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Novel Inhibitors of the Mitochondrial Respiratory Chain: Oximes and Pyrrolines Isolated from *Penicillium brevicompactum* and Synthetic Analogues

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The capacity of inhibition of the mammalian mitochondrial respiratory chain of brevioxime **5a**, a natural insecticide compound isolated from *Penicillium brevicompactum* culture broth, and another 15 analogue compounds, other oximes **5b** and **5c**; two diastereomeric pyrrolidines **1c′** and **1c′′**; five pyrrolines **3c′**, **3c′′** (diastereomers between them), **3a**, **3b**, and **6**; two oxazines **4c′** and **4c′′** (also diastereomers between them); and four pyrrol derivatives **7**−**10**, are analyzed in this paper. Compounds **3b**, **3c′**, **3c′′**, **4c′**, **4c′′**, **5b**, **5c**, **6**, and **10** were found to be inhibitors of the integrated electron transfer chain (NADH oxidase activity) in beef heart submitochondrial particles (SMP), establishing that all of them except compound **3b** and **6** only affected to complex I of the mitochondrial respiratory chain. The most potent product was **5b**, with an IC_{50} of 0.27 μM, similar to the IC_{50} values of other known complex I inhibitors. The diastereomeric pairs **1c′**/**1c′′**, **3c′**/**3c′′**, **4c′**/**4c′′**, and **5c** have not been previously described. Chemical characterization, on the basis of spectral data, is also shown.

KEYWORDS: ***Penicillium brevicompactum*; brevioxime; oximes; oxazines; pyrrols; pyrrolines; pyrrolidines; inhibitors of mitochondrial respiratory chain; complex I; insecticide; fungicide**

INTRODUCTION

Over the past years, new potent natural and synthetic insecticides have been described as inhibitors of complex I of the mitochondrial respiratory chain (*1*−*7*). The present knowledge of complex I is primarily based on studies with bovine heart and *Neurospora crassa* enzymes, whereas little is known about the structure of insect complex I (*1*). Enzymatic and immunochemical evidence indicate a high degree of similarity to their mammalian and fungal counterparts. The study of mammalian complex I inhibition by new potential insecticides is relevant by two opposite reasons. On one hand, an inhibitory effect would explain their mechanism of action similarly to other previously described insecticides. On the other hand, lack of inhibition could represent low toxicity against mammals, at this level.

In 1997, Moya et al. isolated from *Penicillium brevicompactum* a natural product, brevioxime **5a**, which was demonstrated as an inhibitor of juvenile hormone biosynthesis (**Figure 1**) (*8*). Thereafter, derivative compounds of **5a**, showing important insecticidal and fungicidal activities, were found (*9*−*14*). The interesting nature of these compounds has encouraged other research groups to synthesize **5a** and other analogues (*15*−*20*). In this report, we analyze whether brevioxime and analogues can inhibit the mitochondrial electron transport system.

MATERIALS AND METHODS

General Experimental Procedures. Chemicals were obtained from commercial suppliers and used without further purification. IR spectra were obtained with a 710FT spectrophotometer (Nicolet, Madison, WI). ^{1}H, ^{13}C, and COSY H−H NMR spectra were recorded on a Gemini 300 MHz instrument (Varian, Walnut Creek, CA). The assignment of ^{13}C signals is supported by DEPT experiments. Mass spectra were obtained under electron impact with a VG AutoSpec spectrometer (Fisons, Manchester, United Kingdom). Thin-layer chromatography was run on silica gel F_{254} precoated plates (Merck), and spots were detected under UV light.

Biological Material. Submitochondrial particles (SMP) for biological assays were obtained from beef heart following Fato et al. (*21*). Extensive ultrasonic disruption of frozen-thawed mitochondria was carried out to produce open membrane fragments.

Biological Methods. The inhibitory potency of the compounds was assayed by using SMPs from beef heart. SMPs were diluted to 0.5

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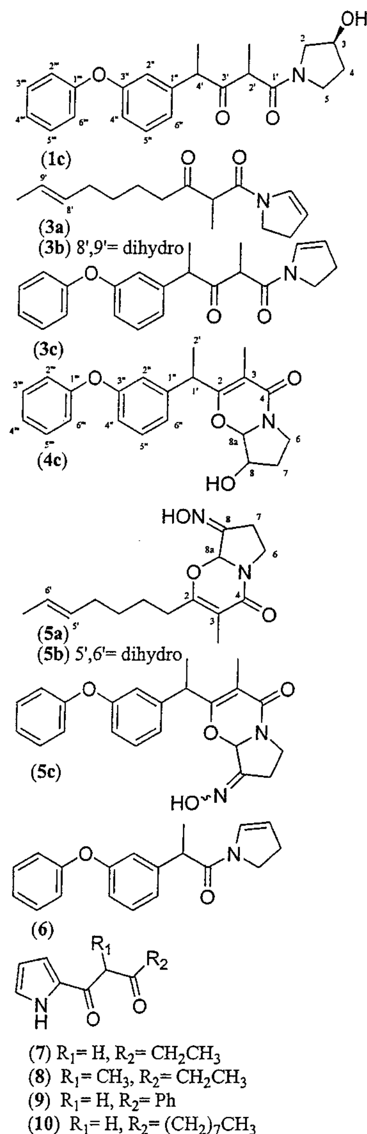


