrum of biological activities (Gavryushina et al., 2021; Zhao et al., 2019). Despite that, applications in agriculture have lagged behind their clinical counterparts. Thus, only a few molecules have been assayed against plant pathogens (Table 1). In addition, only a reduced number of phytopathogens have been screened from an overwhelmingly large pool of bacteria, fungi and viruses that affect crops worldwide (Brauer et al., 2019; Panno et al., 2021; Rosa et al., 2022). Indeed, phytopathogenic-specific works are scarce.

Most of the tested molecules are naturally synthesized by fungi (Table 1). However, technology has enabled the chemical synthesis of peptaibiotics (De Zotti et al., 2020). In that sense, several works have tested synthetic peptides against notorious phytopathogens with promising results not only for their bioactivity, but also due to the increased hydrophilicity (Baccelli et al., 2022; Bolzonello et al., 2023; Caracciolo et al., 2023; De Zotti et al., 2020; De Zotti et al., 2009; Sella

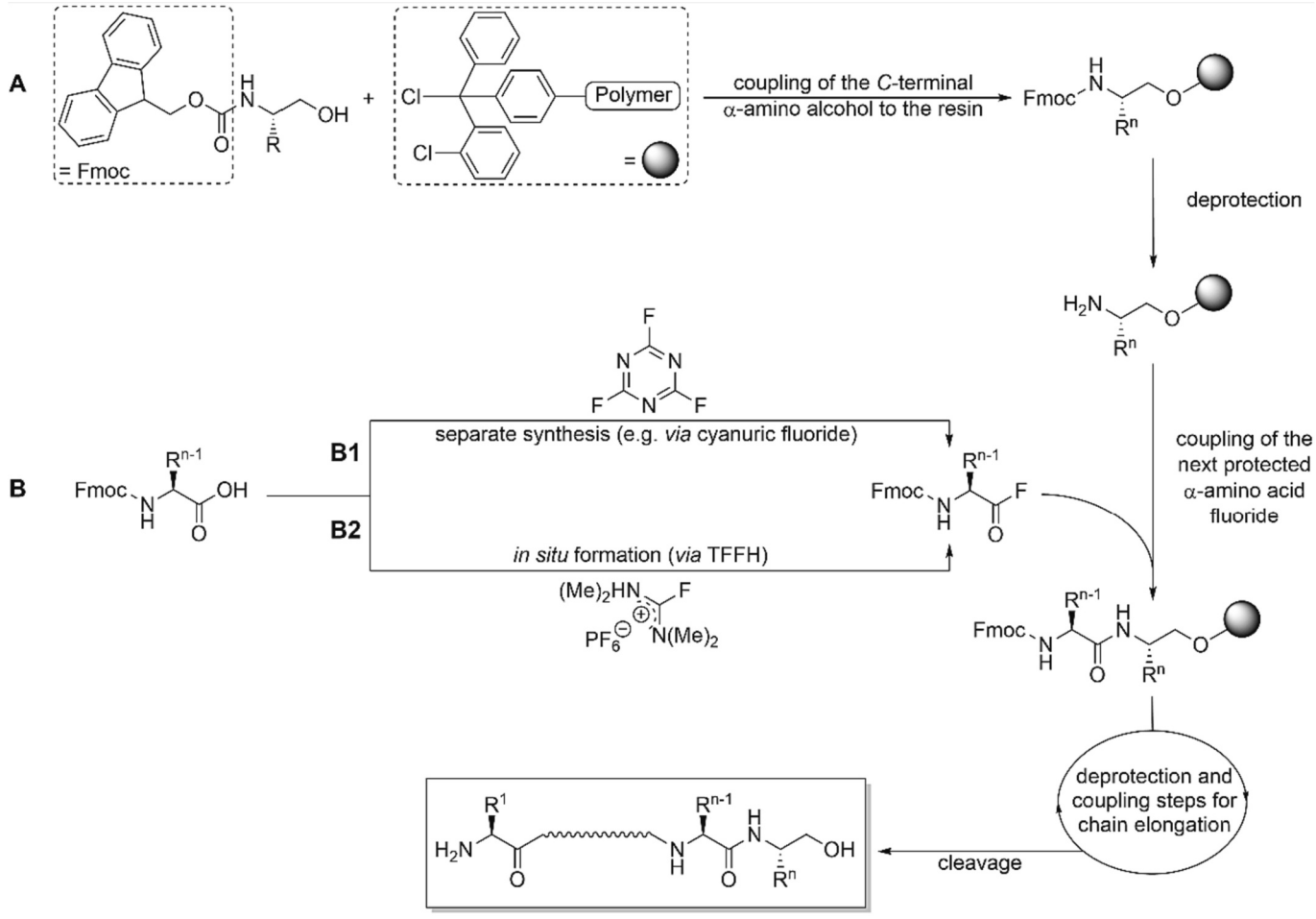

Fig. 5. Schematic representation of the SPPS of peptaibols *via* (A) anchoring the *C*-terminal amino alcohol onto a 2CTC resin (Wenschuh et al., 1995) and (B) using reactive N^{α}-protected amino acid fluorides, either synthesized separately using, e.g., cyanuric fluoride (B1) (Carpino et al., 1990) or formed *in situ* by addition of TFFH to the reaction medium (B2) (Bertelsen et al., 2007; Lam et al., 2021).

et al., 2021). In some cases, chemical synthesis served as a means to elucidate the compounds' structure and configuration (Lam et al., 2021; Otto et al., 2015). Among the tested peptaibiotics, long-sequence molecules have been the preferred subject (Table 1). While long- and medium-sequence peptides have shown promising results, short peptaibiotics were less promising (De Zotti et al., 2009; Otto et al., 2015), although showing antimicrobial potential after tweaks to their amino acid composition (Baccelli et al., 2022; Caracciolo et al., 2023; De Zotti et al., 2020). Sella et al. (2021) reported that replacing Gly by Lys residues in positions 2 and 6 greatly increased the antimicrobial activity due to a strengthened amphiphilic structure. Micro-heterogeneous extracts from fungal cultures have also been interrogated regarding their antimicrobial potential (Table 1). These mixtures are composed of isoforms/dimers, showing single to multiple mutations (Dornberger et al., 1995; Maddau et al., 2009; Marik et al., 2019; Otto et al., 2016b). Their antimicrobial potential is often associated with one or a few dimers in their composition (Balázs et al., 2023; Tamandegani et al., 2020; Viterbo et al., 2007). In some cases, synergism among dimers underlies their efficacy, showing a broader spectrum of antimicrobial activity (Maddau et al., 2009). The application of extracts can be a cheap way to test if the bioactive potential of a particular strain is indeed linked to its peptaibiotics profile, and only then proceed to later stages of purification and testing of individual molecules (Maddau et al., 2009; Marik et al., 2013).

4.1. Assays studying the bioactivity of peptaibiotics against phytopathogens

Ten works assayed peptaibiotics against gram-positive and/or gram-negative phytopathogenic bacteria. Boletusin (Lee et al., 1999a), chrysospermins (Kim et al., 2000), peptaivirins (Yeo et al., 2002), tylopeptins (Lee et al., 1999b), trichokonins (Song et al., 2006) have all inhibited gram-positive bacteria *Corynebacterium lilium* and *Clavibacter michiganensis in vitro* with as low as 30 μg/paper disc of peptide in agar diffusion assays (Table 1). Likewise, *C*-terminal-modified Trichogin analogue 4r, carrying a terminal amide, completely inhibited *B. subtilis in vitro* at 3 μM. Finally, micro-heterogeneous extracts from *T. effusum*, *T. longibrachiatum* f. *bissettii*, *T. longibrachiatum*, *T. reesei*, and *T. saturnisporum* have been reported to inhibit *C. michiganensis* and *R. fascians* growth at concentrations of 156 to 5000 μg/mL (Balázs et al., 2023) (Table 1 and Supplementary Data – Table S1).

