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Isolation, Structural Assignment and Synthesis of N-(2-Methyl-3-Oxodecanoyl)-2-Pyrroline, a New Natural Product from *Penicillium brevicompactum* with *in vivo* Anti-Juvenile Hormone Activity

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Introduction

One of the most important and challenging aspects of pesticide research is the urgent need to develop new and effective methods of controlling plagues with no harm to human health and environment and which are accepted as safe by the general public[1]. Natural products, with their tremendous structural diversity, are an important source of new alternatives. Many natural products showing fungicidal, bactericidal or insecticidal activities are isolated every year. If their properties allow, and if sufficient quantities can be obtained from natural sources or by synthesis, such compounds may be used as agricultural chemicals. Alternatively, they may constitute useful starting points as lead molecules for the synthesis of analogues with improved biological and physical properties[2].

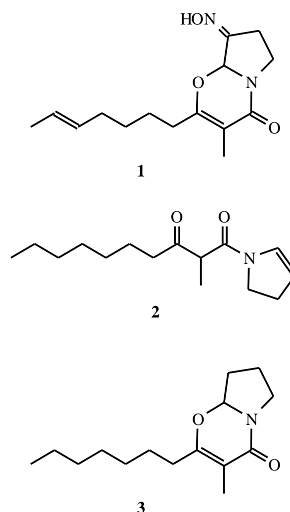
In recent times, investigations of several research groups have shown that one of the most important sources of bioactive compounds are fungi. The secondary metabolites of fungal origin exhibit a wide range of potentially useful biological activities[3,4].

Penicillium is one of the genera, together with *Aspergillium* and *Fusarium*, producing metabolites known to be toxic to insects[5]. Particularly, the fungus *Penicillium brevicompactum* Dierckx has been described as one of the most prolific producers of secondary metabolites. These include mycophenolic acid and related compounds[6], the Raistrick phenols[7-9], the pebrolides[10] or the N-benzoyl derivatives of phenylalanine, phenylalaninol and their ester, asperphenamate[11]. In addition, the fungus also produces brevigellina[12], several piperazine-2,5-dione derivatives, a drimane diterpenoid[13], the brevianamides[14,15] and compactin[16]. The latter is a reported hypocholesterolaemic agent that was shown to be a reversible, competitive inhibitor of 3-hydroxy-3-methylglutaryl-

coenzime A reductase (HMG-CoA reductase). Previous work on the effects of this product on insects has shown that it is able to produce a potent *in vitro* JH biosynthesis inhibition, with IC_{50} of the order of 10^{-7}-10^{-9} M in lepidoptera[17,18] and dictioptera[19,20]. However, as a general rule, the morphological effects of compactin are scarce.

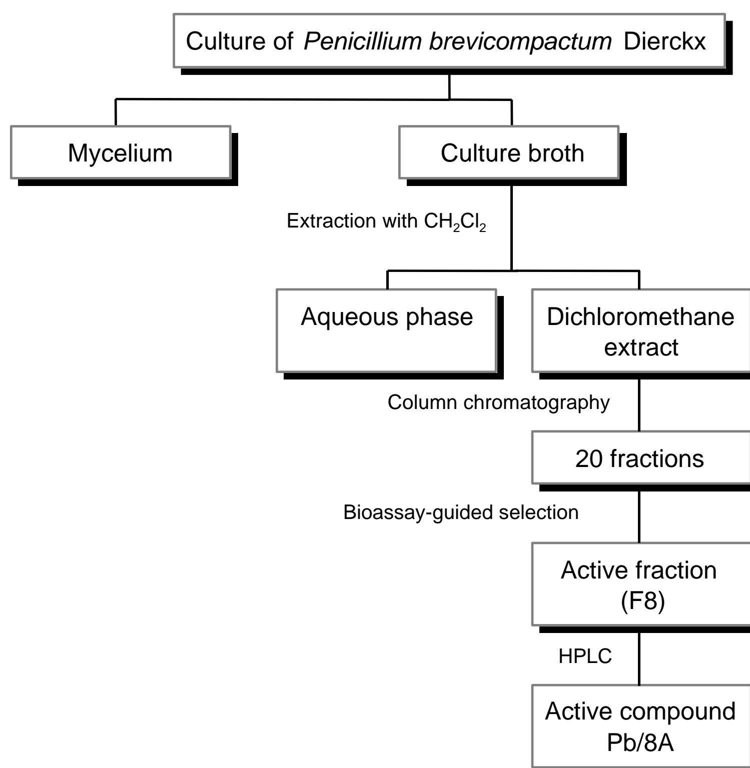
Recently we have reported the isolation and identification of brevioxime (**1**), a new metabolite from *P. brevicompactum,* which exhibits a very high activity as JH biosynthesis inhibitor[21]. Its chemical structure contains an unusual heterobicyclic skeleton and an oxime funtionality.

