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Synthesis and Biological Evaluation of New Analogues of the Active Fungal Metabolites *N*-(2-Methyl-3-oxodecanoyl)-2-pyrroline and *N*-(2-Methyl-3-oxodec-8-enoyl)-2-pyrroline.

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Running title: Bioactive analogues of fungal metabolites.

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ABSTRACT

In order to evaluate the effect of simplifying the β-ketoamide system present in active isolated metabolites from *Penicillium brevicompactum* (**2, 3**) on the activity, new analogues with a monocarbonylic amide functionality have been obtained. This way, the insecticidal and fungicidal activities have been improved in relation to the natural products taken as lead molecules. Thus, two of the synthetic analogues (**5a** and **5b**) showed very important insecticidal activities against third instar nymphs of *Oncopeltus fasciatus* Dallas, with acute LD_{50} values of 3.0 and 1.5 μg/cm^2, respectively. Moreover, some analogues showed good levels of fungicidal activity against a wide range of commercially important and taxonomically diverse fungi; remarkably, compound **7c** has proved to be highly active against *Colletotrichum gloesporoides* and *Colletotrichum coccodes*, with ED_{50} values of 2.04 and 11.7 μg/mL, respectively.

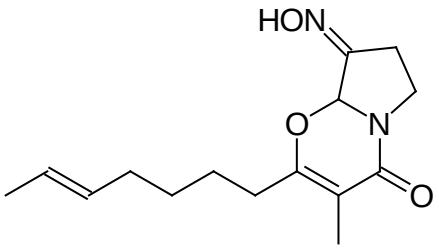
Keywords: Amide, fungicidal, insecticidal.

INTRODUCTION

The independent synthesis of bioactive natural products is often necessary to confirm their structures and activities as these compounds are secondary metabolites usually isolated in minimal quantities. Such work also leads to a series of synthetic intermediates, chemically related to the natural compounds, which are potentially active; sometimes these analogues are even more active than the isolated compound. Thus, active natural products can be used as lead molecules in order to design different derivatives with similar functionalities but with improved biological activities .

Recently, we have achieved the isolation and identification of a new family of bioactive metabolites from fungal extracts. Thus, brevioxime (**1**), isolated from *Penicillium brevicompactum*, exhibits a very high activity as JH biosynthesis inhibitor (Moya et al., 1997; Castillo et al., 1998). The related compounds, *N*-(2-methyl-3-oxodecanoyl)-2-pyrroline (**2**), *N*-(2-methyl-3-oxodec-6-enoyl)-2-pyrroline (**3**) and 2-hept-5-enyl-3-methyl-4-oxo-6,7,8,8a,-tetrahydro-*4H*-pyrrolo[2,1-b]-1,3-oxazine (**4**), isolated from the same source, show *in vivo* JH antagonistic and insecticidal activity (Moya et al., 1998; Cantín et al., 1999). Two inactive pyrrolic metabolites, presumably belonging to the same biosynthetic pathway, were used as starting point to obtain active analogues, upon introduction of simple structural changes (Cantín et al., 1998).

Now we wish to report the synthesis and biological activities of several derivatives of the enamides **2** and **3**, which were prepared as part of a programme aimed at improving the activities exhibited by these natural products.



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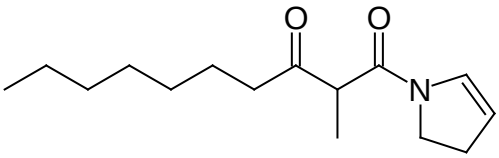

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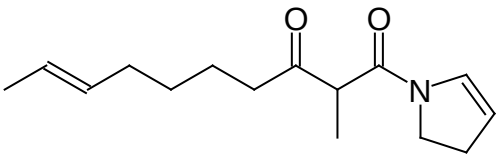

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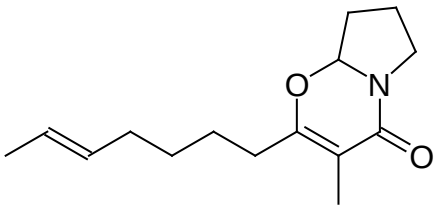

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MATERIALS AND METHODS

All chemicals were obtained from commercial suppliers and used without further purification. IR spectra were obtained as liquid films; ν_{max} is given for the main absorption bands. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, in $CDCl_3$ solvent; chemical shifts are reported in δ (ppm) values, using TMS as internal standard. The assignement of ^{13}C signals is supported by DEPT experiments. Mass spectra were obtained under electron impact or chemical ionization;

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.