Attempts to control gram-negative bacteria were less successful initially, due to the lipopolysaccharides of the bacterial outer membrane forming a strong diffusion barrier (Marik et al., 2018; Yeo et al., 2002). However, encouraging results have been observed when plants were treated with a peptaibol solution (trichokonins at 9.6 nmol and 18-mer peptaibol at 0.3 μg/mL) prior to the infection with *P.s.* pv. *lachrymans* and *P. carotovorum* (Table 1 and Supplementary Data – Table S1). Aversely, the direct application of these peptaibols to the media had no inhibitory effect, implying that such compounds do not have antimicrobial activity against these bacteria but instead induce the plant's resistance mechanisms (Li et al., 2014; Viterbo et al., 2007). Recently, trichokonins were also reported to reduce pathogenicity of *X. oryzae* after rice plantlets were dipped in a solution containing those peptaibols at 27 μg/mL (Zhang et al., 2022). Strikingly, trichokonins also showed antibacterial activity at 38-100 μg/mL when applied to the culture medium (Zhang et al., 2022). Trichokonin A offered the best results with a MIC of 38 μg/mL. Interestingly, Song et al. (2006) had previously reported no activity for trichokonins against gram-negative *R. solanacearum* and *E. carotovora* (Table 1 and Supplementary Data – Table S1). Trichogin synthetic analogues have also been reported to completely inhibit the growth of gram-negative *X. campestris* with doses as low as 1 μM (Caracciolo et al., 2023). Peptide 4r was further able to decrease the severity of *X. campestris* disease by spraying leaves prior to the infection with peptide solutions at 50 μM. The same lipopeptaibol was also able to inhibit *A. tumefaciens*, *E. carotovora*, *P. corrugate*, *P. savastanoi*, *R. solanacearum*, and *X. arboricola* at 3-15 μM. Moreover, *T. longibrachiatum* f. *bissettii* extract has been reported to inhibit *R. radiobacter* growth *in vitro* at 1250 μg/mL (Balázs et al., 2023) (Table 1 and Supplementary Data – Table S1).

Fungal phytopathogens have been a major target due to their ubiquity, devastating effects, and difficulty to control (Rosa et al., 2022). Overall, 48 different species have been tested across 27 genera. The most represented genera are *Fusarium* (10 species), *Trichoderma* and *Colletotrichum* (4 species), and *Alternaria*, *Botryosphaeria* and *Phytophthora* (3 species) (Table 1). Broad-host range ascomycete and model organisms *B. cinerea* and *F. oxysporum* have been frequent targets. Zhao et al. (2018) used *B. cinerea* as a model to assess the mechanism behind trichokonins' bioactivity, whereas Shi et al. (2012) used *F. oxysporum* for a similar purpose. The first was antagonized in 13 works. Ampullosporin F (4.6 μM), chilenopeptin A (5.3 μM), leucinostatin Z (5000 μg/mL), trichorzianins (200 μg/mL), trichokonins (25 μM, 80-200 μg/mL, 320 AU/mL), tylopeptin A (5.3 μM), tulasporin D (3.7 μM), and trichotoxins (800 μg/mL) have completely or strongly inhibited *B. cinerea* growth, whereas albupeptins (25-50 μM) have shown moderate activity (Alfaro-Vargas et al., 2022; Lam et al., 2021; Liu et al., 2020; Lorito et al., 1996; Otto et al., 2016a, 2016b; Otto et al., 2015; Schirmbock et al., 1994; Song et al., 2006). Synthetic trichogin GA IV analogues were also able to detain *B. cinerea*'s growth in both *in vivo* and *in vitro* experiments at 50 μM and 5-15 μM, respectively (Baccelli et al., 2022; De Zotti et al., 2020) (Table 1 and Supplementary Data – Table S1).

Fusarium oxysporum was antagonized by trichorzianins (200 μg/mL), atroviridins and neoatroviridins (50 μg/paper disc), trichokonins (25 μM), and trichotoxins (800 μg/mL) showing moderate to strong susceptibility (Alfaro-Vargas et al., 2022; Oh et al., 2002; Schirmbock et al., 1994; Shi et al., 2012; Song et al., 2006). Likewise, the micro-heterogeneous extract of *T. cerinum* showed moderate activity against *F. ciceris* when applied directly to the growth medium, but strongly reduced disease severity when used for seed priming (Khare et al., 2018) (Table 1 and Supplementary Data – Table S1). Contrarily, synthetic helical and non-helical 8-meric and 4-meric, *C*-terminal short peptides showed no activity against this and other phytopathogenic fungi (De Zotti et al., 2009).

Phoma destructiva has also been a model due to the easy-to-spot effects on this pathogen's cells (i.e., the induction of pigmentation and morphogenesis) (Grigoriev et al., 2003). 2000 μg/mL alamethicin (Grigoriev et al., 2003), 30-50 μg/paper disc or agar well of ampullosporins A-D (Kronen et al., 2001; Ritzau et al., 1997), 250 μg/mL of chrysospermin D (Dornberger et al., 1995), 2000 μg/mL of bergofungins, cephaibols, paracelsin, and texenomycin (Grigoriev et al., 2003), and 1000 μg/mL of trichofumins have been reported to induce said effects in *P. destructiva*, indicating membrane perturbing activity (Table 1 and Supplementary Data – Table S1).

Phytophthora infestans has been successfully inhibited by 14.7 μM of ampullosporin A (Lam et al., 2021), 50 μg/paper disc of atroviridins and neoatroviridins (Oh et al., 2002), 84 μM of albupeptin C (Otto et al., 2015), 10 μM of chilenopeptin A (Otto et al., 2016b), 60/agar well μg of leucinostatins (Wang et al., 2016), 4 μM of tulasporin D (Otto et al., 2016a), 13.34 μM of tylopeptins (Otto et al., 2016b). *Phytophthora capsici* was strongly inhibited by 60 μg/agar well of leucinostatins A-B (Wang et al., 2016), while *P. parasitica* was susceptible to trichokonins (25 μM). *Pyricularia oryzae* has been antagonized with several trichogin-derived synthetic peptaibols, carrying different modifications to their sequence. Several of these have been effective *in vitro* and *in vivo* at 50 μM (Sella et al., 2021). Similarly, these synthetic molecules have been effective against *P. viticola in vitro* (30 μM) and under open-field conditions at 100 μM or 64.7 g/ha (Bolzonello et al., 2023). Again, peptide 4r outstood for its efficacy (Table 1 and Supplementary Data – Table S1).

Furthermore, atroviridins and neoatroviridins showed strong inhibition against *Cladosporium* sp., *C. demantium*, *C. inaequalis*, and *V. dahliae* at 50 μg/paper disc in agar diffusion assay (Oh et al., 2002). Harzianins HC and trichorzins' application reduced *S. cepivorum* growth *in vitro* by 40 and 75% at 100 μg/mL, respectively (Goulard et al., 1995).

Helioferins A-B were able to inhibit *F. culmorum* growth at concentrations as low as 6.25 μg/mL (Gräfe et al., 1995). Trichokonins have been reported to inhibit *A. citrullina* (20 μM), *B. sorokiniana*, *C. lagenarium*, *C. lunata*, *R. solani*, *V. mali*, and *V. dahliae* (320 AU/mL) (Shi et al., 2012; Song et al., 2006). Trichotoxins were able to inhibit the growth of *C. gloeosporioides* and *A. alternata* by 92 and 58%, respectively, at 800 μg/mL (Alfaro-Vargas et al., 2022). Likewise, trilongins B (I-IV) have shown promising results against *C. gloeosporioides* at concentrations between 40 and 320 μM. Trilongins B I showed the best results, needing only 40 μM to reduce mycelial growth by 41%. Lastly, TvB I and II peptaibol had low bioactivity against *R. solani* at 200 μg/mL *in vitro* (Viterbo et al., 2007) while synthetic trichogin analogues strongly inhibited the growth of *B. sorokiniana* (15 μg/mL), *F. graminearum*, and *P. expansum* at 5-15 μM (Table 1 and Supplementary Data – Table S1).

