Noreupenifeldin, a Tropolone from an Unidentified Ascomycete[⊥]

Sloan Ayers,[†] Deborah L. Zink,[†] Joanne S. Powell,[‡] Christine M. Brown,[‡] Alan Grund,[§] Gerald F. Bills,[△] Gonzalo Platas,[△] Donald Thompson,[‡] and Sheo B. Singh*,[†]

Natural Products Chemistry, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, Merck and Co., Inc., Branchburg Farm, 203 River Road, Somerville, New Jersey 08876, Bio-Technical Resources, 1035 South Seventh Street, Manitowoc, Wisconsin 54220, and CIBE, Merck, Sharp & Dohme de Espana, S.A., Josefa Valcarcel 38, 28027 Madrid, Spain

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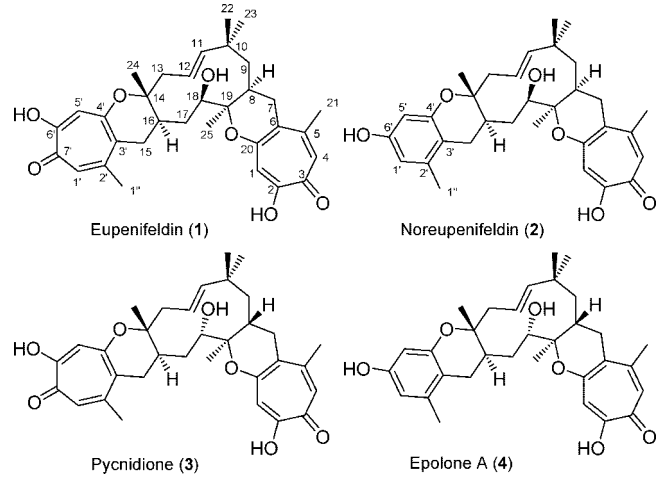
Noreupenifeldin (**2**), a new monotropolone derivative of the bistropolone eupenifeldin (**1**), was isolated from an unidentified ascomycete by bioassay-guided fractionation as part of our search for new anthelmintics. The structure of **1** was confirmed by comparison with literature data. The structure of **2** was elucidated from MS and 1D and 2D NMR data. Compounds **1** and **2** are diastereomers of pycnidione (**3**) and epolone A (**4**), respectively. Compounds **1**−**3** were evaluated for their anthelmintic activity against the parasitic worm *Hemonchus contortus*. Compounds **1** and **3** exhibited modest *in vitro* activity, showing EC_{90} 50 and 83 μg/mL, respectively, in reducing motility of L3 larvae of *H. contortus*. Compound **2** was inactive, indicating that the second tropolone moiety is required for activity.

Great success has been achieved in the development of anthelmintic drugs in the last 50 years. During this period all current classes of anthelmintic synthetic drugs were developed, including the benzimidazoles and imidazothiazoles (such as levamisole). The discovery and development of the macrolactone natural product avermectin from *Streptomyces avermitilis* was the most significant advance made during the 1980s, leading to the regulatory approval of ivermectin for treatment of parasitic infections. Ivermectin exhibited superior potency and excellent broad-spectrum activity. However, resistance to ivermectin and all other classes of antiparasitic drugs has been observed, and infection by internal parasites such as *Hemonchus contortus* remains one of the greatest economic threats to the sheep and goat industry. Increased emergence of resistance to current drugs necessitates the continued need for further research to discover new classes of anthelmintics, especially those with novel modes of action.[1]

Our strategy to discover new anthelmintics has been to screen extracts of terrestrial plants[2–4] and bacterial[5] and fungal fermentations using *in vitro* activity against *H. contortus*,[6] which is one of the most prevalent parasitic worms that infect small ruminants. Evaluation of *in vivo* activity was accomplished using *Heligmosomoides polygyrus* (a related organism) in mice.[7] An acetone extract of the liquid fermentation of an ascomycetous fungus showed *in vitro* activity and was selected for further study. Bioassay-guided fractionation using the *in vitro H. contortus* assay led to the isolation of a known bistropolone, eupenifeldin (**1**), and a new monotropolone derivative of **1** named noreupenifeldin (**2**). The isolation, structure elucidation, and anthelmintic activities of these compounds are described.