General Synthetic Procedures

Synthesis of *N*-Acylpyrrolidines. To a mixture of pyrrolidine (14.1 mmol) with 1.7 M KOH (9.0 mL) was added a solution of acyl chloride (14.0 mmol) in CH_2Cl_2 (9.0 mL) dropwise (10 min). After being stirred at room temperature for 5 h 30 min, the mixture was extracted with CH_2Cl_2; the resulting organic extracts were washed with brine, dried over Na_2SO_4 and concentrated to dryness to give the *N*-acylpyrrolidine in a straightforward manner as oils.

***N*-Octanoylpyrrolidine (5a).** 91 % yield; obtained as an oil; HRMS (EI): m/z 197.1774 ($C_{12}H_{23}NO$ requires 197.1779); IR: ν_{max} 2900, 2860, 2840, 1605, 1410, 1330, 1230, 1160, 1090, 1030, 905 and 830; ^{1}H NMR: δ_H 3.4 (m, 4H, H-2+H-5), 2.2 (t, *J*= 7 Hz, 2H, H-2'), 2.0-1.8 (m, 4H, H-3+H-4), 1.6 (m, 2H, H-3'), 1.3 (br s, 8H, (CH_2)$_4$$CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_3); ^{13}C NMR: δ_C 171.8 ($C_{1'}$), 46.5 (C_2), 45.5 (C_5), 34.8 ($C_{2'}$), 31.6, 29.4, 29.0, 26.0, 24.9, 24.3, 22.5 (C_3, C_4, $C_{3'}$-$C_{7'}$) and 14.0 (CH_3); MS: m/z 197 (M^+, 11), 168 (12), 154 (9), 140 (13), 126 (73), 113 (100), 98 (46), 85 (56), 71 (72), 70 (80), 57 (30) and 55 (65).

***N*-Oct-6-enoylpyrrolidine (5b).** 85 % yield; obtained as an oil; HRMS (EI): m/z 195.1627 ($C_{12}H_{21}NO$ requires 195.1623); IR: ν_{max} 2910, 2845, 1640, 1430, 1330, 1250, 1220, 1190, 1160 and 960; ^{1}H NMR: δ_H 5.4 (m, 2H, H-6'+H-7'), 3.4 (m, 4H, H-2+H-5), 2.2 (t, *J*= 7 Hz, 2H, H-2'), 2.0-1.7 (m, 6H, H-3+H-4+H-5'), 1.6 (m, 5H, H-3'+H-8') and 1.4 (m, 2H, H-4'); ^{13}C NMR. δ_C 171.5 ($C_{1'}$), 130.9 ($C_{6'}$), 124.7 ($C_{7'}$), 46.4 (C_2), 45.4 (C_5), 34.5, 32.2, 29.2, 25.9, 24.2 (C_3, C_4, $C_{2'}$-$C_{5'}$) and 17.7 (CH_3); MS: m/z

195 (M^+, 87), 180 (6), 166 (12), 152 (7), 140 (30), 127 (95), 126 (57), 113 (58), 99 (36), 98 (65), 85 (42), 70 (100) and 55 (85).

***N*-[2-(3-Phenoxyphenyl)propionyl]pyrrolidine (5c).** 83 % yield; m.p. 80-83° C (from hexane); HRMS (EI): m/z 295.1577 ($C_{19}H_{21}NO_2$ requires 295.1572); IR: ν_{max} 3040, 2960, 2860, 1700, 1630, 1575, 1480, 1420, 1360, 1330, 1240, 1160, 1060, 1020, 950, 920, 750 and 695; ^{1}H NMR: δ_H 7.3 (m, 2H, H-3'''+H-5'''), 7.2 (t, *J*= 8 Hz, 1H, H-5''), 7.1 and 6.9 (m, 5H, H-2''+H-6''+H-2'''+H-4'''+H-6'''), 6.8 (ddd, *J*= 8, 3 and 1 Hz, 1H, H-4''), 3.7 (q, *J*= 7 Hz, 1H, H-2'), 3.5 and 3.4 (m+m, 4H, H-2+H-5), 1.8 (m, 4H, H-3+H-4) and 1.4 (d, *J*= 7 Hz, 3H, CH_3); ^{13}C NMR: δ_C 171.5 ($C_{1'}$), 157.1, 156.8, 143.4 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 129.7, 129.4, 122.9, 122.0, 118.4, 117.9, 116.6 ($C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 46.0 (C_2), 45.7 (C_5), 44.4 ($C_{2'}$), 25.7 (C_3), 23.8 (C_4) and 19.8 (CH_3); MS: m/z 295 (M^+, 81), 242 (5), 224 (3), 197 (18), 181 (4), 104 (8), 103 (7), 98 (100), 91 (7), 77 (10) and 55 (33).

Anodic Oxidation of *N*-Acylpyrrolidines. A solution of amide (1.6 mmol) in methanol (60.0 mL) containing tetrabutylammonium *p*-toluenesulfonate (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) was passed through the solution. After 4.0 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 ethyl acetate as 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, to afford the methoxylated amide.

