Brevioxime: A New Juvenile Hormone Biosynthesis Inhibitor Isolated from *Penicillium brevicompactum*

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Introduction

The discovery of new inhibitors of juvenile hormone (JH) biosynthesis has attracted much attention in the last 20 years because of their potential use as commercial insecticides. Inhibition of the final steps of JH biosynthesis (methyl farnesoate formation and epoxidation) constitutes an especially interesting objective since the involved enzymes are specific for insect isoprenoid biosynthesis, which diverges from that of vertebrates after farnesyl pyrophosphate formation.[1] We report here the isolation and identification of brevioxime from *Penicillium brevicompactum*. This is a new compound with an unusual heterocyclic oxime structure, which exhibits a very high activity as JH biosynthesis inhibitor.

Entomopathogenic fungi constitute an interesting source of active compounds for insect control, which mainly arise from secondary metabolic pathways. The isolation of compactin from several species of *Penicillium* constituted an important step toward the discovery of anti-JHs of natural origin.[2] However, compactin is not insect specific, since it inhibits the first step of isoprenoid biosynthesis instead of the final methylation/epoxidation steps.[2b] By contrast, the new compound brevioxime isolated in the present work from *P. brevicompactum* exhibits a very high specific activity as JH biosynthesis inhibitor.

Results and Discussion

A systematic screening was performed with 118 strains of *Penicillium* from fungal contamination of cereals. The most interesting results were obtained with a strain of *P. brevicompactum*. Thus, the dichloromethane extract obtained from its culture medium exhibited the highest entomotoxicity to *Oncopeltus fasciatus* (20% mortality and 40% precocious adults at 10 μg/cm^2 following the method of Bowers et al.[3]). For in vitro studies, a radiochemical assay of the *corpora allata* (CA) biosynthetic activity, based on the stoichiometric incorporation of radioactivity from labeled methionine into the newly synthesized hormone, was employed. The dichloromethane extract completely blocked spontaneous JH biosynthesis in vitro in the radiochemical assay [4] at a dose of 120 μg/mL. This assay served as guide for silica gel column separation, which led to a fraction (F9) with remarkable in vitro activity. Its analysis by HPLC revealed the presence of several components. Again, bioassay-guided fractionation by semipreparative HPLC allowed separation of the pure active compound Pb/9B. Its structure was assigned by means of combined spectral data and X-ray diffraction of a carefully recrystallized sample. On this basis, the structure of a disubstituted heterocyclic oxime (Figure 1) was assigned to Pb/9B, and hence, it was named brevioxime. A view of the crystal structure showing the atomic numbering scheme is depicted in Figure 2. This confirmed the bicyclic skeleton and the type, number, and position of substituents and location of the double bonds.

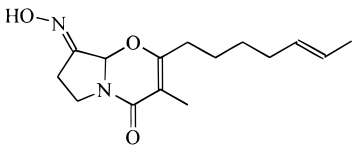

Figure 1. Structure of brevioxime.

Brevioxime was able to prevent spontaneous JH biosynthesis in *Locusta migratoria* CA in vitro. It completely blocked hormone production at 100 μM and caused 60% inhibition at 0.5 μM. The presence in CA culture medium of late intermediates of the hormone biosynthetic pathway, such as farnesol or farnesoic acid, did not restore JH production, even with farnesol doses as high as 200 μM. This clearly indicates that at least the final steps of the hormone biosynthesis (methylation and epoxidation) are inhibited by the chemical. Such steps are insect specific,[1,5] although different orders perform them in different sequence. As the bioassays were performed in *L. migratoria*, in which methylation occurs prior to epoxidation by methyl farnesoate epoxidase, it was not possible to disclose which of the two steps was blocked by brevioxime. To circumvent this problem, the JH inhibitory activity of brevioxime was determined on *Agrotis ipsilon* male CA, since in this case only JH epoxyacid is produced without further methylation.[6] Our

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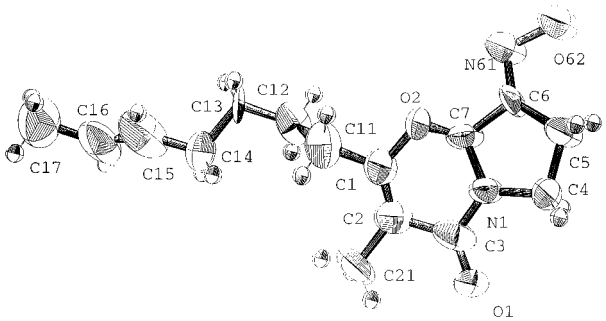

Figure 2. Crystal structure of brevioxime showing the atomic numbering scheme. The absolute configuration shown is arbitrary.

preliminary results showed complete inhibition of the process when the chemical was present at 100 μM. This is an indication that brevioxime could be a P450-linked enzyme inhibitor.

In summary, isolation of the new anti-JH compound brevioxime from *P. brevicompactum* nicely illustrates the possibility of discovering leading active molecules from hitherto relatively unexplored natural sources such as fungi. This appears to be a promising strategy for the development of biorational insecticides.

Experimental Section

Culture Conditions. The fungus was isolated from contaminated corn and was classified by The International Mycological Institute (IMI, Surrey, U.K.) as *P. 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 containing antibiotic test broth (1:9 volume ratio), and the mixture was incubated for 15 days in the dark at 28 °C.