In this paper we report the isolation, identification and alternative synthesis of a new natural product (**2**) from *P. brevicompactum*, with a high *in vivo* anti-JH activity. Although at first sight the structures of **1** and **2** are unrelated, chemical studies have shown that **2** can be converted into the bicyclic isomer **3** upon acid catalysis. As the basic skeletons of **1** and **3** are the same, it appears that the new natural product **2** can be biogenetically related to brevioxime.



Results and Discussion

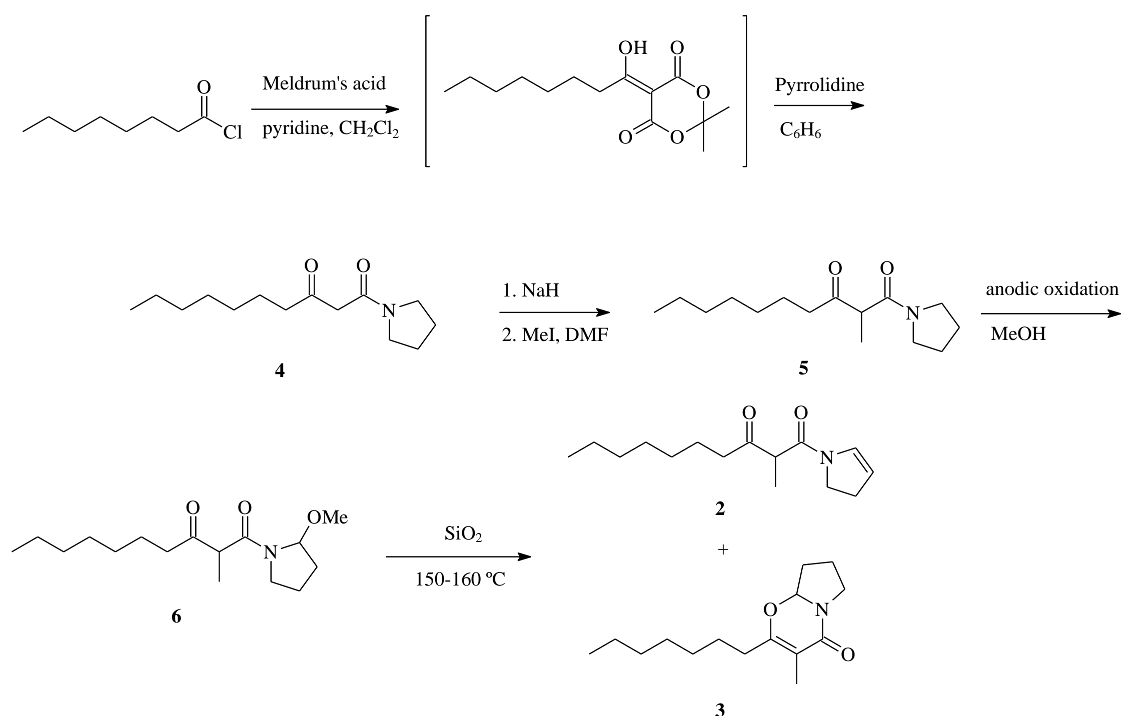
A systematic screening was performed with 118 strains of *Penicillium* isolated from fungal contamination of cereals. The most promising results were obtained with a strain of *P. brevicompactum*. A summary of the procedure followed to isolate the active compound is illustrated in Scheme 1. The dichloromethane extract obtained from the culture medium exhibited the highest entomotoxicity and anti-JH activity to *Oncopeltus fasciatus* (20 % mortality and 40 % precocious adults at 10 μg/cm^2, following the method of Bowers et al.[22]). In addition, the extract showed an important fungicidal activity against *Colletotrichum gloesporoides*, *Alternaria tenuis*, *Fusarium culmorum* and *Trichoderma viride* at 500 μg/mL.