2-Methoxy-*N*-octanoylpyrrolidine (6a) 45 % yield; obtained as an oil; HRMS (CI): m/z 228.1965 ($M+H^+$, $C_{13}H_{26}NO_2$ requires 228.1963); IR: ν_{max} 2910, 2840, 1650, 1410, 1350, 1330, 1240, 1160, 1110, 1040, 990, 910 and 830; ^{1}H NMR: δ_H 5.4 and 5.0 (d+d, *J*= 4 Hz, 1H, H-2), 3.6 (m, 2H, H-5), 3.4 and 3.3 (s+s, 3H, OMe), 2.3 (m, 2H, H-2'), 2.2-1.8 (m, 4H, H-3+H-4), 1.6 (m, 2H, H-3'), 1.3 (br s, 8H, $(CH_2)_4CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_3); ^{13}C NMR: δ_C 173.5 ($C_{1'}$), 88.7 and 86.9 (C_2), 56.4 and 53.9 (OMe), 46.1 and 45.4 (C_5), 34.6, 34.0, 31.6, 31.3, 30.9, 29.4, 29.3, 29.0, 25.1, 24.5, 22.9, 22.5, 21.0 (C_3, C_4, $C_{2'}$-$C_{7'}$) and 14.0 (CH_3); MS (CI): m/z 228 ($M+H^+$, 61), 214 (74), 195 (73), 184 (8), 180 (7), 173 (9), 142 (12), 129 (100), 113 (29) and 111 (85).

2-Methoxy-*N*-oct-6-enoylpyrrolidine (6b). 25 % yield; obtained as an oil; HRMS (CI): m/z 226.1813 ($M+H^+$, $C_{13}H_{24}NO_2$ requires 226.1807); IR: ν_{max} 2920, 2860, 1645, 1400, 1350, 1310, 1230, 1170, 1090, 1070, 1060, 960, 910, 810 and 720; ^{1}H NMR: δ_H 5.4 (m, 2H, H-6'+H-7'), 5.0 (d, *J*= 4 Hz, 1H, H-2), 3.6 (m, 2H, H-5), 3.4 and 3.3 (s+s, 3H, OMe), 2.3 (m, 2H, H-2'), 2.1-1.8 (m, 6H, H-3+H-4+H-5'), 1.6 (m, 5H, H-3'+H-8') and 1.4 (m, 2H, H-4'); ^{13}C NMR: δ_C 173.1 and 173.0 ($C_{1'}$), 131.0 and 130.9 ($C_{6'}$), 124.9 and 124.8 ($C_{7'}$), 88.6 and 86.8 (C_2), 56.3 and 53.8 (OMe), 46.0 and 45.3 (C_5), 34.4, 33.8, 32.2, 31.3, 30.8, 29.2, 29.1, 24.5, 23.9, 22.8, 20.9 (C_3, C_4, $C_{2'}$-$C_{5'}$) and 17.8 (CH_3); MS (CI): m/z 226 ($M+H^+$, 9), 212 (22), 194 (100), 165 (5), 142 (10), 129 (16), 124 (9) and 111 (10).

2-Methoxy-*N*-[2-(3-phenoxyphenyl)propionyl]pyrrolidine (6c). Two diastereomers. Combined yield: 27 %; obtained as oils.

Spectral data of the first eluted diasteromer **6c1**: HRMS (EI): m/z 325.1689 ($C_{20}H_{23}NO_3$ requires 325.1678); IR: ν_{max} 3060, 2975, 2931, 2888, 1658, 1581, 1488, 1403, 1238, 1163, 1083, 917 and 694; ^{1}H NMR: δ_H 7.4-6.8 (m, 9H, Ar-H), 5.5 and 4.8 (d+d, *J*= 5 Hz, 1H, H-2), 3.9 (q, *J*= 7 Hz, 1H, H-2'), 3.6-3.3 (m, 2H, H-5), 3.3 and 3.2

(s+s, 3H, OMe), 2.1-1.7 (m, 4H, H-3+H-4) and 1.4 (m, 3H, C*H*$_3$); ^{13}C NMR: δ_C 173.8 and 173.3 ($C_{1'}$), 157.5, 156.9, 143.9 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 130.1, 130.0, 123.5, 122.2, 118.8, 117.1, 117.0 ($C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 88.0, 87.4 (C_2), 56.5, 55.9 (OMe), 45.9, 45.7 (C_5), 44.7, 44.0 ($C_{2'}$), 31.3, 30.6 (C_3), 22.9 (C_4) and 20.4, 19.8 (CH_3); MS: m/z 325 (M^+, 23), 310 (41), 294 (72), 224 (15), 197 (46), 181 (10), 128 (100), 103 (18), 91 (20), 85 (84), 77 (30), 70 (32) and 55 (29).