Figure 1. Structures of assayed oximes (**5a**–**c**), diastereomer pyrrolidines (**1c′** and **1c″**), pyrrolines [**3c′**, **3c″** (diastereomers), **3a**, **3b**, and **6**], diastereomer oxazines (**4c′** and **4c″**), and pyrrols (**7**–**10**).

mg/mL in 250 mM sucrose and 10 mM Tris-HCl buffer, pH 7.4, and treated with 300 μM NADH in order to activate complex I before starting the experiments. The enzymatic activity was assayed at 22 °C in 50 mM potassium phosphate buffer, pH 7.4, and 1 mM EDTA with SMP diluted to 6 μg/mL. Stock solutions (15 mM in absolute EtOH) of **1c′**, **1c″**, **3a**, **3b**, **3c′**, **3c″**, **4c′**, **4c″**, **5a**, **5b**, **5c**, and **6**−**10** were prepared and kept in the dark at −20 °C. Each compound was added to the diluted SMP preparation and incubated, during 5 min, in ice. NADH oxidase activity was measured as the aerobic oxidation of 75 μM NADH. Reaction rates were calculated from the linear decrease of NADH concentration (λ 340 nm, ϵ 6.22 mM^{-1} cm^{-1}) in an end window photomultiplier spectrophotometer ATI-Unicam UV4-500. For each compound, three experiments were carried out.

Cytochrome *c* reductase activity sustained by succinate (integrated activity of complexes II and III) was measured while monitoring the reduction of 40 μM ferricytochrome *c* at 550 nm (ϵ 19.1 mM^{-1} cm^{-1}) in the presence of 2 mM KCN to block its reoxidation. The reaction was started with 13.5 mM succinate.

NADH:ubiquinone oxidoreductase (specific complex I activity) was measured with 75 μM NADH and 30 μM decylubiquinone (DB) as a soluble short chain analogue of ubiquinone in the presence of 2 μM antimycin and 2 mM potassium cyanide to block any reaction downstream of complex I, following the NADH oxidation rate. Similarly, succinate:ubiquinone oxidoreductase (specific complex II activity) was measured by changing NADH for 12.5 mM succinate and following the reduction of DB at 378 nm (ϵ 14.0 mM^{-1} cm^{-1}). SMPs were preincubated for 10 min in the presence of 1.25 mM succinate to fully activate complex II.

Synthesis of Natural Products and Their Analogues. The synthesis of compounds **3a**, **3b**, and **6**−**10** was carried out as described in our previous work (*9*−*11*, *13*). In addition, **Scheme 1** shows the new brevioxime synthetic route, which is based on that previously described by Clark (*18*) and from which byproducts **1c′**/**1c″**, **3c′**/**3c″**, **4c′**/**4c″**, **5a**, **5b**, and **5c** were obtained. This route presents the following five steps.

First Step (i, ii): Synthesis of Hydroxyketoamides (1). To a 2.0 M solution of lithium diisopropylamide (LDA) in anhydrous tetrahydrofuran (THF) (30 mmol) at −78 °C was slowly added 3-triethylsililoxy-*N*-propionylpyrrolidine (20 mmol) dissolved in anhydrous THF (15 mL) (*13*). After the mixture was stirred for 2 h, the corresponding ester (10 mmol) was added dissolved in anhydrous THF (10 mL) shaking for 6 h. Then, the mixture was quenched by addition of a saturated solution of ammonium chloride, warmed at ambient temperature, and extracted with diethyl ether. At that point, β-ketoamide signals were observed in the NMR spectra; therefore, no further purification was carried out. Thus, the crude was dissolved in anhydrous THF (60 mL), and acetic acid (10 mmol) and a 1.0 M solution of Bu_4NF (10 mmol) were added. The mixture was stirred at ambient temperature before being diluted with ethyl acetate and washed with water and a saturated solution of $NaHCO_3$. The combined organic extracts were concentrated and purified by column chromatography over silica gel (gradient elution with mixtures of Hex and EtOAc, with 50−100% EtOAc). According to this step, two compounds, **1c′**/**1c″**, were synthesized.