Scheme 1. Isolation and purification of the anti-JH compound **2** from *Penicillium brevicompactum*

The entomotoxicity and anti-HJ activity bioassay served as guide for silica-gel column separation, which led to a fraction (F8) with remarkable *in vivo* anti-JH activity. Preparative HPLC of this fraction allowed isolation of the active pure compound Pb/8A. Its structure was tentatively assigned, by means of combined spectral data, as N-(2-methyl-3-oxodecanoyl)-2-pyrroline (**2**).

In order to confirm the structure and to prepare higher amounts of compound for further biological assays, we designed an alternative synthesis based on the approach outlined in Scheme 2. If successful, slight modifications of this approach could allow to synthesize a number of related intermediates and/or derivatives with enhanced biological activities.



Scheme 2. Synthesis of the natural ketoamide **2**

The synthetic scheme involves the use of commercial starting materials such as pyrrolidine and octanoyl chloride. The first step was acylation[23,24] of Meldrum's acid[25,26] with octanoyl chloride. This treatment gave an intermediate, which was submitted to aminolysis without previous purification, by reaction with pyrrolidine in

refluxing benzene [27]. This led to the β-keto amide **4** in 61 % overall yield from octanoyl chloride.

The next step was introduction of a methyl group[28,29] between both carbonyls. Thus, after using NaH to generate the carbanion, methylation was achieved by treatment with iodomethane. The desired alkylated β-keto amide **5** was obtained in 79 % yield.

In order to achieve the pyrrolidine to pyrroline conversion of **5**, a methoxy group was introduced at C_2 by means of anodic oxidation, following the method previously described by Shono[30-35]. A methanolic solution of β-keto amide **5** was subjected to a constant electric current of 20 mA, in the presence of tetrabutylammonium *p*-toluenesulfonate as a supporting electrolyte, until 3.7 F/mol had passed through the solution. In this way, the methoxylated β-keto amide (**6**) was obtained in 45 % yield, and 50 % of starting material was recovered. The two diasteromers of **6** (**a** and **b**) were resolved by column chromatography. Both of them were present in solution as a mixture of the two posible amide conformations, which gave separate signals in NMR.

Finally, elimination of methanol was carried out by adsorption of **6** on SiO_2 and subsequent heating at 150-160 ºC[31,36-40]. Under these conditions, a 1:1 mixture of the desired pyrroline **2**, and the isomeric bicyclic product **3** was obtained.

Insecticidal, anti-JH and antimicrobial activities of the natural product **2**, as well as the bicyclic isomer **3** and the synthetic intermediates **4-6**, have been evaluated. Table 1 contains the relevant data for the compounds showing activity against the milkweed bug *O. fasciatus*. The natural product (**2**) exhibited an important antagonistic JH activity that induced precocious metamorphosis. The effects of this product on treated nymphs were of the same type as those described for the precocenes[22,41]. In addition, **2** has been shown to be a true anti-JH agent according to Staal,[42] because its coadministration with

a juvenoid, (methoprene) is able to reverse the action. Experiments are under way to determine the mechanism of action of this compound. On the other hand, two intermediates in the synthesis of **2**, compounds **4** and **5**, have shown a strong knockdown toxicity to *O. fasciatus* (Table 1). Compound **5** was more active than **4**, clearly suggesting that introduction of a methyl group in the molecule enhances the entomotoxicity.

Table 2 shows fungicidal activities of the compounds. In all cases, minimun inhibitory concentration (MIC) values were superior to 100 μg/mL, so none of the compounds were strongly effective in inhibiting the growth of the tested microorganisms. However, it could be of some interest the selective activity exhibited by compounds **2**, **4** and **6** on *Colletotrichum* genus. Under certain conditions, a selective fungicide may be very useful for controlling a particular microorganism; thus, these compounds could be used as lead molecules for the synthesis of analogues with improved fungicidal activity against *Colletotrichum*, a very important phytopathogen.