An organic extract of the liquid fermentation was partitioned with methylene chloride and ethyl acetate. The methylene chloride and ethyl acetate extracts were combined and chromatographed on a C_{18} preparative HPLC column to yield compounds **1** (57.6 mg/L) and **2** (10.5 mg/L) (see Experimental Section for details).

Compound **1** was obtained as an off-white solid. Low-resolution MS analysis indicated a molecular weight of 548. ^{1}H NMR data were essentially identical to literature data reported for eupenifeldin,[8] allowing assignment of **1** as eupenifeldin rather than pycnidione (**3**).[9]



Noreupenifeldin (**2**) was also obtained as an off-white solid. Its formula, $C_{32}H_{40}O_6$, was deduced from the molecular ion of 521.2877 $[M + H]^+$ obtained by HRESIFTMS. This formula differs from eupenifeldin (**1**) by a loss of CO. The carbon count was confirmed by the ^{13}C NMR spectrum, which showed 32 signals. The ^{1}H NMR data of **2** were very similar to eupenifeldin (**1**), except for a few significant chemical shift differences. The aromatic resonances H-1′ and H-5′ were shifted upfield by approximately 1 ppm, and the aromatic methyl singlet H_3-1″ was shifted upfield by 0.34 ppm. These shifts were consistent with the substitution of the tropolone with a benzene ring, supported by the absence of an electron-withdrawing carbonyl group in the ring. The substitution of a benzene ring for a tropolone ring was also evident from the ^{13}C NMR spectrum, where two carbonyl signals were present from δ_C 169–171 in **1**, while only one signal was present in this region for **2**. The skeleton of **2** was confirmed by the COSY, HMQC, and HMBC spectroscopic data. The relative configuration of **2** was confirmed to be the same as that of eupenifeldin (**1**) by the ROESY spectrum. Selected ROESY correlations are shown in Figure 1. The key correlations included H-8 to H-25, H-16, and H-11, confirming the *cis*-ring fusion at C8−C19. Similar key ROESY correlations were from H-18 to H-9β and H-24. These correlations suggested that the relative configuration of **2**, at these positions, was identical to that of eupenifeldin (**1**) and that **2** was a diastereomer of epolone A (**4**).[6] The numbering scheme used here is different than in ref 6

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* To whom correspondence should be addressed. Tel: (732) 594-3222. Fax: (732) 594-6880. E-mail: sheo_singh@merck.com.

[†] Natural Products Chemistry, MRL.

[‡] Branchburg Farm.

[§] Bio-Technical Resources.

[△] CIBE.

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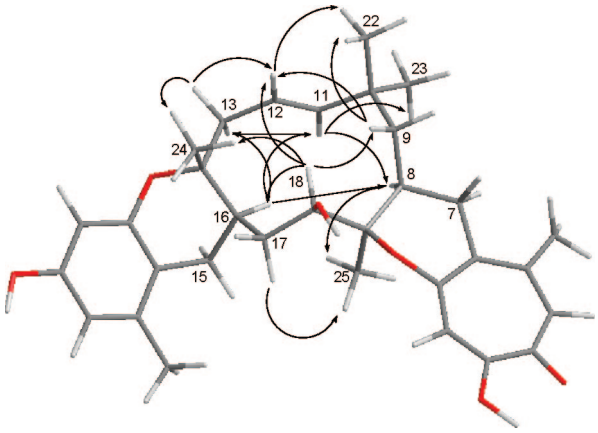

Figure 1. ChemDraw 3D model and selected ROESY correlations for **2**.

to keep the positions of the benzene ring of **2** consistent with the tropolone ring of **1**, for ease of comparison.

Compounds **1**−**3** were tested in the *in vitro H. contortus* assay as described previously.[6] *In vitro* activity is reported as EC_{90}: the concentration at which L3 larval motility is reduced by 90%. Compounds **1** and **3** reduced motility of L3 larvae with EC_{90} values of 50 ± 10 and 83 ± 12 μg/mL, respectively. These compounds were much less potent than the ivermectin control (EC_{90} = 0.18 μg/mL). Compound **2** was inactive *in vitro*. In the *in vivo* test against *H. polygyrus*-infected mice,[7] neither compound (**1** or **3**) gave any meaningful reduction of worm counts when dosed intramuscularly at 50 mg/kg.

