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# COPROPHILIN: AN ANTICOCCIDIAL AGENT PRODUCED BY A DUNG INHABITING FUNGUS

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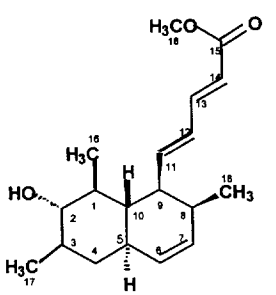
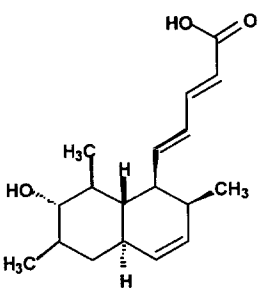
**Abstract:** Coprophilin, a decalin pentanedienoic acid methyl ester, was isolated from an unidentified fungus by bioassay guided separation. It inhibited (MIC = 1.5 μM) the growth of *Eimeria tenella* in an in vitro assay. The isolation, structure elucidation, absolute stereochemistry and biology are described. 

Coccidiosis, a poultry disease caused by the protozoan *Eimeria tenella* and other *Eimeria* species, is of major economic concern to the chicken farmers worldwide. Many of the current drugs used to prevent coccidiosis are polyether ionophore antibiotics such as salinomycin[1] and narasin.[2] The effectiveness of these drugs is diminishing due to the emergence of rapid resistance. Therefore, new anticoccidial agents are needed to control ionophore-resistant strains. Recently, we reported[3,4] the discovery of apicidin which is highly active against *E. tenella* and a host of other apicomplexans.

During the course of continued screening of natural products, we discovered coprophilin, a new decalin pentanedienoic acid methyl ester (**1**), from an unidentified, non-sporulating, fungus (MF 5773). It inhibited the growth of *E. tenella* in MDBK 441 cells and showed a MIC value of less than 1.5 μM without formation of schizonts. We describe, herein, the isolation, structure elucidation, absolute stereochemistry and biological activity of coprophilin (**1**) and its derivatives.



**1**: Coprophilin



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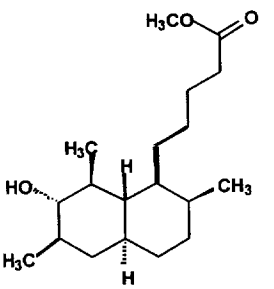

**3**

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**Isolation**

Silica gel chromatography of a methylethylketone extract of the fermentation broth of the nonsporulating fungus[5] grown on a solid or in a liquid nutrient medium, followed by either size exclusion chromatography on sephadex LH-20 or precipitation from chilled hexane afforded coprophilin (**1**).[6,7]

**Structure Elucidation**

FAB mass spectral analysis of coprophilin gave a pseudo molecular ion at *m/z* 305 $(M + H)^+$ which upon high-resolution (found *m/z* 304.2053) analysis provided a molecular formula $C_{19}H_{28}O_3$ (calcd 304.2038). EI mass spectral analysis of coprophilin gave a very weak molecular ion at *m/z* 304 but gave a strong ions at *m/z* 286 ($M-H_2O$, HR, found 286.1935, calcd for $C_{19}H_{26}O_2$: 286.1933) and at *m/z* 273 ($M-OCH_3$). Trimethylsilylation gave a mono-TMS derivative indicating the presence of only one exchangeable proton (IR: 3400 $cm^{-1}$).

The molecular formula derived from mass spectral analysis was corroborated by the $^{13}C$ NMR spectrum and suggested that coprophiln has six degrees of unsaturation. APT spectrum of **1** in $CDCl_3$ revealed the presence of four methyl groups including a methoxy, one methylene, 13 methines (seven aliphatic and six olefinic) and an ester carbonyl (IR: 1720 $cm^{-1}$). The $^1H$ NMR spectrum was assigned by a $^1H$-$^1H$ COSY experiment (Table 1). The $^1H$ NMR (500 MHz) spectrum of **1** in $CDCl_3$ exhibited three methyl doublets and a methoxy singlet, an olefinic doublet and five olefinic doublets of doublets. This molecule exhibited a proton–proton spin network which was completely assigned by $^1H$-$^1H$ COSY spectrum and this enabled us to step through the molecule from one end to the other.