Bactericidal activities have been determined against six selected Gram-positive and Gram-negative bacteria. Only compound **3** showed a moderate activity on *Bacillus cereus*, with an inhibition zone of 11 mm at the dose of 20 μg/mm^2.

Conclusion

N-(2-methyl-3-oxodecanoyl)-2-pyrroline (**2**), a new natural product, has been isolated from *Penicillium brevicompactum*. Its structure has been tentatively assigned based on spectral data and unambiguously confirmed by alternative synthesis. Compound **2** and its bicyclic isomer **3** arise from a common precursor (**6**) in the

synthetic sequence; this reveals that **2** can be biogenetically related to the recently discovered brevioxime (**1**). The biological activities of **2** and **3** and their synthetic intermediates **4-6** suggest that these compounds can be useful as lead molecules for the development of new biorational pesticides.

Experimental Section

All chemicals were obtained from commercial suppliers and used without further purification. IR spectra were obtanined as liquid films (or KBr plate for the natural product); ν_{max} is given for the main absorption bands. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz (or 400 and 100 MHz for the natural product), respectively, in $CDCl_3$ solvent; chemical shifts are reported in δ (ppm) values, using TMS as internal standard. Mass spectra were obtained under electron impact (or chemical ionization for the natural product); the ratios *m/z* and the relative intensities are reported. Isolation and purification were done by flash column chromatography on silica gel 60 (230-400 mesh). Analytical TLC was carried out on precoated plates (silica gel 60 F_{254}), and spots were visualized with UV light and in a I_2 chamber.

Isolation and characterization of the active compound. The procedure was similar to that previously reported for brevioxime[21]. Briefly, the fungus was isolated in our laboratories and classified by The International Mycological Institute (IMI, Surrey, UK) as *Penicillium brevicompactum* Dierckx. A sample of the strain is filed in the "Colección de Cultivos de la Cátedra de Microbiología" of the Department of Biotechnology (Universidad Politécnica de Valencia). It is codified as P79 and kept in agar slants with potato dextrose agar (PDA) as culture medium.

The strain was seeded in Petri dishes with PDA culture medium and incubated for 7 days at 28 °C. Then, sterile distilled water with Tween 80 (0.05 %) was used to obtain a suspension containing ca. 10^6 conidia/mL. This suspension was added to an erlenmeyer flask with antibiotic test broth (1:9 volume ratio) and the mixture was incubated for 15 days, in the dark, at 28 °C.

After incubation, the culture medium was extracted three times with CH_2Cl_2 (1:3, v/v). The resulting extract was dried over $CaCl_2$, filtered and evaporated in vacuo. The residue (2.0 g from 20 L of culture) was submitted to column chromatography on silica-gel (1:60, w/w) using mixtures of CH_2Cl_2, AcOEt, Me_2CO and MeOH (stepwise gradient) as eluent. This led to the separation of 20 fractions. Using the method of Bowers et al[22] (see below) it was possible to localize a significant biological activity in fraction number 8, whose yield was 125.6 mg.

Preparative HPLC chromatographic resolution of fraction 8 was achieved using the following conditions: column Lichrosorb Si-60, 7 μm (25.0 x 2.5 cm); mobile phase CH_2Cl_2:AcOEt (70:30, v/v); flow 8mL/min; detection by UV (254 nm) and refraction index, simultaneously. A fraction was obtained (rt: 24.9 min) consisting in 12.6 mg of the pure active compound, whose structure was tentatively assigned to be N-(2-methyl-3-oxodecanoyl)-2-pyrroline on the basis of spectral data. $[\alpha]^{20}_D$= 27° (c 0.07, $CHCl_3$); HRMS: m/z 251.1888 ($C_{15}H_{25}NO_2$ requires 251.1885); IR: ν_{max} 2952, 2924, 2855, 1718, 1642, 1610, 1457, and 1419; ^{1}H-NMR: δ_H 6.9 and 6.6 (m+m, 1H, H-2), 5.3 (m, 1H, H-3), 3.9 (m, 2H, H-5), 3.6 and 3.5 (q+q, *J*= 7 Hz, 1H, H-2'), 2.8-2.4 (m, 4H, H-4'+H-4), 1.6 (m, 2H, H-5'), 1.4 (d, *J*= 7 Hz, 3H, $CHCH_3$), 1.2 (m, 8H, $(CH_2)_4CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_2CH_3); ^{13}C-NMR: δ_C 207.2 ($C_{3'}$), 165.6 ($C_{1'}$), 129.3 and 128.3 (C_2), 113.1 and 111.6 (C_3), 53.3 ($C_{2'}$), 45.5 (C_5), 39.3 ($C_{4'}$), 31.7, 29.0, 28.1, 23.5, 22.6