In conclusion, we have isolated a new tropolone named noreupenifeldin (**2**), which is an analogue of eupenifeldin (**1**) and a diastereomer of epolone A (**4**). Eupenifeldin (**1**) was isolated as the major metabolite and is a diastereomer of pycnidione (**3**). Compounds **1** and **3** showed *in vitro* anthelmintic activity, but were inactive *in vivo*. Compound **2** was inactive *in vitro*, which indicated that the second tropolone moiety was required for the *in vitro* anthelmintic activity. Compound **3** isolated from a *Phoma* sp. was reported to inhibit the cleavage of β-casein by stromelysin with an IC_{50} of 31 μM.[9] Compounds **3** and **4**, isolated from an unknown fungus (ATCC 74390), are shown to induce erythropoietin gene expression 5-fold at a concentration of 1–1.6 μM.[10] The presence of two tropolone moieties in the molecule is not required for this activity.

Experimental Section

General Experimental Procedures. All reagents were obtained from Sigma-Aldrich and were used without further purification. The NMR spectra were obtained on a Varian Inova 500 MHz spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei, and solvent shifts were used for internal reference. IR data were obtained on a Perkin-Elmer Spectrum One instrument. UV/vis spectra were taken on a Perkin-Elmer Lambda 35 UV/vis spectrometer. Low-resolution mass spectra were obtained on an Agilent MSD, and high-resolution mass spectra were obtained on a Thermo Finnigan LTQ-FT with the standard Ion Max API source (without the sweep cone) and ESI probe. Pycnidione (**3**) was a gift from Dr. Guy Harris.[9] Ivermectin was used as the commercial product Ivomec injection (1%) for cattle and swine (Merial, Duluth, GA).

Producing Organism and Fermentation. The fungus (an ascomycete, deposited as F-150626 in CIBE, Spain) was isolated from rabbit dung collected near Yepes, Toledo, Spain. Cultures of the fungus produced vegetative hyphae only in agar culture and formed perithecial initials only when inoculated onto sterile plant stems. Perithecial initials contained pseudoparaphyses, but never formed mature asci or ascospores, which prevented positive identification. Nuclear DNA was extracted from living mycelia, and the intertranscribed spacers 1 and 2 and the 5.8S region of the rDNA were PCR-amplified, purified, and sequenced. Similarity searches in GenBank and EMBL indicated some similarity to ascomycetes in the Pleosporales. The strain was maintained on plates of potato dextrose agar (Difco) at 22 °C and preserved as mycelium in 10% glycerol at −80 °C. Agar plugs were used to inoculate 250 mL baffled flasks containing 50 mL of KFA seed medium, which consisted of corn steep liquor (5 g/L of distilled H_2O), tomato paste (40 g/L), oat flour (10 g/L), glucose (10 g/L), and a trace element mix (10 mL/L). The trace element mix consisted of $FeSO_4 \cdot 7H_2O$ (1 g/L), $MnSO_4 \cdot 4H_2O$ (1 g/L), $CuCl_2 \cdot 2H_2O$ (25 mg/L), $CaCl_2$ (100 mg/L), H_3BO_3 (56 mg/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (19 mg/L), and $ZnSO_4 \cdot 7H_2O$ (200 mg/L), adjusted to pH 6.8. The flasks were incubated at 28 °C at 220 rpm for 2–5 days to obtain vegetative cells. Seed flasks were used to inoculate unbaffled flasks containing CYS80 medium (20% volume), which consisted of sucrose (80 g/L), yellow corn meal (50 g/L), and yeast extract (1 g/L), with no pH adjustment. Liquid cultures were incubated at 22 °C at 220 rpm for 13 days.