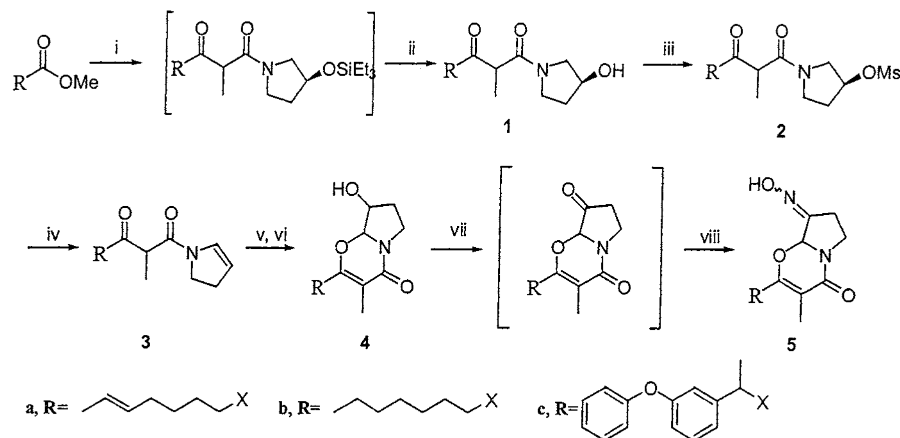
3-Hydroxy-*N*-[2-methyl-4-(3-phenoxyphenyl)-3-oxopentanoyl]-pyrrolidine (1c). In these conditions, only two diastereomeric alcohols could be clearly resolved. The first eluted diastereomer (**1c′**, $R_f = 0.78$ in EtOAc) was obtained with a 18% yield and exhibited the following spectral data. HRMS *m*/*z* 367.1717 ($C_{22}H_{25}NO_4$ requires 367.1783). IR: γ_{max} 3500−3100, 2970, 2929, 2878, 1721, 1613, 1495, 1429, and 1244. ^{1}H NMR: δ_H 7.3−6.8 (m, 9H, Ar−H), 4.3 (m, 1H, H-3), 3.8 (m, 1H, H-2a), 3.5−2.8 (m+m+m, 5H, H-2b, H-5, H-2′, H-4′), 1.8 (m, 2H, H-4), 1.4, and 1.3 (d+d, J = 7 Hz, 6H, 2 × CH_3). ^{13}C NMR: δ_C 206.3 ($C_{3'}$), 169.6 ($C_{1'}$), 157.7, 156.6, 142.8 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 130.3, 129.8, 123.7, 122.8, 118.9, 118.9, 117.2 ($C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 70.9 (C_3), 54.4 (C_2), 51.1, 49.7 ($C_{2'}$, $C_{4'}$), 44.5 (C_5), 34.0 (C_3), 18.4, and 13.2 (2 × CH_3). MS *m*/*z* 367 (M^+, 100), 224 (6), 197 (27), 170 (29), 143 (72), and 114 (84).

The second eluted diastereomer (**1c″**, $R_f = 0.73$ in EtOAc) obtained in 40% yield showed the following spectral data. HRMS *m*/*z* 367.1784 ($C_{22}H_{25}NO_4$ requires 367.1783). IR: γ_{max} 3500−3100, 2970, 2929, 2878, 1721, 1628, 1495, 1429, and 1239. ^{1}H NMR: δ_H 7.4−6.8 (m, 9H, Ar−H), 4.4 (m, 1H, H-3), 4.0 (m, 1H, H-2a), 3.7−2.9 (m+m+m, 5H, H-2b, H-5, H-2′, H-4′), 1.9 (m, 2H, H-4), 1.4, and 1.3 (d+d, J = 7 Hz, 6H, 2 × CH_3). ^{13}C NMR: δ_C 206.0 ($C_{3'}$), 171.0 ($C_{1'}$), 158.1, 157.0, 143.5 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 130.6, 130.2, 124.0, 123.2, 119.3, 118.9, 117.9 ($C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 71.3 (C_3), 55.0 (C_2), 50.8, 50.5 ($C_{2'}$, $C_{4'}$), 45.0 (C_5), 34.7 (C_3), 18.8, and 13.5 (2 × CH_3). MS *m*/*z* 367 (M^+, 100), 224 (8), 197 (32), 170 (30), 143 (69), and 114 (75).

Second Step (iii): Synthesis of Mesylates (2). To a solution of the corresponding alcohol (1.0 mmol) in anhydrous CH_2Cl_2 (7.0 mL) was added Et_3N (1.3 mmol) and methane sulfonyl chloride (1.1 mmol). The mixture was stirred at ambient temperature overnight and then diluted with 1.0 M HCl. After it was extracted with CH_2Cl_2, the crude was purified by column chromatography over silica gel (gradient elution with mixtures of Hex and EtOAc, with 50−100% EtOAc). According to this step, two mesylates, **2c′**/**2c″**, were synthesized.

3-Methanesulfonyloxy-*N*-[2-methyl-4-(3-phenoxyphenyl)-3-oxo-pentanoyl]pyrrolidine (2c). The mesylates corresponding to the previously described alcohols were obtained with 80−85% yields. The mesylate obtained from the first described alcohol (**2c′**, $R_f = 0.63$ in EtOAc) showed as spectral data. HRMS *m*/*z* 445.1591 ($C_{23}H_{27}NO_6S$ requires 445.1559). IR: γ_{max} 2970, 2934, 2883, 1726, 1644, 1480, 1429, 1352, 1249, 1162, and 886. ^{1}H NMR: δ_H 7.4−6.8 (m, 9H, Ar−H), 5.2 (m, 1H, H-3), 3.9 (m, 1H, H-2a), 3.8−3.2 (m, 5H, H-2b, H-5, H-2′,

Scheme 1. General Scheme of the New Synthesis of Brevioxime **5a** and the Other Analogues, Based on the Clark Method



Reagents and conditions: (i) 3-Triethylsililoxy-N-propionylpyrrolidine, LDA, THF, −78 °C. (ii) Bu_4NF, AcOH, THF. (iii) $MeSO_2Cl$, Et_3N, CH_2Cl_2. (iv) *t*-BuOK, DMSO. (v) Oxone, $NaHCO_3$, CF_3COCF_3, MeOH. (vi) PPTS, toluene. (vii) Dess–Martin periodinane, CH_2Cl_2. (viii) NH_2OH HCl, Et_3N, MeOH.