(C_4, $C_{5'}$-$C_{9'}$), 14.1 and 13.1 ($2xCH_3$); MS: m/z 251 (M^+, 2), 167 (1), 126 (5), 125 (6), 96 (4), 70 (6), 69 (100), 68 (20), 57 (10), 55 (4) and 41 (3).

Synthesis of N-(2-methyl-3-oxodecanoyl)-2-pyrroline.

N-(3-oxodecanoyl)pyrrolidine (4). To a cooled solution (0 ºC) of 2,2-dimethyl-1,3-dioxane-4,6-dione (5.43 g, 36.9 mmol) in dichloromethane (35.0 mL), were added pyridine (6.0 mL, 74.2 mmol) and octanoyl chloride (5.2 mL, 30.7 mmol) *via* syringe, dropwise, under nitrogen. The solution was stirred at 0 ºC for 1 h 25 min, after which it was allowed to warm to room temperature for an additional period of 2 h 10 min. The dichloromethane solution was washed with dilute HCl, water and brine, dried and concentrated to dryness, to give the acylated Meldrum's acid, which was used for the aminolysis without further purification.

A solution of the acylated Meldrum's acid and pyrrolidine (5.7 mL, 68.9 mmol) in benzene (180.0 mL) was refluxed for 12 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (gradient elution with mixtures of EtOAc and hexane, between 30 and 40 % of the former) to provide the β-keto amide **4** (4.50 g, 61 %) as a brown oil. HRMS: m/z 239.1891 ($C_{14}H_{25}NO_2$ requires 239.1885); IR: ν_{max} 2940, 2920, 2840, 1710, 1630, 1450, 1420, 1360, 1330, 1295, 1250, 1220, 1190, 1160, 1105, 910, 860, 780 and 720; ^{1}H-NMR: δ_H 3.5 (s, 2H, H-2'), 3.5-3.4 (m, 4H, H-2+H-5), 2.6 (t, *J*= 8 Hz, 2H, H-4'), 2.0-1.9 (m, 4H, H-3+H-4), 1.6 (m, 2H, H-5'), 1.3 (br s, 8H, $(CH_2)_4CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_3); ^{13}C-NMR: δ_C 204.6 ($C_{3'}$), 171.8 ($C_{1'}$), 50.3 ($C_{2'}$), 47.0 (C_2), 45.7 (C_5), 43.0 ($C_{4'}$), 31.5, 28.9, 25.8, 24.2, 23.3, 22.4 (C_5, C_4, $C_{5'}$-$C_{9'}$) and 13.9 ($C_{10'}$); MS: m/z 239 (M^+, 23), 222 (3), 210 (5), 197 (10), 196 (5), 182 (6), 168 (53), 155 (67), 140 (32), 127 (4), 113 (85), 112 (100), 98 (80), 85 (43), 72 (41), 71 (56), 70 (96), 69 (31), 57 (27), 56 (28), 55 (50) and 43 (45).