Extraction and Isolation. Three liters of liquid fermentation broth was mixed with 3 L of acetone and shaken for 1 h on a platform shaker. The extract was filtered in a sintered-glass funnel with Celite as filter aid. The cells were washed with 500 mL of MeOH and 500 mL of MEK. The MeOH and MEK washes were combined with the acetone/water extract, which was then concentrated to aqueous (~2.8 L). This aqueous concentrate was partitioned with $CHCl_3$ (2 × 1.2 L), then with EtOAc (2 × 1.2 L). All organic phases were dried over Na_2SO_4, combined, and evaporated to afford a brown oil. This oil was brought up to 40 mL with acetone, and an 8 mL aliquot was fractionated by preparative HPLC (Waters XTerra RP C_{18}, 300 × 19 mm, 7 μm, 10 mL/min, 40 min 40–100% MeCN in H_2O (both with 0.1% TFA) gradient) in eight 1 mL injections. Fractions eluting at 23.0 min were lyophilized to afford eupenifeldin (**1**, 34.6 mg, 57.6 mg/L), and fractions eluting at 25.8 min gave noreupenifeldin (**2**, 6.3 mg, 10.5 mg/L).

Noreupenifeldin (2): off-white solid: IR (ZnSe film) ν_{max} 3241, 2956, 2926, 2869, 1619, 1592, 1462, 1443, 1428, 1170, 1141, 1087, 910, 732 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 205 (4.65), 256 (4.49), 362 (3.99) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.29 (1H, s, H-4), 7.09 (1H, s, H-1), 6.33 (1H, s, H-1′), 6.17 (1H, s, H-5′), 5.82 (1H, d, J = 16.0 Hz, H-11), 5.65 (1H, ddd, J = 16.0, 11.0, 3.5 Hz, H-12), 4.19 (1H, dd, J = 11.0, <1 Hz, H-18), 3.45 (1H, dd, J = 17.5, 12.5 Hz, H-15β), 2.75 (1H, dd, 13.5, 3.5 Hz, H-13β), 2.70 (1H, dd, 16.0, 4.5 Hz, H-7α), 2.54 (1H, dd, J = 13.5, 11.0, H-13α), 2.46 (3H, s, H-21), 2.44 (1H, m, H-15α), 2.32 (1H, dd, J = 16.0, <2.0, H-7β), 2.23 (1H, m, H-17α), 2.19 (1H, m, H-16), 2.16 (3H, s, H-1″), 1.78 (1H, dd, J = 14.5, <2.0, H-9β), 1.75 (1H, m, H-8), 1.55 (1H, ddd, J = 13.0, 11.0, <2.0, H-17β), 1.42 (3H, s, H-24), 1.10 (3H, s, H-23), 1.09 (3H, s, H-25), 1.05 (3H, s, H-22), 0.83 (1H, dd, J = 14.5, 4.5, H-9α); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.3 (C-3), 163.7 (C-2), 162.1 (C-20), 154.7 (C-6′), 153.6 (C-4′), 151.8 (C-5), 144.7 (C-11), 139.5 (C-2′), 125.2 (C-6), 124.9 (C-12), 124.3 (C-4), 115.2 (C-1), 110.9 (C-3′), 110.0 (C-1′), 101.4 (C-5′), 81.4 (C-14), 80.4 (C-19), 70.9 (C-18), 46.2 (C-9), 46.1 (C-13), 41.3 (C-16), 34.9 (C-10), 33.2 (C-15), 32.2 (C-8), 29.6 (C-17), 29.5 (C-7, C-23), 27.4 (C-21), 27.1 (C-22), 19.4 (C-1″), 19.2 (C-24), 15.6 (C-25); HRESIFTMS m/z 521.2877 (calcd for $C_{32}H_{40}O_6$ + H, 521.2904).

Biological Assays. The *in vitro* assay against *Hemonchus contortus* was used as described in ref 6. The *in vivo* mouse assay was modified from ref 3 and performed as follows: Swiss-Webster mice (approximately 30 g, Taconic Laboratories, Germantown, NY) were inoculated with 200–400 L3 *H. polygyrus* larvae. The mice were checked for infection around day 12 postinoculation and then dosed im (intramuscularly) with test compound in triplicate. On day 3 post-treatment, mice were euthanized and the intestine was collected (from below stomach to above cecum), opened, and placed in 5 mL of sterile water. The mucosa was scraped and rinsed through a 200-mesh screen. The rinseate was then examined for the presence of worms, and worms were counted. Worm counts for treated mice were then compared to infected, untreated mice as negative controls and infected mice treated with ivermectin (10 mg/kg) as positive controls.

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