H-4′), 3.0 (s, 3H, SO_2CH_3), 2.2 (m, 2H, H-4), 1.4, and 1.3 (d+d, J = 7 Hz, 6H, 2 × CH_3). ^{13}C NMR: δ_C 205.0 ($C_{3'}$), 166.8 ($C_{1'}$), 156.3, 155.3, 140.4 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 128.7, 128.4, 122.2, 121.4, 117.5, 117.4, 116.1 ($C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 77.8 (C_3), 50.6 (C_2), 49.6, 48.5 ($C_{2'}$, $C_{4'}$), 42.2 (C_5), 37.3 (SO_2CH_3), 31.4 (C_3), 16.8, and 11.9 (2 × CH_3). MS m/z 445 (M^+, 100), 248 (44), 221 (86), and 192 (96).

The spectral data of the mesylate obtained from the second alcohol (**2c″**, R_f = 0.58 in EtOAc) were as follows. HRMS m/z 445.1590 ($C_{23}H_{27}NO_6S$ requires 445.1559). IR: γ_{max} 2990, 2929, 2868, 1730, 1644, 1480, 1429, 1352, 1249, 1168, and 896. ^1H NMR: δ_H 7.4−6.8 (m, 9H, Ar−H), 5.2 (m, 1H, H-3), 4.0 (m, 1H, H-2a), 3.9−3.3 (m, 5H, H-2b, H-5, H-2′, H-4′), 3.0 (s, 3H, SO_2CH_3), 2.2 (m, 2H, H-4), and 1.3 (m, 6H, 2 × CH_3). ^{13}C NMR: δ_C 206.9 ($C_{3'}$), 168.7 ($C_{1'}$), 158.2, 157.1, 142.2 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 130.4, 130.3, 124.0, 123.2, 119.4, 118.8, 117.9 ($C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 79.7 (C_3), 52.5 (C_2), 52.3, 50.5 ($C_{2'}$, $C_{4'}$), 44.1 (C_5), 39.3 (SO_2CH_3), 33.3 (C_3), 18.7, and 14.0 (2 × CH_3). MS m/z 445 (M^+, 10), 221 (28), 197 (76), and 149 (100).

Third Step (iv): Synthesis of Pyrrolines (3). To a solution of the corresponding mesylate (0.6 mmol) in dimethyl sulfoxide (5 mL) was added *t*-BuOK (12.6 mmol), and the mixture was stirred at ambient temperature for 4 h. Ice was then added followed by a saturated solution of ammonium chloride. The mixture was extracted twice with ethyl ether, and the combined organic extracts were washed with water. After that, the organic extract was purified by column chromatography over silica gel (gradient elution with mixtures of Hex and EtOAc, with 50−100% EtOAc). According to this step, two compounds, **3c′**/**3c″**, were synthesized.

***N*-[2-Methyl-4-(3-phenoxyphenyl)-3-oxopentanoyl]-2-pyrroline (3c).** The pyrrolines corresponding to the previously described mesylates were obtained in 30−40% yields as oils. The spectral data of the first one (**3c′**, R_f = 0.41 in Hex/EtOAc:70/30) were as follows. HRMS m/z 349.1646 ($C_{22}H_{23}NO_3$ requires 349.1677). IR: γ_{max} 3067, 2990, 2924, 2863, 1721, 1644, 1590, 1490, 1255, and 1152. ^1H NMR: δ_H 7.3−6.8 (m, 9H, Ar−H), 6.3 (m, 1H, H-2), 5.3 (m, 1H, H-3), 4.0 (m, 1H, H-4′), 3.7−3.4 (m, 2H, H-5), 3.1 (m, 1H, H-2′), 2.6 (m, 2H, H-4), and 1.3 (m, 6H, 2 × CH_3). ^{13}C NMR: δ_C 205.6 ($C_{3'}$), 166.0 ($C_{1'}$), 158.6, 145.2, 142.0 ($C_{1''}$, $C_{3''}$, $C_{1'''}$), 130.2, 130.1, 123.7, 122.7, 119.4, 118.1, 117.4, 109.4 (C_2−C_3, $C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 52.5, 51.2 (2 × $CHCH_3$), 41.8 (C_5), 30.5(C_4), 17.8, and 14.6 (2 × CH_3). MS m/z 349 (M^+, 36), 242 (5), 224 (14), 211 (9), 197 (87), and 83 (100).

The second 2-pyrroline obtained (**3c″**, R_f = 0.25 in Hex/EtOAc:70/30) showed as spectral data. HRMS m/z 349.1601 ($C_{22}H_{23}NO_3$ requires 349.1677). IR: γ_{max} 2990, 2925, 1735, 1660, 1655, 1587, 1501, 1298, and 1190. ^1H NMR: δ_H 7.3 (m, 2H, H-3‴+H-5‴), 7.2 (m, 1H, H-5″), 7.1 (t, J = 8 Hz, 1H, H-4‴), 7.0−6.8 (m, 5H, H-2″, H-4″, H-6″, H-2‴, H-6‴), 6.2 (m, 1H, H-2), 5.2 (m, 1H, H-3), 3.9 (q, J = 7 Hz, 1H, H-4′), 3.8−3.6 (m, 2H, H-5), 3.5 (q, J = 7 Hz, 1H, H-2′), 2.5 (m, 2H, H-4), and 1.4 (d+d, J = 7 Hz, 6H, 2 × CH_3). ^{13}C NMR: δ_C 205.6 ($C_{3'}$), 166.0 ($C_{1'}$), 157.6, 142.4, 142.1 ($C_{1''}$,$C_{3''}$, $C_{1'''}$), 130.0, 129.8, 127.9, 123.5, 122.6, 118.9, 118.4, 117.1, 112.9 (C_2-C_3, $C_{2''}$, $C_{4''}$-$C_{6''}$, $C_{2'''}$-$C_{6'''}$), 50.6, 45.5 (2 × $CHCH_3$), 45.2 (C_5), 28.1(C_4), 18.3, and 12.9 (2 × CH_3). MS m/z 349 (M^+, 35), 279 (6), 242 (10), 224 (18), 211 (10), 197 (100), and 83 (43).