N-(2-methyl-3-oxodecanoyl)pyrrolidine (5). To a stirred slurry of pre-washed NaH (60% dispersion oil; 0.79 g, 19.8 mmol) in DMF (30.0 mL) at 0 ºC was added a solution of the β-keto amide **4** (4.50 g, 18.8 mmol) in DMF (5.0 mL), *via* double ended needle, dropwise. After hydrogen evolution had ceased, the mixture was warmed to room temperature, stirred for 1 h 30 min and then re-cooled to 0 ºC. Then iodomethane (1.3 mL, 21.7 mmol) was added. After being stirred at room temperature for 3 h 10 min the mixture was diluted with water and extracted with CH_2Cl_2. The combined extracts were washed with brine, dried and concentrated to dryness. Chromatography of the residue on silica gel (gradient elution with mixtures of EtOAc and hexane, between 20 and 30 % of the former) provided the β-keto amide 5 (3.74 g, 79%) as a yellow oil. HRMS: m/z 253.2050 ($C_{15}H_{27}NO_2$ requires 253.2041) ;IR: ν_{max} 2930, 2900, 2840, 1710, 1630, 1445, 1420, 1360 and 1330; ^{1}H-NMR: δ_H 3.6-3.4 (m, 5H, H-2+H-5+H-2'), 2.5 (m, 2H, H-4'), 2.1-1.8 (m, 4H, H-3+H-4), 1.6 (m, 2H, H-5'), 1.4 (d, *J*= 7 Hz, 3H, CHCH_3), 1.3 (br s, 8H, $(CH_2)_4CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_2CH_3); ^{13}C-NMR: δ_C 207.1 ($C_{3'}$), 168.4 ($C_{1'}$), 52.8 ($C_{2'}$), 46.6 (C_2), 45.8 (C_5), 39.3 ($C_{4'}$), 31.4, 28.8, 25.8, 24.0, 23.2, 22.3 (C_3, C_4, $C_{5'}$-$C_{9'}$), 13.8 and 13.0 (2xCH_3); MS m/z 254 (M^++1, 2), 253 (M^+, 8), 207 (1), 182 (6), 169 (6), 154 (1), 127 (100), 126 (48), 112 (6), 99 (10), 98 (27), 71 (17), 70 (51), 57 (42), 55 (39) and 43 (39).

2-Methoxy-N-(2-methyl-3-oxodecanoyl)pyrrolidine (6). A solution of β-keto amide **5** (0.39 g, 1.6 mmol) in methanol (60.0 mL) containing tetrabutylammonium *p*-toluenesulfonate (1.84 g, 4.4 mmol) as a supporting electrolyte was placed into an electrolysis cell equipped with carbon electrodes (8.5 cm^2). A constant current (20 mA) were passed through the solution. After 3.7 F/mol of electricity were passed, the solvent was evaporated under reduced pressure. Water was added to the residue and the product

was extracted with CH_2Cl_2. The combined organic layer was dried over anhydrous sodium sulfate. Thereafter, the drying agent was removed by filtration, the solvent was evaporated to dryness and the residue was filtered through silica gel using OEtAc as a eluent, in order to eliminate the supporting electrolyte. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel, using a hexane/EtOAc mixture (75:25) as eluent, to afford two diastereomers of the methoxylated β-keto amide **6** (0.13 + 0.07, 45%) as a yellow oil and the unreacted β-keto amide **5** (0.20 g, 0.8 mmol).

Spectral data of the first eluted diastereomer **6a**. HRMS: m/z 283.2140 ($C_{16}H_{29}NO_3$ requires 283.2147); IR: ν_{max} 2910, 2840, 1710, 1650, 1450, 1405, 1360, 1170, 1075, 1060 and 810; ^{1}H-NMR: δ_H 5.5 and 5.0 (d+d, *J*= 4 Hz, 1H, H-2), 3.7-3.3 (m, 3H, H-5+H-2'), 3.4 and 3.3 (s+s, 3H, OMe), 2.5 (m, 2H, H-4'), 2.2-1.8 (m, 4H, H-3+H-4), 1.5 (m, 2H, H-5'), 1.4 and 1.3 (d+d, *J*= 7 Hz, 3H, CHC*H*$_3$), 1.3 (br s, 8H, (C*H*$_2$)$_4$CH$_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C-NMR: δ_C 206.9 ($C_{3'}$), 170.5 and 169.9 ($C_{1'}$), 88.5 and 87.2 (C_2), 56.6 and 54.0 ($C_{2'}$), 53.0 (OMe), 46.2 and 45.9 (C_5), 39.4 and 39.2 ($C_{4'}$), 31.5 and 31.3, 30.6, 29.0 and 28.9, 23.4, 22.7 and 22.5, 20.8 (C_3, C_4, $C_{5'}$-$C_{9'}$), 14.0 and 13.9, 13.0 (2xCH_3); MS: m/z 283 (M^+, 2), 268 (10), 253 (23), 252 (16), 251 (20), 199 (4), 183 (9), 167 (8), 156 (34), 127 (64), 126 (30), 125 (61), 100 (22), 97 (52), 85 (17), 70 (100), 69 (26), 57 (20) and 155 (16)