Spectral data of the second eluted diasteromer **6c2**: HRMS (EI): m/z 325.1672 ($C_{20}H_{23}NO_3$ requires 325.1677); IR: ν_{max} 3060, 2973, 2931, 2884, 1658, 1579, 1489, 1442, 1400, 1242, 1084, 926 and 694; ^{1}H NMR: δ_H 7.4-6.8 (m, 9H, Ar-H), 5.4 (d+d, *J*= 5 Hz, 1H, H-2), 3.9 (q, *J*= 7 Hz, 1H, H-2'), 3.8-3.5 (m, 2H, H-5), 3.4 and 3.1 (s, 3H, OMe), 2.2-1.7 (m, 4H, H-3+H-4) and 1.4 (m, 3H, C*H*$_3$); ^{13}C NMR: δ_C 173.5 ($C_{1'}$), 157.4, 144.1, 143.6 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 130.0, 129.6, 123.2, 122.3, 118.6, 118.2, 117.1 ($C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 88.5, 87.6 (C_2), 56.9 (OMe), 45.7 (C_5), 44.9 ($C_{2'}$), 31.1 (C_3), 22.7 (C_4), 20.7 and 19.8 (CH_3); MS: m/z 325 (M^+, 34), 310 (15), 294 (10), 224 (19), 197 (30), 181 (7), 128 (100), 103 (10), 91 (10), 85 (50), 77 (12), 70 (13) and 55 (8).

Synthesis of Enamides. The corresponding methoxy derivative (0.05 mmol) and silica gel (0.05 mmol) were 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. Under those conditions enamides were obtained; when the reaction was carried out with β-oxoamides, bicyclic oxazines were also formed.

***N*-Octanoyl-2-pyrroline (7a).** 30 % yield; obtained as an oil; HRMS (EI): m/z 195.1619 ($C_{12}H_{21}NO$ requires 195.1623); IR: ν_{max} 2960, 2920, 2860, 1630, 1550, 1410, 1350, 1160, 1110, 1050 and 840; ^{1}H NMR: δ_H 7.0 and 6.5 (m+m, 1H, H-2), 5.2 (m, 1H, H-3), 3.8 (dd, *J*= 9 Hz, 2H, H-5), 2.7 and 2.6 (m+m, 2H, H-4), 2.3 and 2.2 (t+t, *J*= 7 Hz, 2H, H-2'), 1.6 (m, 2H, H-3'), 1.3 (m, 8H, $(CH_2)_4CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_3); ^{13}C NMR: δ_C 169.1 ($C_{1'}$), 129.3 and 128.9 (C_2), 111.3 and 111.0 (C_3), 45.5 and 44.7 (C_5), 34.5, 34.2 (C_4), 31.6, 29.3, 29.0, 25.0, 22.5 (C_4, $C_{2'}$-$C_{7'}$) and 14.0 (CH_3); MS: m/z 195 (M^+, 12), 156 (5), 145 (33), 141 (98), 129 (48), 127 (52), 111 (39), 98 (26), 86 (45), 70 (73), 69 (64), 57 (100) and 55 (37).

***N*-Oct-6-enoyl-2-pyrroline (7b).** 28 % yield; obtained as an oil; HRMS (EI): m/z 194.1463 ($C_{12}H_{19}NO$ requires 193.1466); IR: ν_{max} 2920, 2850, 1647, 1224, 1333, 1106, 964, 734 and 612; ^{1}H NMR: δ_H 7.0 and 6.5 (m+m, 1H, H-2), 5.4 (m, 2H, H-6'-H-7'), 5.2 (m, 1H, H-3), 3.8 (dd, *J*= 9 Hz, 2H, H-5), 2.7 and 2.6 (m+m, 2H, H-4), 2.3 and 2.2 (t+t, *J*= 7 Hz, 2H, H-2'), 2.0 (m, 4H, H-4+H-5'), 1.6 (m, 5H, H-3'+H-8') and 1.4 (m, 2H, H-4'); ^{13}C NMR: δ_C 169.0 ($C_{1'}$), 130.9 ($C_{6'}$), 130.9 and 129.2 (C_2), 125.0 ($C_{7'}$), 111.4 and 110.1 (C_3), 45.5 and 44.8 (C_5), 34.1(C_4), 32.2, 30.1, 29.2, 24.4 ($C_{2'}$-$C_{5'}$) and 17.9 (CH_3); MS: m/z 193 (M^+, 37), 138 (5), 124 (21), 111 (17), 97 (11), 96 (11), 84 (18), 81 (35), 69 (72), 68 (100) and 55 (98).