Fourth Step (v, vi): Synthesis of Alcohols (4). 1,1,1-Trifluoroacetone (1 mL), sodium bicarbonate (1.48 mmol), and oxone (1.00 mmol) were added to a 0° C solution of acyl enamine (1.00 mmol) in methanol (10 mL). The resulting mixture was warmed at room temperature and stirred overnight. Then, it was diluted with water, extracted with CH_2Cl_2, and dried over Na_2SO_4. Without further purification, the mixture was dissolved in toluene (40 mL) and PPTS (0.12 mmol) was added to the resulting solution, which was heated at reflux for 3 h. After it was cooled, the mixture was concentrated and purified by column chromatography over silica gel using gradient elution with mixtures of Hex and EtOAc (30−100% EtOAc). According this step, we synthesized the compounds **4a′**/**4a″**, **4b′**/**4b″**, and **4c′**/**4c″** [**4a′**/**4a″** and **4b′**/**4b″** were synthesized from compounds **3a** and **3b**, respectively, which we had previously obtained by other known route (*9*−*11*)].

2-Hept-5-enyl-6,7-dihydro-8(8a*H*)-hydroxy-3-methyl-4H-pyrrole[2,1-b]-[1.3]oxazine-4-one (4a). In the above-described conditions, two different alcohols were obtained. Their spectral data were fully coincident with those described by Clark (*18*).

2-Heptyl-6,7-dihydro-8(8a*H*)-hydroxy-3-methyl-4H-pyrrole[2,1-b]-[1.3]oxazine-4-one (4b). In these conditions, two different alcohols were obtained; the less polar one (**4b′**, R_f = 0.32 in EtOAc) was obtained with a 56% yield for the two steps. It showed as spectral data. ^1H NMR: δ_H 5.0 (d, J = 4 Hz, 1H, H-8a), 4.5 (m, 1H, H-8), 3.6−3.4 (m 2H, H-6), 2.3−2.1 (m, 2H, H-7), 2.0 (m, 2H, H-1′), 1.8 (s, 3H, CH_3), 1.5 (m, 2H, H-2′), 1.3 [m, 8H, $(CH_2)_4CH_3$], and 0.9 (t, J = 7 Hz, 3H, CH_2CH_3). ^{13}C NMR: δ_C 171.1 (C_4), 163.6 (C_2), 106.0 (C_3), 92.2 (C_{8a}), 74.9 (C_8), 60.3 (C_6), 41.8 ($C_{1'}$), 31.6, 30.5, 28.9, 26.7, 22.5, 20.9 (C_7, $C_{2'}$-$C_{6'}$), 14.0, and 9.9 (2 × CH_3).

The more polar isomer (**4b″**, R_f = 0.23 in EtOAc) was obtained with a 24% yield for the two steps. It showed as spectral data. ^1H NMR: δ_H 5.2 (d, J = 4 Hz, 1H, H-8a), 4.4 (m, 1H, H-8), 3.7−3.5 (m 2H, H-6), 2.4−2.0 (m, 4H, H-7, H-1′), 1.8, (s, 3H, CH_3), 1.5 (m, 2H, H-2′), 1.3 [m, 8H, $(CH_2)_4CH_3$], and 0.9 (t, J = 7 Hz, 3H, CH_2CH_3). ^{13}C NMR: δ_C 171.0 (C_4), 163.0 (C_2), 106.4 (C_3), 87.6 (C_{8a}), 70.4 (C_8), 60.3 (C_6), 41.5 ($C_{1'}$), 31.6, 30.5, 28.8, 22.5, 20.9 (C_7, $C_{2'}$-$C_{6'}$), 14.0, and 9.8 (2 × CH_3).

2-[1-(Phenoxyphenyl)ethyl]-8(8a*H*)-hydroxy-3-methyl-4H-pyrrole[2,1-b]-[1.3]oxazine-4-one (4c). In these conditions, two different alcohols were obtained; the less polar one (**4c′**, R_f = 0.19 in Hex/EtOAc: 5/5) was obtained with a 24% yield for two steps. It showed the following spectral data. HRMS m/z 365.161259 ($C_{22}H_{23}NO_4$ requires 365.162708). IR: γ_{max} 3400−3100, 2888, 1634, 1485, 1444, 1234, 922, and 692. ^1H NMR: δ_H 7.4−6.8 (m, 9H, Ar−H), 4.9 (d, 1H, J = 3 Hz, H-8a), 4.5 (m, 1H, H-8), 3.9 (q, 1H, J = 7 Hz, H-1′), 3.6 (m, 2H, H-6), 2.5 (m, 1H, H-7a), 2.2 (m, 1H, H-7b), 1.8 (s, 3H, CH_3), and 1.4 (d, 3H, J = 7 Hz, CH_3). ^{13}C NMR: δ_C 165 (C_4), 158 (C_2),145, 144, 130, 125, 124, 123, 122, 119, 118, 117 ($C_{1''}$-$C_{6''}$, $C_{1'''}$-$C_{6'''}$), 107 (C_3),

93 (C_{8a}), 75 (C_8), 42 (C_6), 40 ($C_{1'}$), 30 (C_7), 17 (CH_3), and 10 (CH_3). MS m/z 365 (M^+, 21), 280 (10), 252 (15), 224 (34), and 197 (100).