Micro-heterogeneous extracts have also showed potential to control the growth of fungi. *Clonostachys rosea* extract strongly inhibited *C. truncatum*, *F. solani*, *M. phaseolina*, *R. solani*, and *S. sclerotiorum* (Rodríguez et al., 2011). *Trichoderma asperellum* and *T. longibrachiatum* extracts were shown to halt spore germination and growth of *A. solani*, *F. culmorum*, *F. graminearum*, *F. moniliforme*, and *R. solani*, at 100 and 200 μg/mL, respectively (Tamandegani et al., 2020). *T. citronoviride* extract was able to completely stop the growth of *B. parva*, *B. obtusa*, *D. pinea*, and *D. scrobiculata* as well as to strongly inhibit that of *A. quercina*, *B. corticola*, and *B. mediterranea* at 100 μg/mL (Maddau et al., 2009). Also *T. gamsii* and *T. koningiopsis* (Marik et al., 2018) were able to completely or strongly inhibit mycelial growth of *A. alternata*, *F. solani*, *P. cucurbitacearum*, *R. solani*, *T. aggressivum* f. *europaeum* and *T. pleuroti*, at concentrations ranging from 1600-100000 μg/mL. Finally, *T. reesei* extract strongly inhibited *A. alternata*, *A. fumigatus*, *F. falciforme*, *F. keratoplasticum*, *F. solani*, and *P. cucurbitacearum* at 200 μg/mL (Marik et al., 2019) (Table 1 and Supplementary Data – Table S1).

The application of peptaibols against plant viruses has shown good results when plants were primed before the infection rather than as a curative measure (Table 1 and Supplementary Data – Table S1). This was only tested on Tobacco Mosaic Virus (TMV) and Cucumber Mosaic Virus (CMV), but results yielded the same conclusion. Thus, Kim et al. (2000) showed that chrysospermins D inhibited by 93% the formation of TMV-derived lesions on plant tissues at 100 μg/mL, whereas Yeo et al. (2002) and Yun et al. (2000) observed 70-80% fewer TMV-derived lesions at 10 μg/mL and almost 100% fewer lesions at 100 μg/mL. Similarly, Luo et al. (2010) observed a 50% decrease in TMV-derived lesions in tobacco plants when these were pre-treated with trichokonins at 200 ng/mL. Finally, trichorzins were tested against CMV by priming cowpea plantlets prior to the infection. The authors reported a 90% reduction of lesions, particularly for trichorzins HA V, at 10 μM (Kai et al., 2018).

4.2. Methods employed to study the bioactivity of peptaibiotics

Testing the bioactive properties of a molecule or extract should be as straightforward and reliable as possible to provide researchers with information on the most promising candidates for further testing and comparative assessments (Marik et al., 2013; Rush et al., 2021). Bioactive strains are often identified through the dual-culture method to search for any growth inhibition ability (Fig. 6). If there is growth inhibition, researchers will often proceed to test if it is linked to any component of the fungal extract (Wang et al., 2016).

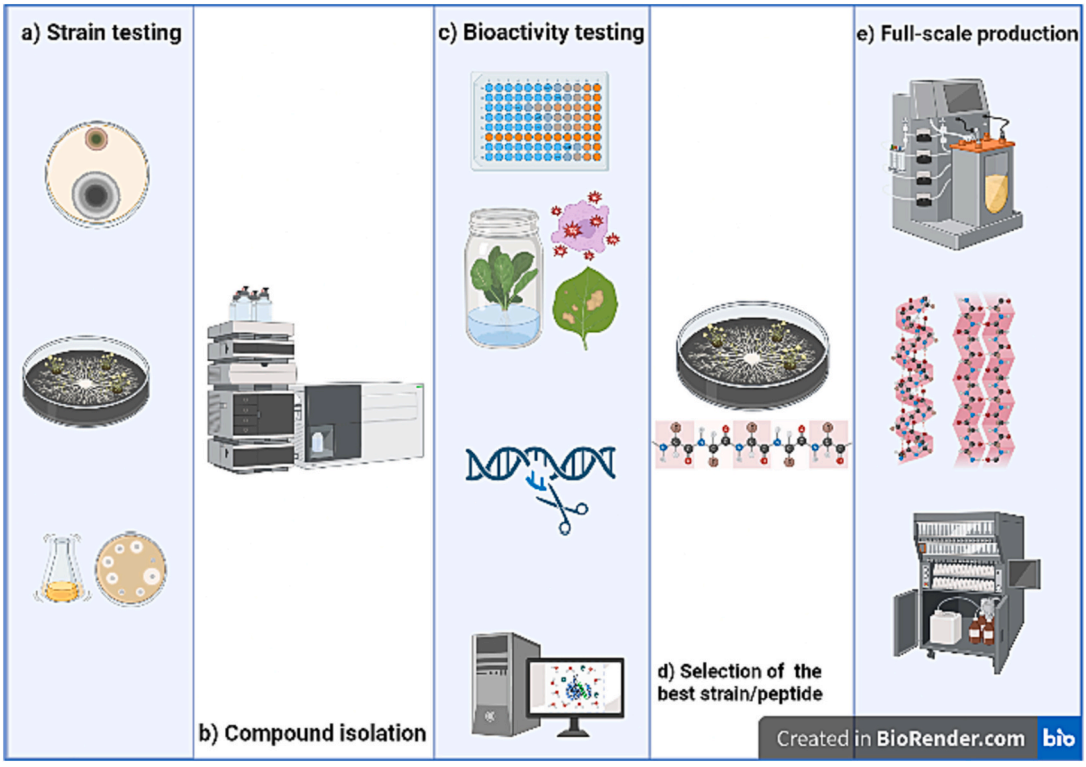

Fig. 6. Schematic representation of the workflow from strain identification to peptaibiotic mass production. a) Illustrates the identification and testing of a strain's antimicrobial activity through the dual-culture method. The extract's testing testifies the activity is due to a compound in its composition. b) HPLC and its derivations are the preferred method for the identification and purification of the peptaibiotics fraction present in the strain's crude extract. c) After purification, the pure compound is tested to characterize its bioactivity against phytopathogens, toxicity to plants and animals, delivery efficiency, and mode of action. Recently, genetic transformation of strains has enabled to test the compound's bioactivity without the need for purification by silencing/expression of genes controlling peptaibiotics biosynthesis. Likewise, *in silico* models offer the possibility to pre-test the peptide's properties without purifying it. d) Finally, after selecting an ideal strain or molecule, we can step into mass production and structure and conformation analysis. Liquid or solid-state fermentation and chemical synthesis are the options to produce large amounts of the desired peptides. Fermentation could be used in combination with genetic transformation, epigenetics, and elicitation to increase yields. Chemical synthesis, on the other hand, can be coupled with *in silico* design to produce tailored molecules to a specific target organism and high purity levels.

The detection of antimicrobial activity is usually assessed through low-cost biological screenings (Fig. 6). This is usually tested through the agar diffusion method, consisting of adding a pre-established concentration (minimal inhibitory concentration, MIC) of the peptide to the medium (Alfaro-Vargas et al., 2022; Balázs et al., 2023; De Zotti et al., 2020; Goulard et al., 1995; Rebuffat et al., 1995; Tamandegani et al., 2020; Zhang et al., 2022) or by placing a peptide-imbibed paper disc onto the cultivation medium (De Zotti et al., 2009; Grigoletto et al., 2020; Liu et al., 2020; Oh et al., 2002). This is usually carried out on a Petri dish or a microtiter plate (Otto et al., 2016b; Tamandegani et al., 2020) (Supplementary Data – Table S1).

In vivo experiments are also common (Fig. 6). The most usual iterations are spraying (or dipping) plantlets (Baccelli et al., 2022; Caracciolo et al., 2023; Kim et al., 2000; Li et al., 2014; Luo et al., 2010; Sella et al., 2021; Viterbo et al., 2007; Yeo et al., 2002; Yun et al., 2000; Zhang et al., 2022) or detached organs (e.g., leaf, fruit) (Alfaro-Vargas et al., 2022; Baccelli et al., 2022; Khare et al., 2018; Sella et al., 2021) with the peptaibiotic and compare with a mock-treated sample (control) for disease symptoms. Zhao et al. (2018) evaluated the inhibitory effect of TK VI by spraying the leaves after inoculation, whereas De Zotti et al. (2020) sprayed detached leaves and berries before inoculating with *B. cinerea*. Kai et al. (2018) added the peptaibols to the hydroponic medium where plants were grown, while Bolzonello et al. (2023) sprayed a vineyard with synthetic lipopeptaibol against *P. viticola* (Supplementary Data – Table S1).