Spectral data for the second eluted diastereomer **6b**. HRMS: m/z 283.2146 ($C_{16}H_{29}NO_3$ requires 283.2147); IR: ν_{max} 2920, 2840, 1710, 1650, 1455, 1405, 1370, 1170, 1090, 1070 and 810; ^{1}H-NMR: δ_H 5.4 and 4.9 (d+d, *J*= 4 Hz, 1H, H-2), 3.8-3.2 (m, 3H, H-5+H-2'), 3.3 and 3.2 (s+s, 3H, OMe), 2.5 (m, 2H, H-4'), 2.2-1.8 (m, 4H, H-3+H-4), 1.5 (m, 2H, H-5'), 1.4 and 1.3 (d+d, *J*= 7 Hz, 3H, CHC*H*$_3$), 1.2 (br s, 8H,

(CH_2)$_4$$CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_2CH_3); ^{13}C-NMR: δ_C 206.5 ($C_{3'}$), 170.7 ($C_{1'}$), 88.7 and 87.3 (C_2), 56.5 and 53.6 ($C_{2'}$), 52.8 and 52.2 (OMe), 46.1 and 45.9 (C_5), 39.8 and 39.3 ($C_{4'}$), 31.5 and 31.2, 30.4, 29.0 and 28.9, 23.3, 22.7 and 22.4, 20.9 (C_3, C_4, $C_{5'}$-$C_{9'}$), 13.9 and 13.8, 14.0 (2xCH_3); MS: m/z 283 (M^+, 1), 268 (26), 253 (15), 252 (12), 251 (12), 199 (1), 183 (13), 167 (4), 157 (13), 156 (12), 127 (32), 126 (21), 125 (46), 100 (35), 97 (57), 85 (23), 70 (100), 69 (25), 57 (22) and 55 (17).

N-(2-methyl-3-oxodecanoyl)2-pyrroline (2) and 2-heptyl-3-methyl-4-oxo-6,7,8,8a-tetrahydro-4H-pyrrolo[2,1-b]-1,3-oxazine (3). A mixture of the two diastereomeric α-methoxy-amides **6** (13 mg, 0.05 mmol) and silica gel (8 mg, 0.14 mmol) was heated at 150-160 ºC in a flask, under reduced pressure and nitrogen atmosphere. After 2 h 45 min, water was added to the residue and the slurry was extracted with CH_2Cl_2. The combined organic layer was dried over anhydrous sodium sulfate. Then, the drying agent was removed by filtration, the solvent was evaporated to dryness and the residue was purified by column chromatography on silica gel (gradient elution with mixtures of EtOAc and hexane between 15 and 20 % of the former) to provide, in order of elution, the enamine **2** (3 mg, 29%), the starting β-keto amide **6** (4 mg, 0.01 mmol) and the bicyclic enone **3** (4 mg, 37%).

The enamine **2** was a yellow oil which showed spectral data identical to those reported above for the natural product.

The enone **3** was a yellow oil. HRMS: m/z 251.1883 ($C_{15}H_{25}NO_2$ requires 251.1885); IR: ν_{max} 2920, 2840, 1720, 1655, 1425, 1370, 1345, 1080 and 760; ^{1}H-NMR: δ_H 5.2 (dd, *J*= 6 and 5 Hz, 1H, H-8a), 3.7 and 3.4 (m+m, 2H, H-6), 2.4-1.8 (m, 6H, H-7+H-8+H-1'), 1.8 (s, 3H, CH_3), 1.5 (m, 2H, H-2'), 1.3 (br s, 8H, (CH_2)$_4$$CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_2CH_3); ^{13}C-NMR: δ_C 168.2 (C_2), 163.8 (C_4), 106.4 (C_3), 87.5

(C_{8a}), 44.3 (C_6), 31.8, 31.7, 30.6, 29.3, 29.0, 26.8, 22.6, 21.9 (C_7, C_8, $C_{1'}$-$C_{6'}$), 14.1 and 10.1 (2xCH_3); MS: m/z 251 (M^+, 39), 250 (92), 223 (7), 210 (12), 183 (53), 166 (16) 152 (25), 141 (100), 140 (65), 139 (42), 127 (97), 126 (97), 113 (97), 112 (98), 111 (81), 98 (93), 97 (81), 83 (99) and 71 (94).