***N*-[2-(3-Phenoxyphenyl)propionyl]-2-pyrroline (7c).** 29 % yield; obtained as an oil; HRMS (EI): m/z 293.1416 ($C_{19}H_{19}NO_2$ requires 293.1416); IR: ν_{max} 3060, 2931, 2888, 1647, 1573, 1489, 1420, 1258, 1110 and 610; ^{1}H NMR: ^{1}H NMR: δ_H 7.4-6.8 (m, 9H, Ar-H), 6.5 (m, 1H, H-2), 5.2 and 5.1 (m+m, 2H, H-3), 4.0-3.5 (m, 3H, H-5+H-2'), 2.8-2.5 (m, 2H, H-4) and 1.5 (d, *J*= 7 Hz, 3H, CH_3); ^{13}C NMR: δ_C 169.1 ($C_{1'}$), 157.4, 157.3, 143.9 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 130.1, 129.7, 128.4, 123.2, 122.0, 118.7, 117.2, 111.8, 110.4 (C_2, C_3, $C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 45.2 (C_5), 44.9 ($C_{2'}$), 27.9 (C_4) and 19.9, 19.7

(CH_3); MS: m/z 293 (M^+, 52), 250 (2), 197 (72), 104 (14), 91 (17), 77 (20) and 69 (100).

Synthesis of Imides. A mixture of 2-pyrrolidinone (2.7 mmol), acyl chloride (1.8 mmol) and Et_3N (2.0 mmol) in benzene (20 mL) was refluxed for 8 h. The reaction mixture was extracted with CH_2Cl_2; the resulting organic extracts were washed with brine, dried over Na_2SO_4 and concentrated to dryness, providing a residue which was purified by column chromatography on silica gel to afford the corresponding imide.

***N*-Octanoyl-2-pyrrolidinone (8).** 68 % yield; obtained as an oil; HRMS (EI): m/z 211.1570 ($C_{12}H_{21}NO_2$ requires 211.15.72); IR: ν_{max} 2952, 2922, 2853, 1738, 1694, 1460, 1360, 1324, 1250 and 593; ^{1}H NMR: δ_H 3.8 (t, *J*= 7 Hz, 2H, H-5), 2.9 (t, *J*= 7 Hz, 2H, H-3), 2.6 (t, *J*= 8 Hz, 2H, H-2'), 2.0 (m, 2H, H-4), 1.6 (m, 2H, H-3'), 1.3 (br s, 8H, (CH_2)$_4$$CH_3$) and 0.9 (t, *J*= 7 Hz, 3H, CH_3); ^{13}C NMR: δ_C 175.4 (C_2), 174.4 ($C_{1'}$), 45.4 (C_5), 36.7, 33.7, 31.6, 29.1, 29.0, 24.1, 22.5, 17.1 (C_3, C_4, $C_{2'}$-$C_{7'}$) and 14.0 (CH_3); MS: m/z 211 (M^+, 35), 182 (7), 154 (23), 140 (98), 127 (100), 112 (14), 99 (88), 86 (53), 69 (26), 57 (91) and 55 (58).

Biological activity.

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

Target Microorganisms. Fungicidal activity was measured against thirteen agronomically important phytopathogens: *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).

The strains were provided by the "Colección Española de Cultivos Tipo" (CECT) or by the "Colección de la Cátedra de Microbiología" (CCM) of the Department of Biotechnology (Universidad Politécnica de Valencia).

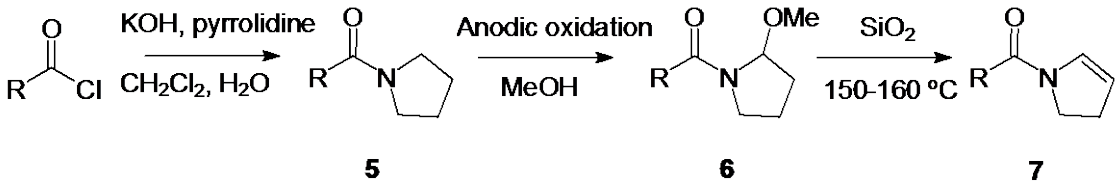
Entomotoxicity and anti-JH activity. The test was carried out basically according to the contact method of Bowers et al. (1976). Briefly, 15 third-instar *O. fasciatus* nymphs were confined to a 9 cm Petri dish coated , across the bottom, with 10 µg/cm^2 of the product. Chemicals showing high activity at 10 µg/cm^2 were retested at 7.5, 6, 5, 4, 2.5 and 1 µg/cm^2. Toxicity effects were considered according to the number of insects dead after 72 h of exposure to the chemicals and probit analysis (Finney, 1971) was applied to determine the LD_{50}. 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 when precocious metamorphosis occurred. Controls were run in parallel and received the same amount of acetone as treated insects.