Likewise, genetic transformation has been proven a great tool to test the antimicrobial properties of a strain (Fig. 6). Wang et al. (2016) demonstrated the role of leucinostatins in *P. lilacinum* bioactivity against *P. capsici* and *P. infestans*, by producing both silenced- and over-expressing-*lcs* isolates, encoding a putative bZIP transcription factor associated with secondary metabolism, and growing them in dual-culture. In the absence of leucinostatins, the phytopathogens were able to grow whereas in the presence of overexpressing-*lcs* isolates they showed limited growth (Wang et al., 2016).

By using *in vivo* models, researchers can assess if the peptaibols have any undesired effect on the plant (Fig. 6). This is a critical step for any active substance being considered for commercial formulation, as many microorganisms produce harmful metabolites that could affect non-target organisms (Rodrigo et al., 2022; Rush et al., 2021). Thus, the peptaibol extract of *T. reesei* was tested on *A. thaliana* to assess potential toxicity effects. Authors reported plant growth inhibition at concentrations as low as 0.1 mg/mL. Above 0.3 mg/mL, chlorophyll and carotenoid levels decreased, and at 1 mg/mL primary roots showed deformations. The concentration of 0.1 mg/mL had no impact on photosynthetic pigments and increased anthocyanin levels (Marik et al., 2019). Likewise, testing peptaibols against animal cells has been a standard practice to assess possible nefarious effects (Rodrigo et al., 2022). Marik et al. (2019) showed inhibition of spermatozoa and porcine kidney PK-15 cells at 0.1 mg/mL, possibly indicating toxicity of peptaibol extract of *T. reesei*. De Zotti et al. (2009) tested the cytotoxicity of four peptaibols against mammalian cells and observed a low hemolytic ability of the two longer ones and no activity by the shorter two. Trichofumins' neuroleptic activity was evaluated in mice and found to induce hypothermia (Berg et al., 2003). Peptavirin A was shown to exhibit cytotoxic effects against tumor cell lines (Yeo et al., 2002). Maddau et al. (2009), tested the toxicity of peptaibol mixture against *A. salina*, resulting in an LC_{50} of 1.24 mg/mL. Fortunately, there are also examples where no phytotoxic effects were detected, as is the case of the engineered lipopeptaibol trichogin GA IV, even though it effectively inhibited fungal growth *in vitro* (De Zotti et al., 2020).

Another advantage of using *in vivo* assays is to test how efficient the delivery of the peptide is and how effective is its protective effect (Sella et al., 2021) (Fig. 6). Several factors can affect the interaction between peptaibiotics and pathogens. Innate plant defense mechanisms, such as proteolytic activity or tissue absorption, may decrease the effective concentration of the active compound (Huan et al., 2020; Rosa et al., 2022). Sella et al. (2021) raised the issue that cuticle wax of leaves and fruits could affect how the solution interacts with tissue surfaces after observing different behaviors between barley and rice. On barley leaves, the solution drops tended to cluster together, forming a spherical bead, whereas on rice leaves they appeared more uniformly distributed throughout the surface. Thus, the effectiveness of the treatment could be at least in part affected by the uneven distribution of the peptide (Sella et al., 2021).

5. Mechanisms behind peptaibiotics antimicrobial activity

Despite their antimicrobial potential, peptaibiotics' mechanisms of action (MoA) are still poorly understood (Dam et al., 2018; Shi et al., 2012). Based on the literature, peptaibiotics are known to exert their effect on microorganisms mainly through two MoA: i) direct action on the biological membranes (Bortolus et al., 2013; Khare et al., 2018; Shi et al., 2012), or ii) intracellular action by inhibiting or disrupting metabolic processes (e.g., synthesis of nucleic acids, essential enzymes or functional mechanisms) (Sella et al., 2021; Zhao et al., 2012, 2018) and through the induction of systemic plant defenses (Li et al., 2014; Lorito et al., 1996). These MoA are the reason behind peptaibols potent and remarkably fast bioactivity, hampering any attempt from the pathogens to counteract their action (Dam et al., 2018; Lorito et al., 1996).

5.1. Evidence of membrane permeating ability

5.1.1. Pore formation ability on the pathogen's cell membranes

Peptaibiotics exert their effect through the formation of pores by assembling into helical bundles which form channels in the phospholipid bilayers, altering the inward current of ions and allowing an uncontrolled exchange of cytoplasmatic material that can induce apoptosis of the cells (Bortolus et al., 2013; Shi et al., 2012; Zhao et al., 2018). In this way, Zhang et al. (2022) showed that trichokonins A were able to rupture *X. oryzae* cell membrane, leading to leakage of intracellular nucleic acids and proteins.

Pore formation is usually linked to the toroidal and barrel-stave models (Fig. 7) (Matsuzaki, 2019; Zhang et al., 2021). In the toroidal-pore model, high peptide concentration can induce deformation of lipid molecules, which allows the peptides and lipid head groups to embed themselves vertically inside of the lipid hydrophobic center, which will bend the membrane and form pores (Matsuzaki, 2019; Zhang et al., 2021). In the barrel-stave model the pores are only composed of peptide aggregates, which create a water-filled channel. The size of the pore is defined by the peptide-to-lipid molar ratio, which after reaching the threshold (10^{-6} to 10^{-8} M) in the bilayer region, parallel to the phospholipids, will lead to the formation of a channel, allowing cytoplasmatic outflow (Huan et al., 2020; Matsuzaki, 2019). Both are hypothesized to last milliseconds, which is enough to destabilize the membrane and for the translocation of peptide through the bilayer (Matsuzaki, 2019). The same characteristics difficult the study of the molecular details pertaining to this process (Matsuzaki, 2019). The "carpet-like" mechanism, on the other hand, has been described to lead to membrane disruption and formation of mixed lipid-peptide aggregates after the accumulation of flat-oriented peptides on the lipid bilayer surface (Fig. 7). High concentration of peptide covers the membrane in clusters and causes the membrane to rupture, without pore formation or peptide insertion, inducing the complete or partial cell membrane lysis (Zhang et al., 2021).

Alamethicin is the most studied peptaibol, hence, its mode of action has been better dissected than those of other peptaibiotics (Leitgeb et al., 2007; Tyagi et al., 2019). Thus, it has been established that 3-12 molecules self-associate into hexameric barrel–stave transmembrane helices resulting in the creation of channels in the biological membranes (Leitgeb et al., 2007). Studies have corroborated the membrane permeating ability for many other members of the peptaibiotics family

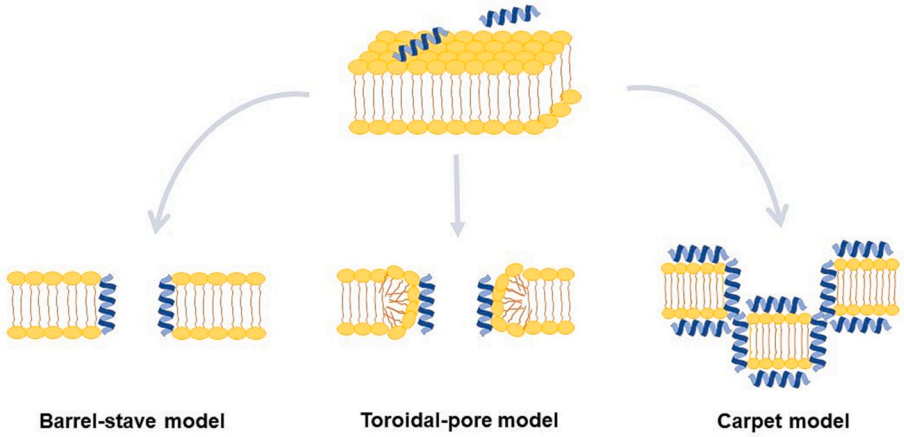

Fig. 7. Schematic representation of the barrel-stave, toroidal-pore and carpet modes of peptides' action onto the phospholipidic membrane. In the barrel-stave mode the pore is composed only of peptide aggregates, whilst in the toroidal-pore mode the pore is formed both by the peptide and the membrane lipids. In the carpet model, peptides cover the membrane in clusters and cause its rupture in a surfactant-like manner. Neither channel formation nor insertion of the peptides into the membrane occurs in this model. Peptides are represented in blue, whereas lipids of the membrane bilayer are colored in yellow.