The more polar isomer (**4c″**, R_f = 0.13 in Hex/EtOAc:5/5) was obtained with a 15% yield for the two steps and showed as spectral data. HRMS m/z 365.160703 ($C_{22}H_{23}NO_4$ requires 365.162708). IR: γ_{max} 3400−3100, 2883, 1644, 1486, 1440, 1245, 922, and 687. ^{1}H NMR: δ_H 7.4−6.8 (m, 9H, Ar−H), 5.0 (d, 1H, J = 3 Hz, H-8a), 4.4 (m, 1H, H-8), 4.0 (q, 1H, J = 7 Hz, H-1′), 3.6 (m, 2H, H-6), 2.4−2.2 (m, 2H, H-7), 1.8 (s, 3H, CH_3), and 1.4 (d, 3H, J = 7 Hz, CH_3). ^{13}C NMR: δ_C 164 (C_4), 158 (C_2),143, 130,123, 122, 119, 118, 117 ($C_{1''}$-$C_{6''}$, $C_{1'''}$-$C_{6'''}$), 107 (C_3), 93 (C_{8a}), 75 (C_8), 42 (C_6), 40 ($C_{1'}$), 30 (C_7), 18 (CH_3), and 9 (CH_3). MS m/z 365 (M^+, 39), 280 (15), 252 (19), 224 (35), and 197 (100).

Fifth Step (vii, vii): Synthesis of Oximes (5). To a solution of the less polar alcohol (0.55 mmol) in CH_2Cl_2 (10.0 mL), a 15 wt % solution of Dess−Martin periodinane in CH_2Cl_2 (0.60 mmol) was added. After it was stirred at room temperature for 41 h, the mixture was concentrated and extracted with diethyl ether. The resulting crude was dissolved in methanol (10 mL) and stirred overnight after adding hydroxylamine hydrochloride (0.66 mmol) and triethylamine (0.66 mmol). The mixture was diluted with ethyl acetate, washed with water, and dried over Na_2SO_4. A white dust was obtained in this manner, which was recrystallized with Hex/EtOAc mixtures. According to this last step, the compounds **5a**, **5b**, and **5c** were synthesized.

2-Hept-5-enyl-6,7-dihydro-3-methyl-4H-pyrrole[2,1-b]-[1.3]oxazine-4,8(8a*H*)-dione 8-Oxime (5a). It was obtained with a 27% yield (two steps). All data of the resulting product were coincident with the literature (*18*).

2-Heptyl-6,7-dihydro-3-methyl-4H-pyrrole[2,1-b]-[1.3]oxazine-4,8(8a*H*)-dione 8-Oxime (5b). It was obtained with a 23% yield (two steps). Again, data of the resulting product were coincident with the literature (*16*).

2-[1-(Phenoxyphenyl)ethyl]-3-methyl-4H-pyrrole[2,1-b]-[1.3]oxazine-4,8(8a*H*)-dione 8-Oxime (5c). It was obtained with a 13% yield (two steps). Compound **5c** was purified by semipreparative high-performance liquid chromatography using the following conditions: 25.0 cm × 1.0 cm i.d., 5 μm, Kromasil column; mobile phase, Hex/EtOAc (3/7, v/v); flow, 2 mL/min; detection by photodiode array and refraction index, simultaneously; retention time (R_t) = 17.1 min. It had the following spectral data. HRMS m/z 378.156342 ($C_{22}H_{22}N_2O_4$ requires 378.157957). IR: γ_{max} 3400−3100, 2970, 2924, 1644, 1588, 1495, 1434, 1250, 1209, 1163, 978, 758, and 702. ^{1}H NMR: δ_H 7.9 (s, 1H, N−O*H*), 7.4−6.8 (m, 9H, Ar−H), 5.4 (s, 1H, H-8a), 4.2−4.0 (m, 2H, H-6), 3.4 (q, 1H, J = 7 Hz, H-1′), 2.9 (m, 2H, H-7), 1.9 (s, 3H, CH_3), and 1.4 (d, 3H, CH_3). ^{13}C NMR: δ_C 164 (C_2), 163 (C_4), 158 (C_2),157, 144,130, 123, 121, 119, 117 ($C_{1''}$-$C_{6''}$, $C_{1'''}$-$C_{6'''}$), 107 (C_3), 84 (C_{8a}), 42 (C_6), 40 ($C_{1'}$), 24 (C_7), 17 (CH_3), and 9 (CH_3). MS m/z 378 (M^+, 12), 280 (9), 252 (12), 224 (19), and 197 (100).

RESULTS AND DISCUSSION

Recently, the isolation of brevioxime (**5a**) and other bioactive metabolites from the *P. brevicompactum* culture broth has been reported (*8*). During the chemical synthesis of these compounds in order to confirm their structures, a number of intermediates showing interesting insecticidal and fungicidal activities were obtained (**Figure 1**) (*8−14*). The important nature of these compounds has encouraged other research groups to synthesize **5a** and other analogues (*15−20*).