Antifungal activity. The products, dissolved in acetone, were added to PDA, in a concentration 100 µg/mL. PDA plates containing only acetone were used as control plates and a positive control with Benomyl (methyl-1-[butylcarbamoyl]-2-benzimidazolecarbamate; Sigma, Germany) at 2.5 µg/mL was performed in order to appraise the level of activity of the synthesized compounds. Spores from seven day-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 (6

days for *R. necatrix* and *V. dahliae*), at 28 °C. The antifungal activity of each product was determined three times. When minimun inhibitory concentration (MIC) values were lesser than 100 µg/mL, the effective dose to inhibit 50% (ED_{50}) of the mycelial growth was estimated by linear regression analysis of the percentage of inhibition versus log of compound concentration. Analysis of variance (ANOVA) was performed for fungicidal data (Table 1) and the least significant difference (LSD) test was used to compare means (Statgraphics Plus 2.1).

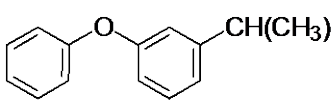
RESULTS AND DISCUSSION

The sequence of reactions previously used to synthesize **2**, **3** and **4** was modified to prepare the related compound **7**, in order to as certain whether the β-ketoamide functionality is esential for the activity. Thus, the side chain presents in the natural products was maintained in the monocarbonylic analogues. Phenoxyphenyl group was introduced, because it is very common in pesticides.



a R= $CH_3(CH_2)_6$

b R= $CH_3CH{=}CH(CH_2)_4$

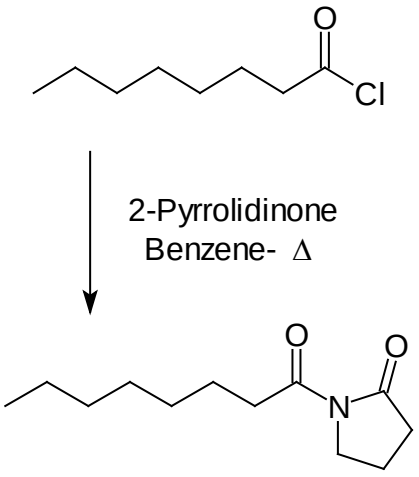
c R= 

Scheme 1. Synthetic sequence with monocarbonylic side chains.

As shown in Scheme 1, the employed synthetic sequence implied formation of amides by means of a Schotten-Baumann reaction, taking pyrrolidine and the

corresponding acid chloride as starting materials in the first step. Subsequent anodic oxidation of the pyrrolidine ring using the method described by Shono (Shono, 1984; Shono et al., 1982; Shono et al., 1982; Shono et al.,1981; Shono et al., 1981) followed by elimination of MeOH upon acid catalysis and heating at 150-160 ºC (Slomczynska et al., 1996; Cornille et al., 1995; Cornille et al., 1994; Moeller et al., 1994; Moeller et al., 1992) afforded the corresponding enamides. This worked is a satisfactory way in the case of **7a** and **7b**; however obtention of **7c** implied in the anodic oxidation a diastereomeric mixture which appeared joined with a series of oxidation products of aromatic rings. This mixture of diastereomers was resoluted by cromatographic column. Lately, both diastereomers were used in the elimination reaction affording the desired enamide **7c**.

Finally, in order to obtain further analogues, 2-pyrrolidinone was introduced as a five member ring instead of pyrrolidine. This was achieved by simple heating of octanoyl chloride with 2-pyrrolidinone and triethyl amine in refluxing benzene (Ostrovskaya, et al., 1993; Sasaki, et al., 1991).



8

Biological activities.

Insecticidal and anti-JH activity. Two of the products (**5a** and **5b**) showed potent insecticidal activity against *O. fasciatus*. In the case of **5a**, acute LD_{50} and LD_{90} values for third-instar nymphs, exposed to the chemical by the contact method, were 3.0 and 5.0 μg/cm^2, respectively. The corresponding values for compound **5b** were 1.5 and 2.0 μg/cm^2. Thus, the latest compound was 2-fold more active than **5a;** this toxicity data unambiguously demonstrates that introduction of an unsaturation in the side chain is associated with an improved entomotoxicity. Insects were unaffected, at test levels, by the other synthetic analogues. Thus, deeper modifications of the side chain or the five membered-ring produce an adverse effect on the toxicity.

Insecticidal activity has been shown to be closely associated with the pyrrolidine moiety. In our previous work on the isolation, synthesis and biological activity of compound **2** (Moya et al., 1998), the study of the insecticidal activity of its synthetic precursors showed that the presence of the pyrrolidine ring was essential for the activity. Particularly, N-(3-oxodecanoyl)pyrroline and N-(2-methyl-3-oxodecanoyl)pyrroline, both with the β-ketoamide functionality, showed LD_{50} of 7.0 and 3.75 μg/cm^2, respectively. As mentioned above, our current results show the same tendency, but in this case there has been a significant improvement of entomotoxicity.