with activity against phytopathogens. Grigoriev et al. (2003) compared the efficiency of pore formation of peptaibols and lipopeptaibols using artificial bilayer models. Alamethicin F30, paracelsin A, chrysospermin C, and texenomycin A, (19-20 residues) had high membrane activity, whereas ampullosporins (A, B and D), trichofumins (A and C), bergofungin B, and cephaibols (B and C) (11-17 residues) had moderate permeabilization abilities. In this last group, there were also peptides with no evident membrane activity, i.e., bergofungin A and cephaibol E (Grigoriev et al., 2003). Likewise, Goulard et al. (1995) showed the membrane permeating ability of trichorzins (HA and MA), Grigoriev et al. (1995) of chrysospermins (A-D), Oh et al. (2002) of atroviridins (A–C), Berg et al. (2003) of trichofumins (A–D), Shi et al. (2012), Zhao et al. (2018) and Zhang et al. (2022) of trichokonins A, and Alfaro-Vargas et al. (2022) of trichotoxins. Generally, the barrel-stave mechanism seems to be preferred for long-sequences, whereas short molecules apparently act according to the carpet model (Peggion et al., 2003; Tyagi et al., 2019). Strikingly, medium-length peptides (12-16-mer) are reported as likely able to switch between the barrel-stave and the carpet mechanisms, depending on membrane composition, thickness and peptide concentration (Bortolus et al., 2016).

Works have shown that small differences in the amino acid sequence, could have an enormous impact on their bioactive properties (Balázs et al., 2023; De Zotti et al., 2020; Grigoriev et al., 2003). For instance, Grigoriev et al. (1995), studying the 19-residue chrysospermins (A-D), highlighted the importance of position 15 to the channel conductance ability, showing higher conductivity for Iva than for Aib. In that way, chrysospermins B and D showed improved conductance abilities over A and C. Strikingly, the same amino acid substitution at position 5 had no impact (Grigoriev et al., 1995) on such ability. Others have since corroborated that the richness in hydrophobic amino acids, such as Aib, Ile, Leu and Val, favors helical 3D structures with greater membrane permeability properties (Baccelli et al., 2022; Balázs et al., 2023; Goulard et al., 1995; Grigoriev et al., 2003; Oh et al., 2002). Goulard et al. (1995) evidenced that the hydrophobicity of helical trichorzins is behind their membrane-permebilizing ability, which can be finely tuned by the helix amphipathicity. Likewise, Gly to Aib, Lol to Leu, Val to Leu, and Ala to Aib substitutions led to stronger helical structures, whereas Gly to Lys substitutions at position 9 increased the hydrophilicity of the molecule (Baccelli et al., 2022; Balázs et al., 2023; De Zotti et al., 2020). Lys residue at position 6 was considered crucial to increase activity against *B. cinerea* and *P. oryzae*, particularly when no other Lys residue was spatially close to cause electrostatic repulsion (De Zotti et al., 2020; Sella et al., 2021). Aib to Leu substitutions and presence of the Gly-Leu-Aib-Pro motif also improved bioactivity, while Aib to Val substitution at position 17 led to decreased activity and backbone rigidity (Balázs et al., 2023). Aib to Api at position 8 did not change the structural stability but perturbed the amphipathicity of the helix structure of trichogin and probably increased the peptide's fungicidal activity, due to the introduction of a positive charge (De Zotti et al., 2020). Lastly, the growth-inhibitory effects against both *B. cinerea* and *P. infestans* increased with the presence of δ-methyl ester of glutamic acid (GluOMe) in ampullosporins F and G, compared to ampullosporin A that lacks this modification on Glu (Lam et al., 2021).

Modifications to the *N*- and *C*-termini are also relevant to the properties of peptaibols. Dam et al. (2018) showed that i) acetylated *N*-groups help to form well-defined channels in membranes and to stabilize the secondary helical structure of the peptide, ii) *N*-terminal acyl groups favor the hydrophobicity of the molecules, and iii) *C*-terminal hydroxyl groups reduce both permeability and antibacterial activity compared to *C*-terminal esters (Dam et al., 2018). This was also hypothesized by Grigoriev et al. (1995) when discussing the role of *C*-terminal tryptophanol in chrysospermins as a facilitator of helical folding and channel formation by providing an additional dipole moment to the molecule. Ampullosporin A increased the membrane conductance and showed morphogenesis on *P. destructiva* as well as hypothermia in mice. However, when its sequence was tweaked to desacetyltryptophanyl-ampullosporin A or to hexamethylated at the δ-carboxylamide groups of Gln residues and the hydroxyl group of leucinol it showed no induction of fungal morphogenesis and hypothermia on mice (Grigoriev et al., 2003). Finally, the replacement of *C*-terminal-Lol moiety with a -Leu-NH_2 residue introduced a cheaper alternative for chemical synthesis without jeopardizing the peptide's antimicrobial ability and stability (Bolzonello et al., 2023; De Zotti et al., 2020).

Likewise, long-sequence molecules have enhanced capabilities, compared to shorter ones (Berg et al., 2003; Grigoriev et al., 2003; Oh et al., 2002). Many refer to 18- to 19-residue sequences as optimal for interacting with biological membranes (Goulard et al., 1995; Grigoriev et al., 2003). Some studies have reported a correlation between biological activity and the extent of the damage caused to the pathogen cells (Grigoriev et al., 2003). High membrane activity, as that of long-sequence molecules, led to enhanced antimicrobial activity but also to high toxicity levels to both to microbial and animal cells. Thereby, the strong membrane permeabilization was able to damage the cells and thus neither fungal morphogenesis nor hypothermia in mice were induced (Grigoriev et al., 2003). Aversely, a moderate membrane activity, like that of shorter molecules, correlated with the induction of pigmentation of *Phoma destructiva* and hypothermia in mice. Accordingly, moderate membrane activities may support the formation of unstable membrane pores and thus the leakage of cells should also be mild (Grigoriev et al., 2003). These findings fit with those of trichofumins (A–D), where the 11- to 13-residue trichofumins A–D were shown to open unstable pores in artificial bilayer lipid membranes (Berg et al., 2003).

It is worth mentioning that differences are expected in the inhibition

effectiveness of a given molecule against different pathogens, since the cell membrane structures can vary in elasticity, lipid composition, and charge. De Zotti et al. (2020) observed a relationship between the size and shape of conidia and the sensitivity to three synthetic trichogin-analogues lipopeptaibols when *B. sorokiniana*, with larger spores, was not completely inhibited compared to the other tested fungi. However, the small number of tested samples hampers any conclusive correlations to be drawn. Also, Bolzonello et al. (2023) reported that synthetic trichogin analogue (carrying Lys residues in positions 2 and 5 instead of Gly) was active against *P. viticola* but not against *B. cinerea*. The same principle applies to different molecules against the same pathogen. Grigoletto et al. (2020) observed only 45% inhibition of *C. gloeosporioides* with trilongins, whereas Alfaro-Vargas et al. (2022) observed 92% growth inhibition for the same pathogen after treatment with trichotoxins. Likewise, alamethicin F30 and chrysospermin C completely inhibited the growth of *P. destructiva* at higher dosages and induced pigmentation when lower doses were applied; in turn, paracelsin A, despite also being able to inhibit fungal growth, did not stimulate pigment formation at low dosages (Grigoriev et al., 2003).

5.1.2. Combined action with cell wall degrading enzymes to destabilize the pathogen's cell membrane

Attempts to explain the MoA of certain fungal species against phytopathogenic fungi showed that two phenomena usually occur: i) the secretion of cell wall hydrolytic enzymes and ii) the production of peptaibiotics. Schirmbock et al. (1994) showed that the trichorzianins of *T. harzianum* interacted with cell wall hydrolytic enzymes (chitinase and β-glucanases) against *B. cinerea*. Lorito et al. (1996) further explored this relationship. The combination of both strategies potentiated the effects of the peptaibols and hydrolytic enzymes by enabling a better interaction of the trichorzianins with the membrane while hampering the ability of the host to repair the cell wall (Lorito et al., 1996; Schirmbock et al., 1994). Likewise, a recent study with *T. cerinum* showed its remarkable ability to control *F. oxysporum* f.sp. *ciceris* (Khare et al., 2018). The *T. cerinum* isolate was able to produce lytic enzymes (endoglucanase, chitinase and protease) and peptaibiotics. The antimicrobial activity was mainly attributed to the peptaibiotics, and to a smaller extent (~1 to 6% more effective when present), to the lytic enzymes (Khare et al., 2018). Thus, as observed by Lorito et al. (1996) and Schirmbock et al. (1994), lytic enzymes and peptaibols acted together to destabilize the pathogen's cell wall (Khare et al., 2018).