In the past years, a new series of natural and synthetic products with important biological activities have been described as inhibitors of mitochondrial respiratory chain complex I (NADH oxidase activity) (*3−7*). In this context, it has been studied here whether the oxime **5a** (a mixture of *E* And *Z*-oxime) and two natural analogues from *P. brevicompactum*, **3a** (*11*) and **3b** (*9*), as well as 13 synthetic analogues [three new pairs of diastereomers **1c′**/**1c″**, **3c′**/**3c″**, and **4c′**/**4c″**, oxime **5c**, and six already known compounds **5b** (*16*), **6** (*13*), and **7−10** (*10*)], could be new inhibitors of the respiratory chain and more specifically which complexes in the chain were affected (**Figure 1**).

Table 1. Inhibitory Potency of Pirrolidines, Pyrrolines, Oxazines, Oximes, and Pyrrol Compounds[a]

inhibitors		NADH oxidase activity IC_{50} (μM)	NADH:ubiquinone oxidoreductase activity IC_{50} (μM)
pyrrolidine skeleton	**1c′**	41.50 ± 14.85	ND
	1c″	36.25 ± 1.06	ND
2-pyrroline skeleton	**3c′**	3.92 ± 0.57	6.57 ± 2.39
	3c″	4.40 ± 0.10	16.45 ± 0.10
	3a	inactive	
	3b	4.55 ± 0.85	ND
	6	6.82 ± 0.37	ND
oxazine skeleton	**4c′**	5.48 ± 0.49	14.31 ± 1.40
	4c″	3.13 ± 0.32	11.93 ± 1.27
oxime skeleton	**5a**	inactive	
	5b	0.27 ± 0.06	1.52 ± 0.84
	5c	0.92 ± 0.09	3.44 ± 0.14
pyrrol skeleton	**7**	inactive	
	8	inactive	
	9	214.50 ± 6.30	ND
	10	8.60 ± 0.20	14.30 ± 0.14

[a] Values are means ± standard deviation of three assays; ND, nondetermined.

Nine of the 16 assayed products, compounds **3b**, **3c′**, **3c″**, **4c′**, **4c″**, **5b**, **5c**, **6**, and **10**, were found to be inhibitors of the integrated electron transfer chain (NADH oxidase activity) in beef heart SMPs with IC_{50} values lesser than 10 μM (**Table 1**). Oximes **5b** and **5c** were the most potent inhibitors.

In the integrated electron transfer chain, electrons are first carried from complex I (NADH:ubiquinone oxidoreductase) or from complex II (succinate:ubiquinone oxidoreductase) to complex III (ubiquinol:cytochrome *c* oxidoreductase) by ubiquinone and then from complex III to complex IV (cytochrome *c* oxidase) by the peripheral membrane protein cytochrome *c*. The effect of **3c′**, **3c″**, **4c′**, **4c″**, **5b**, **5c**, and **10** on the activity of complex I or complex II was assayed by using DB, following the method of Estornell et al. (*22*). None of them affected the specific activity of complex II, but they strongly reduced the specific activity of complex I (**Table 1**). The effect of **3b** and **6** on both complex I and complex II using DB could not be determined, because both compounds showed a poor inhibitory potency.

In addition, we could establish for other assays that **3c′**, **3c″**, **4c′**, **4c″**, **5b**, **5c**, **10**, **3b**, and **6** did not affect complex III. To demonstrate this, these nine compounds were added in the presence of succinate as a substrate to complex II; in these conditions, succinate cytochrome *c* was not affected.

In summary, compounds **3c′**, **3c″**, **4c′**, **4c″**, **5b**, **5c**, **10**, **3b**, and **6** indeed inhibit the mitochondrial electron transfer chain and the first seven compounds affect specifically to complex I. These seven compounds were slightly more active on the couple NADH oxidase as compared to the NADH:DB oxidoreductase. Furthermore, as the substrate concentration was higher in DB-dependent NADH oxidation than in the coupled NADH oxidase reaction, it seems to indicate that these inhibitors compete with ubiquinone binding to complex I. In fact, these compounds have a structural similarity with ubiquinone, with a cyclic “head” resembling the ubiquinone ring and a hydrophobic “tail”. It is the case of some potent natural inhibitors of complex I, as for example, piericidin A (*4*). In this way, oxime, pyrroline, oxazine, and the pyrrol nucleus in **5b, 5c**, **3c′**, **3c″**, **3b**, **6**, **4c′**, **4c″**, and **10**, respectively, should likely be needed for establishing the interaction with the enzyme, since they could mimic the quinoid

head of ubiquinone, whereas the aliphatic part favors the passage through the hydrophobic enviromment of the ubiquinone catalytic site.

To establish some preliminary structure−activity relationship (SAR) of **1c′**, **1c″**, **3c′**, **3c″**, **4c′**, **4c″**, **3a**, **3b**, **5a**, **5b**, **5c**, and **6**−**10** as inhibitors of the mitochondrial complex I, compounds were classified in four groups, according to structural similarity.

The first group was formed by brevioxime **5a**, a compound with a heterocyclic oxime structure, **5b**, its synthetic 5′,6′-dihydro derivative, and **5c**, another oxime with a phenoxyphenyl group as hydrophobic anchor. Whereas **5a** was not active on the respiratory chain, **5b** and **5c** exhibited an interesting bioactivity inhibiting respiratory mitochondrial chain with an IC_{50} of 0.27 and 0.92 μM, respectively. These results seem to suggest that the aliphatic chain in the oxime products plays an important role in the interaction with complex I, taking into account that only the presence of a double bond at the C-5 position in the aliphatic chain (**5a**) removed the inhibitory activity exhibited by **5b**. It is unusual for other inhibitors as acetogenins, very active natural compounds with long aliphatic chains, sometimes with double bonds (*23*). It indicates that the important structural factors that affect the inhibitory potency are not necessarily obvious for each inhibitor (*5*).