Although natural enamides **2** and **3** show an important *in vivo* anti-JH activity, neither their previously reported synthetic precursors nor the new analogues assayed in the present work proved to retain such activity; no precocious metamorphosis or not even slighter symptoms of HJ deficiency, as delayed growth or altered fertility, were detected. It seems that this activity has a very specific structural requeriments.

Fungicidal activity. At a first sight, it is interesting to note that the introduction of an amide group, instead of the β-ketoamide functionality present in the natural

products and their synthetic precursors, resulted in an important increase of the fungicidal activity [for comparative purposes see Moya et al. (1998) and Cantín et al. (1998)]. However, comparatively, the level of activity are clearly lower that those of a conventional fungicide such as Benomyl (Table 1).

Fungicidal activity of the new analogues, expressed as the percentage of growth inhibition against 13 agronomically important plant pathogens, is shown in Table 1.

In general, the products possessing the phenoxyphenyl substituent showed the best fungicidal activity with regard to the percentage of growth inhibition and the number of affected species; compound **7c** has proved to be highly active against *C. gloesporoides* [ED_{50} = 2.04 µg/mL; 95% confidence interval: (1.26 , 4.12)] and *C. coccodes* [ED_{50} = 11.7 µg/mL with a 95% confidence interval of (7.3 , 20.7)], although these results still compare unfavourably with those found for Benomyl [ED_{50} = 0.05 µg/mL; 95% confidence interval: (0.04 , 0.08) against *C. gloesporoides* and ED_{50} = 0.13 µg/mL with a 95% confidence interval of (0.12 , 0.17)]. Besides, compound **7c** strongly affected the growth of *T. roseum* and *P. citrophthora* and, very interestinly, *A. tenuis*, one of the Benomyl resistant species; all the other fungi were also inhibited in some extent. The improvement obtained with this product appears important and warrants further work. It will be used as a starting point in the search for more active analogues and also for studies on its mechanism of action.

The remaining analogues exhibited minimun inhibitory concentration values greater than 100 µg/mL, so none of them were strongly effective against tested microorganisms. In some cases, however, obtained data have been useful to appraise the influence of the different chemical transformations on the ability to control fungi.

Among the products possessing the pyrrolidine ring, compound **5c**, with the phenoxyphenyl substituent, showed the best fungicidal activity. Compounds **5a** and **5b**

exhibited lesser level of activity; **5a** was significantly (P>0.05) more active than **5b** against all microorganisms, except for both subspecies of *F. oxysporium* and *A. tenuis*. This fact suggested that the presence of an unsaturation in the side chain of the molecule had adverse effects on fungal growth, contrary to that observed for insecticidal activity.

Introduction of a methoxy group in the pyrrolidine ring to give the corresponding derivatives (**6a**, **6b** and the diasteromers **6c1** and **6c2**) was associated with a decreased activity. Thus, **6a** and **6b** were aproximately 2-fold lesser active than **5a** and **5b**, respectively; this loss of activity could be observed not only at the level of activity but in the number of affected fungi. Compounds **6c1** and **6c2**, the methoxy by-products of **5c**, were also adversely but not dramatically affected.

Molecules containing a pyrroline ring showed a good fungicidal activity specially against *Colletotrichum* genus. Contrary to the observed trend with the above products, the unsaturation on the side chain of this enamide (**7b**) appears to enhance the fungitoxicity.

Finally, important fungicidal activities were obtained when a 2-pyrrolidinone ring was introduced in the structure, instead of pyrrolidine. The activities were similar, or even higher, to those showed by compound **5c** for *Colletotrichum*, *A. tenuis* and *T. viride*, but the other fungi were lesser affected. Previously, a similar 2-pyrrolidone derivative with antibiotic activity was described (Takeuchi and Yonehara, 1964). The product, named variotin, was isolated from the culture broth of the fungus *Paecilomyces varioti* and exhibits activity against different kinds of fungi with MIC values in the range 10-160 µg/mL. It is interesting to note that, as in our case, variotin is specially effective against various species of the *Colletotrichum* genera, with MIC values from 0.2 to 2.0 µg/mL (Yonehara et al., 1959; Takeuchi et al., 1959; Abe et al., 1959).

In summary, good insecticidal and fungicidal activities have been achieved and preliminary structure-activity relationships have been established. In this context, the improvements obtained so far on the biological activities and the simplicity of structures are encouraging to pursue on the search of new, more effective, analogues as promising pesticidal candidates.