5.2. Evidence of disruption of metabolic processes

5.2.1. Disruption of metabolic processes in the pathogen's cells

Several peptaibiotics have been described to act on cellular components of the antagonized microorganisms and/or even disrupting metabolic processes (Sella et al., 2021; Zhao et al., 2012, 2018). Lorito et al. (1996) and Schirmbock et al. (1994), for example, demonstrated that trichorzianins inhibited the β-D-glucan synthase, thus inhibiting the *de novo* synthesis of cell wall β-glucans. Despite that, there is still a huge knowledge gap regarding this type of MoA.

The study of the MoA of trichokonin VI provided evidence that this peptaibol was able to induce metacaspase-independent apoptotic cell death in *F. oxysporum* cells (Shi et al., 2012). This was achieved through physiological changes, such as phosphatidylserine externalization, loss of mitochondrial transmembrane potential, accumulation of reactive oxygen species (ROS), and nuclease-mediated DNA breakage. The authors proposed that after permeating the cell membrane, trichokonin VI promotes the elevation of cytosolic Ca^{2+} which initiates the apoptotic process followed by the accumulation of cytoplasmic vacuoles (Shi et al., 2012). Also working with trichokonin VI, Zhao et al. (2018) aimed at disclosing the mechanism leading to the inhibition of hyphal growth and sporulation of *B. cinerea*. Their conclusions aligned well with those of Shi et al. (2012), showing that trichokonin VI was indeed inducing apoptosis through severe physiological and metabolic changes, respectively, to the membrane and to organelles (Zhao et al., 2018).

More evidence in support of these findings came from transcriptomics studies of *P. oryzae* (Sella et al., 2021). After treatment with a synthetic analogue of lipopeptaibol trichogin GA IV, genes involved in oxidative stress response, detoxification, autophagic cell death, cell wall biogenesis, degradation and remodeling, melanin and fatty acid biosynthesis, and ion efflux transporters were significantly upregulated, suggesting that this peptaibol leads to cell wall and membrane damage and induces autophagic cell death (Sella et al., 2021). Moreover, the authors confirmed that, as mentioned above in regard to the action of trichokonin VI against *F. oxysporum*, trichogin's analogue could induce metacaspase-independent apoptosis through a burst of ROS production, which was confirmed by the downregulation of genes involved in catalase and glutathione peroxidase. They also disclosed an upregulation of genes involved in DNA repair, suggesting DNA damage, possibly due to high concentration of ROS, contributed to cell death (Sella et al., 2021). Furthermore, the authors showed that the peptide localized at the cell wall of *P. oryzae* conidia, as expected, and intracellularly in the agglutinated cytoplasm. This study offered further evidence that the depolarization of the plasma membrane allows the endocytic internalization of the peptide which, alongside the increasing cytoplasmic concentration of calcium ions, enables its action on intracellular membranes (Sella et al., 2021). Peptides' direct damage to DNA remains unconfirmed.

5.2.2. Induction of systemic plant defenses

Another MoA ascribed to antimicrobial peptaibiotics is their reported ability to induce innate plant defense mechanisms. Viterbo et al. (2007) put to the test the direct involvement of peptaibols in the elicitation of *Trichoderma*–plant-induced resistance. For that, *T. virens*, a well-known biocontrol agent that is an inducer of plant defense responses and producer of 11-, 14- and 18-mer peptaibols, was selected. When cucumber seedlings were treated with two synthetic 18-amino-acid peptaibol isoforms, or inoculated with *T. virens*, the systemic defenses were activated against *P.s.* pv. *lachrymans* (Viterbo et al., 2007). This response was characterized by the accumulation of antimicrobial polyphenols in cucumber cotyledons and by the up-regulation of *Hydroxyperoxide Lyase* (*HPL*), *Phenylalanine Ammonia Lyase* (*PAL1*) and *Peroxidase* (*PRX*) genes' expression. Also, 11- and 14-mer peptaibols alone did not provide full systemic protection, indicating that the 18-mer peptaibols are required to initiate the signaling cascade of the plant defense response, probably due to their membrane channel-forming properties (Viterbo et al., 2007).

Similarly, priming tobacco and Chinese cabbage plants with trichokonins increased their resistance to tobacco mosaic virus (TMV) (Luo et al., 2010) and *P.c.* subsp. *carotovorum* (Li et al., 2014), respectively. In both cases, the peptide was able to activate multiple plant defense pathways. Both research teams observed increased local and systemic production of ROS ($O_2^{\bullet-}$ and H_2O_2); increased expression and activity of defense-related genes and enzymes, namely *PAL1*, *Peroxidases* (*POD*) and *Polyphenol Oxidases* (*PPO*), which increased local accumulation of phenolic compounds, as well as several plant defense genes related to reactive oxygen intermediate (ROI)-mediated signaling pathway (*APX*, *CAT*, *POX SOD*) (Li et al., 2014; Luo et al., 2010). In addition, trichokonins led to the upregulation of the expression of salicylic acid-responsive pathogenesis-related genes *PR-1*a, *PR-3* and *COI1* (Li et al., 2014; Luo et al., 2010).

6. Why are peptaibiotics still far from their real use as phytosanitary agents: handicaps and possible solutions

6.1. Obstacles to the wide adoption of peptaibiotics in agriculture

The *in planta* application of peptaibiotics has been successfully employed in numerous studies (Supplementary Data – Table S1). Unfortunately, this has never been translated into full-scale application

under field or greenhouse conditions, despite the amount of data pointing towards their stability, safety, and efficacy. Peptaibiotics of different classes have been proven extremely resistant to proteolytic degradation, extreme pH levels (from pH 1 up to 10), and a wide range of temperatures (from -20 up to 120 °C) (De Zotti et al., 2020; De Zotti et al., 2009; Song et al., 2006). A recent two-year open-field trial also showed that the effectiveness and duration of protection conferred by lipopeptaibols were comparable to those of cupric fungicides against viticulture pathogen *P. viticola* while producing no phytotoxicity symptoms on leaves or bunches (Bolzonello et al., 2023).

As pointed out by Rodrigo et al. (2022), only a reduced number of biocontrol agents' strains are currently used as a control method against a small number of diseases and no secondary metabolites (e.g., peptaibiotics) are legally recommended for the control of phytopathogens, despite the pressing need for it. This scenario is staggering clear for *Trichoderma* spp., as regardless of the wide use of their spores in agrochemical formulations, a product is yet to be approved that comprises *Trichoderma*-derived peptaibiotics (Rush et al., 2021; Shahriar et al., 2022; Woo et al., 2014) that are the main metabolites produced by the formula strains (Degenkolb et al., 2015). The direct application of peptaibiotics in formulations, as opposed to spores, could eliminate the dependence on the plant host, on the interactions with other microorganisms and/or on the environmental conditions (Fraceto et al., 2018; Jaiswal et al., 2020; Pedrero-Méndez et al., 2021). Furthermore, peptaibiotics can be specifically tailored to fight a particular pathogen or group of pathogens, leaving beneficial microorganisms unaffected, whereas formulations are largely broad-spectrum products (Shahriar et al., 2022). Likewise, peptaibiotics could be applied in the post-harvest stage, where a microbial-free treatment is preferred to avoid contamination of foods. Finally, the direct application of peptaibiotics could elicit plant defense responses and increase their resistance against a wide range of phytopathogens (Li et al., 2014; Luo et al., 2010; Viterbo et al., 2007).