In addition, the activity of **5c** seems to demonstrate that the phenoxyphenyl group promotes the interaction with the respiratory chain, even though **5b** is almost three times more active than **5c**. The IC_{50} values of **5b** and **5c** are similar to those shown by other natural complex I inhibitors as benzopyrans and almuheptolides (*24*, *25*). Compounds **5b** and **5c** are placed in a middle range with respect to the most potent complex I respiratory inhibitors, as rotenone, with an IC_{50} of 4.4 nM (*6*).

The second group was formed by two diastereomeric pyrrolidines, **1c′** and **1c″**, and five pyrrolines, **3c′** and **3c″** (diastereomers), **3a**, **3b**, and **6**. All of them, except **3a**, were able to inhibit the respiratory chain; **3c′**, **3c″**, **3b**, and **6** were slightly less active than **5b** and **5c**, whereas **1c′** and **1c″** (diastereomers) exhibited weak activities.

Again, the only structural difference between **3a** and **3b** is the presence, in the former, of a double bond at the C-8′ position. This confirms that the existence of an insatured chain impedes the appropriate interaction of **3a** with the enzyme, as in the case of **5a**.

Diastereomeric pyrrolines **3c′**/ **3c″** and compound **3b** turned out to be slightly more potent than **6** showing inhibition on respiratory chain (**Table 1**). The only structural difference between **6** and **3c′**/**3c″** is the presence in the latter compounds of another 1-carbonyl-2-methylethyl unit in the hydrophobic tail. This seems to demonstrate that a longer hydrophobic tail in the inhibitor favors their interaction with mitochondrial chain. Also, **6** showed a potent fungicidal activity in previous assays, and according to the current results, its mechanism of action could be the inhibition of the fungal respiratory chain (*10*).

In addition, diastereomers **1c′** and **1c″** have the same hydrophobic chain as **3c′** and **3c″**, but the first ones showed lower affinity for the respiratory chain. This should be explained by the substitution of pyrroline nucleus in **3c′** and **3c″** for a pyrrolidine nucleus with an OH group in **1c′** and **1c″**. This hydroxy-pyrrolidine nucleus difficults the interaction with the enzyme.

The third group was formed by two diastereomeric compounds, **4c′** and **4c″** (**Figure 1**). Both of them have an oxazine nucleus as a cyclic "head" as well as the same hydrophobic tail, a phenoxyphenyl group, as **3c′**/**3c″**, **5c**, and **6**. Compounds **4c′** and **4c″** were able to inhibit the respiratory chain with an IC_{50} value similar to those shown by diastereomeric pyrrolines **3c′**/**3c″** and **6**. These results confirm that the oxazine skeleton, as pyrroline nucleus, favors the interaction with the respiratory mitochondrial chain. However, the fact that **4c′**/**4c″**, **3c′**/**3c″**, and **6** were 3−6 times less active than **5c** demonstrates that the oxime nucleus is much more active to complex I than pyrroline or oxazine nucleus.

Finally, the fourth group was formed by four synthetic pyrrol compounds (**7**−**10**). Compounds **7** and **8** did not show inhibition on the mitochondrial chain of mammalian cell because they have a very short alkyl chain and, thus, they cannot interfere with the respiratory chain. Compound **9** showed a very high IC_{50} value inhibiting the respiratory chain, whereas **10** exhibited a level of activity similar to that of **6**. As in the case of compound **6**, **9** and **10** also show fungicidal activities against 13 phytopathogen fungi, and in view of the present results, a possible mode of action could be the inhibition of the fungal respiratory chain (*10*). The structural difference between **9** and **10** is the presence, in the latter, of a long alkyl chain, substituted in **9** for a phenyl group. This confirms again that the alkyl chain is very important to promote the union with the enzyme, because **10**, **5b**, and **3b**, all of them with this alkyl chain, are able to inhibit the electron transfer.

It is worth noticing that neither **5a** nor **3a** inhibited the respiratory chain of mammalian cells while, according to previous assays (*8*, *11*, *12*), **5a** is able to prevent spontaneous juvenile hormone synthesis in vitro and **3a** shows antijuvenile hormone in vivo activity. This would make these compounds very promising for future application in pest control fields, because they would not induce damage in mammalian mitochondria.

Also, we previously reported the insecticidal activity of **7** and **8** against the hemipteran *Oncopeltus fasciatus* (*10*). As **7** and **8** did not inhibit the mammalian respiratory chain, both of them could be used as potential insecticides without affecting mammals or as lead molecules for the design of new, more potent insecticides.

Finally, compound **3b**, which showed antijuvenile hormone in vivo activity (*9*), could be used as insecticide. Although it inhibits the complex I mitochondrial chain, its IC_{50} is in the micromolar concentration range. In general, the toxicity of mitochondrial chain inhibitors is described for compounds with IC_{50} values in the nanomolar concentration range, such as rotenone (*26*). Between the brevioxime analogues, only the oxymes **5b** and **5c** would come close to the area of toxicological concern.

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