Biological assays.

Insects. *Oncopeltus fasciatus* Dallas were mantained at 28 ± 1 °C, 50-60 % relative humidity, 16h/8h (day/night) photoperiod and a diet based on sunflowers seeds.

Target Microorganisms. Fungicidal activity was measured against thirteen agronomically important phytopathogens. These strains were provided by the "Colección Española de Cultivos Tipo" (CECT) or by the "Coleccion de la Catedra de Microbiologia" (CCM) of the Department of Biotechnology (Universidad Politecnica de Valencia). *Aspergillus parasiticus* (CECT 2681), *Geotrichum candidum* (CCM 245), *Alternaria tenuis* (CECT 2662), *Colletotrichum gloesporoides* (CECT 2859), *Colletotrichum coccodes* (CCM 327), *Fusarium oxysporium* ssp *gladioli* (CCM 233), *Fusarium oxysporum* ssp *niveum* (CCM 259), *Fusarium culmorum* (CCM 172), *Penicillium italicum* (CECT 2294), *Trichoderma viride* (CECT 2423),*Trichothecium roseum* (CECT 2410), *Rosellinia necatrix* (CCM 297), *Verticillium dahliae* (CCM 269). Six different bacterial strains were used in order to determine bactericidal activity: *Staphylococcus aureus* (CECT 86), *Enterococcus faecalis* (CCM 12), *Salmonella typhi* (CECT 409), *Erwinia carotovora* (CECT 225), *Escherichia coli* (CECT 405) and *Bacillus cereus* (CECT 148).

Entomotoxicity and anti-JH activity. The test was carried out basically according to the contact method of Bowers et al.[22]. Briefly, 15 third-instar *O. fasciatus* nymphs were confined to a 9 cm Petri dish coated with 500 µg/cm^2 of the extract, or 100 µg/cm^2 in the case of fractions, being tested lower dosis for high activities. Products

were assayed at 10 μg/cm^2 and, in a parallel way, assays were performed by topical application on newly moulted 4rd-instar nymphs of *O. fasciatus*, at 10 μg/nymph. Toxicity effects were considered according to the number of insects dead after 72 h of exposure to the chemicals. All assays were made three times. The surviving nymphs were transferred to a 500 cm^3 glass flask and held at standard conditions. After metamorphosis occurred and reproduction was successful with the production of viable offsprings, the tests were finished. The tests were considered positive for JH antagonistic activity, either when precocious metamorphosis occurred or when sterility of the resulting adults was detected. Controls were run in parallel and received the same amount of acetone as treated insects.

Antifungal activity. The extract or products, dissolved in acetone, were added to PDA, in a concentration of 500 and 100 μg/mL, respectively. PDA plates containing only acetone were used as control plates. Seven days-old cultures of each fungus on PDA plates were used as an inoculum onto the control and test plates. The radial mycelial growth was measured and the percentage of inhibition was calculated on the basis of growth in control plates, after 4 days of incubation, at 28 °C, except to *R. necatrix* and *V. dahliae*, which were measured after an incubation period of 6 days. The antifungal activity of each sample was determined three times.

Antibacterial activity. Disk method: 15 mL of Mueller-Hinton agar on Petri dishes were inoculated with 1 mL of a 24 h old bacterial cultures in Nutrient broth. The optical density of the resulting solution was 0.2 at 700 nm. Whatman nº 113 paper disks (5 mm in diameter) were saturated with 100 μg/mm^2 of the fungi extract. Also control disks were saturated with acetone. After 24 h of incubation, bactericidal activity was measured and expressed as the width of the clear inhibition zones, in mm, including the disk. All assays were made three times.

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