Researchers pointed out a few reasons for the lack of large-scale adoption of peptaibiotics as phytosanitary agents. On one hand, application of peptaibiotics in agrochemical formulations has been impeded by their poor water solubility, resulting from the high content of non-polar residues in their sequences. This hampers the delivery and often leads to underwhelming crop protection (De Zotti et al., 2020). On the other hand, peptaibiotics' biosynthesis is a hard process to reproduce *in vitro*. Production is tightly dependent on species/strain as well as on environmental factors, such as presence of other organisms, pH, available nutrients, and temperature (Katoch et al., 2019; Song et al., 2007; Tamandegani et al., 2020). In fact, some argue that slight alterations to the environmental conditions may lead to significantly different metabolomic profiles (Katoch et al., 2019) and even that some compounds are impossible to reproduce under artificial conditions (Zeilinger et al., 2016). In addition, the purification of peptaibiotics is not trivial because, firstly, they are secreted as a part of micro-heterogeneous mixtures. Secondly, the production of peptaibiotics needs urgent optimization to be fully-scalable on low-cost systems (Katoch et al., 2019). Due to the richness in non-coding amino acids, production of peptaibiotics through genetic engineering of plants, or other model organisms, as is routine for many other metabolites of interest (Kulshreshtha et al., 2022), is not a feasible option yet (Adhikari et al., 2021).

6.2. Solving solubility issues while increasing potency and selectivity

Production limitations have pushed the development of novel solutions for the fully exploitation of the potential of bioactive peptaibiotics. De Zotti et al. (2020) engineered synthetic analogues of the short-length lipopeptaibol trichogin with increased solubility in aqueous media. The authors aimed at obtaining a synthetic product that could be incorporated into water-based formulations while minimizing environmental contaminants and enhancing reproducibility. Strikingly, despite trichogin showing no activity against any of the trailed pathogens, three of the developed analogues completely inhibited fungal growth at micromolar concentrations *in vitro* and significantly reduced disease symptoms of *B. cinerea in planta* without any phytotoxic effects (De Zotti et al., 2020). This was pushed farther in following works (Baccelli et al., 2022; Bolzonello et al., 2023; Sella et al., 2021). Moreover, chemical synthesis allows for bioactive molecules to be tailored for the target organism and to have improved stability (Fig. 6), while affording crude products that are generally of easier purification than natural extracts. As advanced by Dam et al. (2018), modifying the *N*- and *C*-termini of peptaibols may provide analogues with improved selectivity for pathogens over mammalian cells.

While chemical synthesis may indeed help to overcome many of the limitations of natural peptaibiotics, particularly their hydrophobicity and cytotoxicity, it remains an expensive, highly technical approach. For example, the synthesis of 100 mg of a 11-mer peptaibol could cost anywhere from 2000 to 4000 USD. Longer molecules or tougher-to-synthesize modifications will significantly drive the price up. While replacement of the *C*-terminal alcohol for an amide is a simpler and cheaper option reported to deliver synthetic peptaibiotics with interesting properties (De Zotti et al., 2020), it is not enough to bring the price down considerably. Hence, chemical synthesis offers high-quality products but still at a forbidden price that makes its real implementation currently unfeasible in most of the world. Recently, modification of active pharmaceutical ingredients and AMPs with ionic liquids (IL) has been proposed as a cost-effective way to improve the solubility and/or enzymatic activity of the parent bioactive compounds, while preserving or even enhancing their antimicrobial activity (Ferraz et al., 2017; Gomes et al., 2020). A similar strategy might be applied to peptaibiotics, whereby their conjugation to an IL could suffice to overcome solubility issues. This still underexploited approach is worthy to be pursued soon, although the cost to produce the peptaibiotics would likely remain an issue.

6.3. Lab-produced peptaibiotics' extracts

Regarding the *in vitro* production of peptaibiotics, researchers have also made significant progress, especially for *Trichoderma* isolates (Abdullah et al., 2021; Katoch et al., 2019). Preparative purifications, such as liquid or solid-state fermentation, could address the production bottleneck at a cheaper cost (Song et al., 2007). However, to become a truly scalable solution, both yield and purification efficiency of peptaibiotics must increase significantly. In fact, low yields have been a main hinderance to compound isolation and purification (Katoch et al., 2019). Despite this being usually regarded as the cheap approach, the production of 100 mg of peptaibiotics extract will most certainly achieve prices above those of chemical synthesis, at least for now, while the production protocols are scarce and widely variable. Abdullah et al. (2021) have debated that the industrial production of *Trichoderma* should first focus on the optimization of the culture process at a laboratory scale, to increase yield and active biomass, and then scale up to small-plant level to adapt the process to the engineering variables. Because this has yet to be achieved, the average price per batch is considerably high. After this point, integration of fermentation, bioseparation, and formulation into a single process and industrial plant-scale production are the steps that follow, bringing the average prices down (Fig. 6). For that, it is of paramount importance to have available knowledge on the biosynthetic machinery and the biotic and abiotic factors triggering peptaibiotics production. In that sense, fungal genomics has recently demonstrated that gene clusters regulating the secondary metabolism are mostly transcriptionally silent under laboratorial conditions. In the wild, many of these clusters require external stimuli to be expressed (Zeilinger et al., 2016). To do it under controlled conditions, researchers are making use of co-cultivation, epigenetics, and transcript regulation, among other techniques (Fig. 6).

Studies on *T. lixii* revealed a multi-fold increase in production of 11-residue peptaibols when the strain was grown on Rose Bengal medium

(Katoch et al., 2019). The same authors reported that different media resulted in different molecules being favored or negatively regulated. Hence, it is preferable to optimize the conditions towards a desired metabolite which will, in turn, reduce the heterogenicity of the extract and increase purification yields (Katoch et al., 2019). On a different study, solid-state fermentation was used for the production of trichokonin VI (Song et al., 2007). Parameters such as inoculum size, incubation temperature, initial moisture content, and initial pH significantly affected its production. In fact, the authors were able to highlight that: i) the interaction between temperature and moisture content was relevant, ii) maximum production could be determined as a function of the inoculum size and temperature interaction, and iii) there is a measurable optimum moisture content and pH for trichokonin VI production (Song et al., 2007). In addition, authors observed higher yields and purity when using ethanol as extraction solvent, totaling 3.5 mg of trichokonin VI with 1 L culture broth in 28 days.

Alfaro-Vargas et al. (2022) also attempted at optimizing the fungal growth conditions to increase peptaibiotics production through supplementation with different carbon sources, elicitors, and amino acids. The authors observed that sucrose significantly increased the production of peptaibiotics while the biomass was reduced. This has many advantages from a production point of view. Less biomass with higher peptide content means easier purification processes and thus higher productivity and possibly an easily scalable fermentation process. The independent supplementation with the amino acids Aib, Val and Pro significantly increased the peptaibol production. This effect was particularly significant for Aib (Alfaro-Vargas et al., 2022). Similar results had been reported by Leclerc et al. (1998) for *T. longibrachiatum* and *T. harzianum*. These authors reported that supplementation with either Aib, Glu or Arg led to the simplification of the peptide mixture, often towards a single compound. The supplementation with the charged amino acid Glu led to the biosynthesis of acidic longibrachins, which has a Glu residue at position 18, instead of the neutral isoform with a Gln in the sequence (Leclerc et al., 1998). Komon-Zelazowska et al. (2007) reported that starvation-induced sporulation could trigger peptaibol synthesis for *H. atroviridis*.

Greater production of peptaibiotics was also achieved when phytopathogen's cell debris (e.g., *C. gloeosporioides*, *F. oxysporum* or *B. cinerea*) were added to the growth medium, especially with *F. oxysporum* (Alfaro-Vargas et al., 2022). This behavior was also observed for *T. asperellum* (Tamandegani et al., 2020). Also, research has unveiled that the host or antagonized species could have a negative impact on the biosynthesis of some compounds while increasing that of others. For instance, *T. aggressivum* f. *europaeum* increased the production of Pept-1717-a-1 during the confrontation with *P. ostreatus* while the amounts of Pept-1733- a-1, Pept- 1717-a-2 and Pept-1745-a-2 were significantly lowered in consequence of the same interaction. A similar trend was observed in the same work with *T. pleuroti*, for which higher levels of tripleurin VIIIb were detected during confrontation with both *A. bisporus* and *P. ostreatus* while a significantly decreased production was observed for tripleurins Ib and XIIb (Marik et al., 2017). Similarly, Tamandegani et al. (2020) observed that the production of longibrachin AII was significantly decreased during the interactions with *F. culmorum* and *R. solani*.