**Table 1**. $^1H$ and $^{13}C$ NMR Assignments and HMBC Correlations of Coprophilin in $CDCl_3$

| Position | δC | Type | δH (mult, *J* in Hz) | HMBC H→C |
|---|---|---|---|---|
| 1 | 43.9 | CH | 1.39, *m* | C-2, 9, 10, 16 |
| 2 | 82.1 | CH | 2.7, *t*, 9.6 | C-1, 3, 16, 17 |
| 3 | 39.6 | CH | 1.45, *m* | C-1, 2, 4, 16 |
| 4 | 39.2 | $CH_2$ | 1.75, *dd*, 3.4, 13.3 | C-2, 3, 5, 6, 10 |
| | | | 0.94, *q*,12.2 | C-17 |
| 5 | 41.7 | CH | 1.85, *m* | C-4, 6, 10 |
| 6 | 131.6 | CH | 5.45, *dt*, 9.4, 2.1 | C-4, 5, 7, 8, 10 |
| 7 | 132.7 | CH | 5.57, *ddd*, 2.7, 4.4, 9.4 | C-5, 6, 8, 9, 10 |
| 8 | 36.3 | CH | 2.2, *m* | C-6, 7, 9, 10, 11, 18 |
| 9 | 49.4 | CH | 2.44, *ddd*, 5.5, 10.2, 9.0 | C-5, 7, 8, 10, 11, 12, 18 |
| 10 | 46.0 | CH | 1.07, *t*, 9.8 | C-4, 9 |
| 11 | 149.7 | CH | 6.18, *dd*, 10.3, 15.4 | C-10, 12, 13 |
| 12 | 126.8 | CH | 6.10, *ddd*, 15.4, 10.3, 9.0 | C-9, 11, 13, 14 |
| 13 | 145.2 | CH | 7.29, *dd*, 10.5, 15.4 | C-11, 12, 14, 15 |
| 14 | 118.8 | CH | 5.80, *d*, 15.4 | C-12, 15 |
| 15 | 167.8 | CO | | |
| 16 | 19.1 | $CH_3$ | 1.06, *d*, 6.2 | C-1, 2, 10 |
| 17 | 17.9 | $CH_3$ | 1.02, *d*, 6.2 | C-2, 3, 4 |
| 18 | 16.51 | $CH_3$ | 0.94, *d*, 7.1 | C-7, 8, 9 |
| 19 | 51.1 | $OCH_3$ | 3.72, *s* | C-15 |

Once all of the proton spin systems were assigned, an HMQC experiment was used to assign the $^{13}C$ shifts in the $^{13}C$ NMR spectrum of **1**. Finally, an HMBC experiment ($J_{CH}$ = 7 Hz) was used to confirm all of the assignments and further corroborate the structural assignments. The HMBC correlations are summarized in Table 1. Especially important were strong HMBC correlations from the three methyl groups. The methoxy group at δ 3.72 gave only one HMBC correlation to the carbonyl group hence establishing it as part of a methyl ester.

The geometry of the olefins were secured by the measurement of the coupling constants between the respective olefinic protons. For example, H-6 showed a coupling of 9.4 Hz with H-7 corresponding to a *cis* relationship between them. Similarly H-11, H-12 and H-13, H-14 each displayed a coupling of 15.4 Hz thus unambiguosly establishing a *trans* relationship between the respective olefinic protons.

**Stereochemistry**

**Relative stereochemistry:** The relative stereochemistry of decalin system of coprophilin was deduced from vicinal coupling constants and NOE difference spectroscopy. The H-2 methine appeared as a triplet and showed a coupling constant of 9.6 Hz indicating that it was axially oriented and consequently the H-1 and H-3 must also be axial in a cyclohexyl chair conformation. Thus the methyl groups at C-1 and C-3 and the hydroxy group at C-2 must have equatorial orientation. This was further supported by the lack of NOE enhancements of H-1 and H-3 when H-2 was irradiated. Irradiation of H-2 gave NOE enhancements to the axial proton H-4 (δ 0.94, *t*, *J* = 12.2 Hz) and H-10 (δ 1.07, *t*, *J* = 9.8 Hz) indicating a 1,3 diaxial orientation of H-2 and H-10. The axial H-4 and H-10 appeared as triplets with large coupling constants implying that H-5 and H-9 must likewise be axial. This was further supported by NOE enhancements of H-5 and H-1 from H-9. A similar effect was also experienced by H-8 from H-9 indicating that H-8 must be in the equatorial position. H-9 also showed NOE to H-11 and H-12. Irradiation of H-8 produced NOE enhancements to H-5 and H-7. The former NOE could be explained by a flip between a psuedo-chair and boat conformations of the cyclohexene ring. The stereochemistry and NOE's of coprophilin is shown in Figure 1.