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Table 1. Analogues Showing Fungicidal Activity

	Radial Mycelial Growth Inhibition [% (mean±SD)[a]]												
Product	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
5a	0	20.6±3.3^{AB}	21.6±0.1^{A}	13.0±0.5^{A}	36.3±3.4^{AE}	67.9±6.6^{A}	34.9±6.9^{AFG}	26.4±1.6^{A}	44.6±4.8^{AE}	41.5±3.2^{A}	23.3±2.3^{A}	15.8±2.8^{AD}	21.9±1.7^{A}
5b	0	21.3±2.6^{B}	24.7±3.4^{A}	0	25.3±1.8^{B}	50.2±2.3^{BC}	48.8±5.3^{B}	22.1±3.4^{AB}	27.3±1.5^{BG}	13.6±4.5^{B}	9.9±0.1^{B}	0	15.5±2.8^{B}
5c	48.8±2.5 ^{A}	52.0±1.0^{DE}	52.2±2.2^{B}	27.5±2.5^{B}	61.4±1.7^{C}	70.5±6.3^{A}	77.4±10.4^{C}	74.3±2.9^{C}	76.1±5.4^{C}	62.0±2.8^{C}	48.0±2.0^{C}	48.9±3.8^{B}	50.9±1.3^{C}
6a	0	0	0	0	11.7±2.1^{D}	44.7±1.8^{B}	14.1±2.3^{D}	19.9±3.0^{B}	17.3±3.7^{D}	15.6±4.6^{B}	0	0	12.5±0.8^{B}
6b	0	0	0	0	0	45.8±2.2^{B}	24.6±0.7^{A}	10.7±1.9^{D}	19.5±2.2^{BD}	0	0	0	7.6±1.0^{D}
6c1	30.9±3.6^{B}	47.3±1.3^{D}	48.0±1.9^{BC}	15.8±3.8^{A}	55.7±2.1^{C}	55.2±3.4^{CD}	78.6±1.5^{C}	61.1±3.5^{F}	48.2±6.9^{E}	60.2±6.6^{C}	33.3±1.0^{D}	24.0±3.5^{C}	47.0±3.2^{CE}
6c2	30.3±5.3^{BC}	33.9±1.8^{C}	31.6±5.2^{D}	16.7±2.9^{A}	43.1±4.2^{A}	44.5±0.6^{B}	63.6±2.7^{E}	44.4±2.7^{E}	37.5±5.4^{AF}	50.6±2.9^{D}	18.2±0.0^{E}	18.9±1.3^{D}	23.2±3.0^{A}
7a	0	15.8±0.3^{A}	13.5±4.3^{E}	0	34.1±4.6^{E}	47.8±3.9^{B}	42.0±7.3^{BF}	38.9±0.6^{G}	19.9±3.2^{BD}	18.0±3.4^{B}	8.7±2.3^{B}	9.0±2.0^{E}	15.0±2.5^{B}
7b	27.6±3.4^{B}	17.9±2.0^{AB}	21.3±4.1^{A}	0	84.0±5.4^{F}	61.3±1.7^{D}	38.9±6.6^{BFG}	26.2±3.7^{A}	34.1±9.3^{FG}	26.4±4.2^{E}	0	12.2±1.9^{AE}	21.9±4.0^{A}
7c	46.7±2.1^{A}	38.3±2.9^{C}	46.1±2.5^{C}	26.7±2.9^{B}	100^{G}	100^{E}	84.8±0.9^{C}	70.0±2.9^{C}	57.1±5.5^{H}	78.2±4.4^{F}	33.1±3.1^{D}	23.6±1.4^{C}	43.6±3.3^{E}
8	23.0±5.1^{C}	56.0±8.2^{E}	36.9±5.4^{D}	40.7±1.6^{C}	77.2±6.8^{F}	68.3±6.0^{A}	34.3±4.3^{AG}	89.0±5.2^{H}	36.8±3.5^{AF}	33.6±2.0^{G}	65.3±1.2^{F}	38.9±1.9^{F}	48.3±1.5^{C}
Benomyl	87.0±1.4	11.1±0.0	100	0	100	100	100	0	100	100	100	100	100

[a] values represent means ± standard deviations of growth inhibitions from three independent experiments. Assays concentration of analogues: 100 μg/mL; Benomyl concentration: 2.5 μg/mL. Within each column, mean values showing the same superscripts (A-H) are not significantly different (P>0.05). Target Plant Pathogens: *1, F. culmorum*; *2, F. oxysporum ssp gladioli*; *3, F. oxysporum ssp niveum; 4, G. candidum; 5, C. gloesporioides; 6, C. coccodes; 7, T. roseum; 8, A. tenuis; 9, V. dahliae; 10, P. citrophthora; 11, T. viride; 12, P. italicum; 13, A. parasiticus*.