6.4. Using genetic engineering to create hyperproducing strains

Genetic engineering approaches have been used to increase peptaibiotics production by targeting different points of the metabolic pathway (Fig. 6). Zhou et al. (2019) were able to increase production of trichokonins A and B by 5- and 2.6-fold, respectively, through the deletion of *T. longibrachiatum* glucose sensor orthologue *Tlstp1*. This resulted in the impaired growth of both hyphal and conidia alongside the increase of transcriptional levels of the two NRPS encoding genes, *tlx1* and *tlx2*, and other fungal transcription factors and putative chromatin modifiers. Likewise, overexpression of the *P. lilacinum* transcription factor *lcs*F increased the production of leucinostatins A and B by 1.5-fold compared to the wild-type strain by upregulating the entire gene cluster controlling leucinostatins biosynthesis, which includes three NRPS encoding genes, translating into higher bioactive ability (Wang et al., 2016). The impairment of the *tmk1* gene, encoding a mitogen-activated protein kinase (MAPK), led to abnormal fungal development and reduced mycoparasitic activity of *T. atroviride*. Strikingly, the defective mutants showed improved bioactivity due to increased production of 6-pentyl-α-pyrone and peptaibols (Reithner et al., 2007). Finally, the formation of atroviridins has been linked to light-induced sporulation of *H. atroviridis* (Komon-Zelazowska et al., 2007). Specifically, researchers were able to associate this behavior to the blue light regulator genes, *blr1* and *blr2*, and to Gα-protein Gna3. The latter is a negative sporulation regulator that positively controls peptaibols' production (Komon-Zelazowska et al., 2007). Through constitutive overexpression of the methyltransferase *Tllae1*, a global regulator involved in the biosynthesis of secondary metabolites and in the regulation of the expression of cellulases and polysaccharide hydrolases in filamentous fungi, researchers were able to increase trichokonins A and B yields in *T. longibrachiatum* by 2-fold (Shi et al., 2020).

The mentioned works are proof that optimization of production with simple tweaks is feasible and the scale-up of this process towards industrial production should come naturally after laboratorial optimization and implementation. Despite all that, the regulation of peptaibol biosynthetic pathways and its epigenetics are still largely unknown. Further studies directed at this should provide the keys for developing peptaibol-hyperproducing strains through genetic engineering in combination with fermentation protocols.

7. Peptaibiotics databases

Thousands of naturally occurring molecules have been identified from a myriad of different source organisms for the development of antibiotics against clinical, animal and crop pathogens (Huan et al., 2020). This information would be impossible to manage if not for the creation of dedicated databases. These are designed to hold specific details regarding a molecule, or group of molecules, enabling researchers to efficiently find relevant information to their works (Ramazi et al., 2022). The advancement of computational technology has even incorporated into these databases the ability to mine and predict *in silico* molecular structures enabling the design of novel peptides with enhanced properties (Rush et al., 2021). Coupled with chemical synthesis, this means that researchers have now the ability not only to replicate virtually any natural peptide, but also to synthesize molecules *de novo* (Huan et al., 2020; Ramazi et al., 2022) (Fig. 6).

The antimicrobial peptide databases are usually classified as general or specific, and while some molecules may be found in more than one, there are still no databases encompassing all of them (Ramazi et al., 2022). Likewise, phytopathogenic-specific databases are yet to be developed, although information regarding this type of trial can be found within the existing databases with the handicap of having the information scattered.

Alamethicin was the first discovered peptaibol (Leitgeb et al., 2007). Since then, a plethora of peptaibiotics has been identified. The most recent estimations indicate that hundreds of peptaibiotics (including natural and synthetic) have been identified (Zhao et al., 2019). Peptaibiotics-specific databases were first introduced as a response to the lack of information in the protein databases due to their peculiar composition (i.e., non-standard amino acids) and short lengths. Currently, peptaibiotics sequences can be retrieved from multiple platforms, although these may present data in different ways as a result of different modes of handling with the non-standard sequences (Whitmore and Wallace, 2004).

The Peptaibol Database was created to store sequence and structural information on peptaibols in a consistent formatting scheme (Whitmore and Wallace, 2004). Information relative to sequence, 3D structure,

biological source and bibliographical data can be found for naturally occurring peptaibols. The database can be queried for name, group, sequence motif, biological origin, and reference. At the time of writing, the site holds information on 317 unique sequences and is one of the most cited peptaibol-related resources.

The Peptaibiotics Database is a specific database for the Aib-containing, fungal NRPs (Neumann et al., 2015). Currently, this database can only be accessed offline through the installation of the original version. At the time of the last update, it held more than 1,000 unique peptaibiotics sequences collected from the literature. It was designed so researchers could upload sequences directly and help maintain the database by eliminating duplicates or correcting already existing entries. Like The Peptaibol Database, it allows for complex queries.

Norine is a specific platform storing information on natural NRPs (Flissi et al., 2020). It holds information on 177 peptaibols. It allows for complex queries combining the aforementioned parameters with Boolean operators. The database has been steadily updated and improved since its creation, allowing for the extraction of new annotations from external databases and automatic validation of those entries. Also, tools to analyze the chemical structures and infer the molecular formulae have been incorporated into the platform.

The Database of Antimicrobial Activity and Structure of Peptides (DBAASP) is a general database containing information on more than 18,000 entries (Pirtskhalava et al., 2021). This database is manually curated from published peer-reviewed articles regarding sequence, *C*- and *N*-terminal modifications, incorporation of unusual amino acids and/or post-translational modifications, antimicrobial/anticancer activity, and cytotoxicity. DBAASP enables the development of models for *de novo* design of peptides. Currently, it holds information on 343 peptaibols of both natural and synthetic origins along with their bioactivity, including against phytopathogens.

There are many more databases which may represent a valuable source of information on peptaibiotics. Many have incorporated different tools to increase the speed and accuracy of searches. In this regard, please refer to the comprehensive review by Ramazi et al. (2022). The characterization of peptaibiotics is time-consuming and expensive. Hence, databases should aim at helping to identify the best candidates, even before stepping into the wet-lab procedures, using *in silico* prediction models (Balázs et al., 2023; Rush et al., 2021) (Fig. 6).

8. Concluding remarks

Peptaibiotics have a tremendous potential to control phytopathogens, ensuring food security, without increasing anthropogenic pressure on the environment. However, for this to become a reality, it is imperative to make efforts on different fronts. Most data have been published during a period when phylogenetics was not recognized as essential to fungal taxonomy. As a result, many peptaibiotics cannot be linked to a source organism in public collections (Neuhof et al., 2007), and most of the producers reported in literature have never been deposited appropriately (Röhrich et al., 2014; Rush et al., 2021). The efforts devoted to investigate new strains and compounds must be increased and protocols should be updated to state-of-the-art methodologies to increase reliability. Databases and omics can help reduce the load by identifying the best candidates using *in silico* prediction models. Moreover, non-membrane modes of action are still poorly studied (Kai et al., 2018; Sella et al., 2021; Zhao et al., 2018). More works should address this topic. Likewise, genomics and genetics of peptaibiotic-producing fungi should play a role towards the understanding of the regulation of peptaibols biosynthesis. This would have a huge impact in increasing yields of large-scale production of peptaibiotics. Genetic engineering coupled with fermentation techniques, along with the chemical synthesis, could have a meaningful impact on the cost and wide adoption of these small and unique peptides in agriculture. Progress should be made towards the optimization of chemical synthesis, so tailored molecules, with enhanced properties, can be readily available at an affordable price. If these conditions are met, peptaibiotics could become an effective, ultra-specific, biodegradable tool for phytopathogens control in both conventional and organic production models.

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Leandro Pereira-Dias: Writing – original draft, Writing – review & editing, Supervision. **Paulo R. Oliveira-Pinto:** Writing – review & editing. **Juliana O. Fernandes:** Writing – review & editing. **Laura Regalado:** Writing – review & editing. **Rafael Mendes:** Writing – review & editing. **Cátia Teixeira:** Conceptualization, Writing – review & editing. **Nuno Mariz-Ponte:** Conceptualization. **Paula Gomes:** Writing – review & editing. **Conceição Santos:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

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