Extraction and Preliminary Fractionation. 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 a previously developed[4] in vitro radiochemical assay of the *corpora allata* biosynthetic activity, it was possible to localize the biological activity exclusively in fraction number 9. Its yield was 590 mg.

Isolation and Characterization of the Active Compound. Preparative HPLC chromatography of the active fraction was achieved using the following separation conditions: column Lichrosorb-Si60, 7 μm (25 × 2.5 cm); mobile phase CH_2Cl_2:AcOEt (25:75, v/v); flow 10 mL/min; detection UV (254 nm). Three subfractions were obtained. The biological activity was associated with the second one (9 mg). Further purification of this subfraction by semipreparative HPLC was achieved using the following conditions: column Lichrospher-Si60, 5 μm (25 × 0.4 cm); mobile phase C_6H_{14}:AcOEt (50:50, v/v); detection UV (254 nm). This led to the isolation of 5 mg of the pure active compound with $[\alpha]^{23}_D = -39°$ (*c* 0.24, $CHCl_3$). Spectral data: HRMS *m*/*z* 278.16185 (calcd for $C_{15}H_{22}N_2O_3$ 278.16304); MS *m*/*z* 278 (3), 261 (5), 179 (2), 151 (6), 111 (16), 99 (100), 98 (26), 83 (71), 81 (26), 55 (42), 41 (21); IR (cm^{-1}) 1640 (C=O), 1450, 1047; UV (nm) 254 (ϵ 680); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.35 (m, 2H), 1.55 (m, 2H), 1.63 (d, J = 2.4 Hz, 3H), 1.83 (s, 3H), 1.98 (m, 2H), 2.28 (m, 2H), 2.88 (m, 2H) 3.47 (m, 1H), 4.05 (m, 1H), 5.38 (m, 2H), 5.50 (s, 1H), 8.02 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 10.0 (CH_3), 17.8 (CH_3), 23.6 (CH_2), 26.2 (CH_2), 29.1 (CH_2), 30.5 (CH_2), 32.1 (CH_2), 41.5 (CH_2), 84.0 (CH), 106.9 (C), 125.1 (CH), 130.8 (CH), 158.0 (C), 163.2 (C), 163.6 (C).

X-ray Analysis. A very small amount of the compound was crystallized by slow diffusion of C_6H_{14} into a CH_2Cl_2 solution. Molecular formula $C_{15}H_{22}N_2O_3$, M = 278.16, orthorhombic, space group $P2_12_12_1$, a = 6.0200(10) Å, b = 9.164(2) Å, c = 27.662(6) Å, Z = 4, U = 1526.0 (5) Å^3 , D_c = 1.27 g cm^{-3}, λ (Cu Kα) = 1.541 78 Å, T = 293(2) K, μ (Cu Kα) = 6.8 cm^{-1}. A well-shaped crystal with approximate dimensions of 0.27 × 0.25 × 0.10 mm was mounted on a Rigaku AFC7 four-circle diffractometer coupled to a Cu target rotating anode X-ray source. Unit cell dimensions were determined from the angular setting of 25 reflections. An orthorhombic cell was obtained, and the space group $P2_12_12_1$ was confirmed from the structure determination. A total of 1789 reflections were measured ($3.20 = 2q = 154.6$) using the $2q/w$ method. The intensity of three standard reflections monitored every 60 min showed no systematic variations. Lorentz and polarization corrections were applied, but no allowance was made for absorption. The structure was solved by direct methods (SHELXTL PLUS) and refined by full-matrix least-squares analysis on F^2 (SHELX-93). The refinement converged at $R1 = 0.099$ ($F < 4s$ (F)) and $R_2 = 0.291$ (all data). Selected bond lengths (Å) and angles (deg): O(1)−C(3) 1.21(2), O(2)−C(1) 1.40(2), O(2)−C(7) 1.42(2), N(1)−C(3) 1.34(2), N(1)−C(4) 1.49(2), C(1)−C(2) 1.28(2), C(2)−C(3) 1.57(2), C(4)−C(5) 1.55(2), C(5)−C(6) 1.51(2), C(6)−N(61) 1.30(2), C(6)−C(7) 1.50(2), C(15)−C(16) 1.30(2), N(61)−O(62) 1.43(2); N(61)−C(6)-C(7) 119(2), N(61)−C(6)-C(5) 128.7(14), C(12)−C(13)-C(14) 111(2), C(16)−C(15)-C(14) 133(3), C(15)−C(16)-C(17) 131(3), C(6)−N(61)-O(62) 107.0(13).[7]

The limited amount of available material did not allow us to determine the absolute configuration by reaction with asymmetric derivatization reagents.

Inhibition of the JH Biosynthesis. The CA were extracted with $CHCl_3$ (1.5 mL), and 20 μL of cold JH-III was added as internal standard. Separation was made with silica gel plates, and the elution solvent was a mixture of xylene−AcOEt 4:1 v/v. Detection of ^{14}C-labeled JH-III was made with a RITA analyzer (Isomess IM3000). For further details see our previous work.[4]

Supporting Information Available: Copies of the original HPLC chromatograms and radiochromatograms of the JH biosynthesis inhibition bioassays and HRMS data and MS, IR, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR spectra of brevioxime (13 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(7) The author has deposited atomic coordinates for this structure with the Cambridge Crystallographic Data Centre. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.