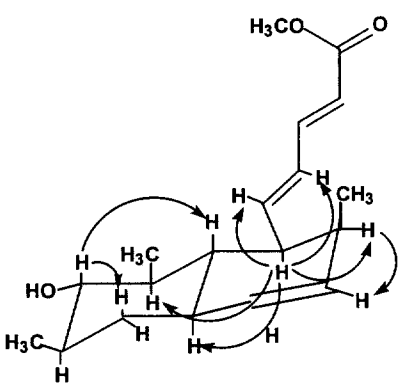


**Figure 1.** Selected NOEs, stereochemistry, and conformation of coprophilin

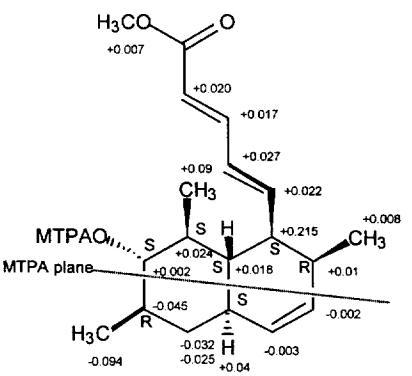


**Figure 2.** Δδ = δ*S*-δ*R* of coprophiln-MTPA esters

**Absolute stereochemistry**: The absolute stereochemistry of coprophilin (**1**) was deduced by application of modified Mosher ester method.[8] Reaction of *S*- and *R*-MTPA chloride with coprophiln gave *R*- and *S*-MTPA esters.[9] The $^1$H NMR spectra in $CDCl_3$ were fully assigned by using $^1$H-$^1$H COSY determinations and the difference in chemical shifts ($\Delta\delta = \delta S\text{-}\delta R$) were measured as shown in Figure 2. As expected, $\Delta\delta$ of one side (top) of the molecule experienced positive values and the other side of the MTPA plane experienced a negative values. Therefore, based on these positive and negative values of $\Delta\delta$ the absolute stereochemistry of 1*S*, 2*S*, 3*R*, 5*S*, 8*R*, 9*S* and 10*S* was assigned to coprophiln as shown in Figure 2.

## Biological Activity

Coprophilin inhibited the growth of *E. tenella* in the MDBK 441 cell line and showed a MIC value of 1.5 μM. In order to evaluate SAR coprophilin was hydrolyzed with LiOH at room temperature over several days to give carboxylic acid (**2**), hydrogenation with 10% Pd/C gave hexahydro ester (**3**). Neither derivatives inhibited the growth of *E. tenella* when tested at concentrations up to 25 μM. Coprophilin is somewhat related to hynapenes[10] and arohynapenes[11] isolated as anticoccidial agents against monensin-resistant *E. tenella*. Surprisingly, several of these compounds, though free carboxylic acids, were reported to have activities at ~35 μM. Coprophilin is a methyl ester and its greater activity may be due to more efficient cell penetration.

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5. The non-sporulating fungus (MF 5773) was isolated from mule deer dung, near sunspot, Otero Co., forest in New Mexico. The vegetative mycelia of MF 5773 was inoculated on either solid corn based production media or liquid based production media to furnish coprophilin 300 mg/L or 375 mg/L, respectively.
6. Analytical HPLC conditions of coprophilin: Eka-Knobel C-18 column (250 mm × 4.6 mm), $CH_3CN$ (70%) - water (30%) at a flow rate of 1 mL/min, temperature 40 °C, photodiode array detection, $t_R$ = 10.3 min.
7. White powder, mp.82–83 °C, $[\alpha]^{22}_D$ +96° (*c* 0.92, MeOH), UV: $\lambda_{max}$ (MeOH); 267 (ε = 3376) nm, IR: $\nu_{max}$ (ZnSe): 3400, 2950, 2850, 1720, 1640, 1450, 1380, 1310, 1270, 1140, 1005, 700 $cm^{-1}$.
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9. $^1$H NMR (400 MHz) assignment of *R* and *S* MTPA esters: *R*-ester (δ, $CDCl_3$): 1.615 (1H, *m*, H-1), 4.608 (1H, *t*, *J* = 10 Hz, H-2), 1.79 (1H, *m*, H-3), 1.845 (1H, *dt*, *J* = 3.2, 12.8 Hz, H-$4_{ax}$), 1.095 (1H, *q*, *J* = 12.2 Hz, H-$4_{eq}$), 1.85 (1H, *m*, H-5), 5.473 (1H, *dt*, *J* = 2, 9.6 Hz, H-6), 5.621 (1H, *ddd*, *J* = 2.8, 4.4, 9.6 Hz), 2.22 (1H, *m*, H-8), 2.446 (1H, *dt*, *J* = 5.6, 9.6 Hz, H-9), 1.115 (1H, *q*, *J* = 10.5 Hz, H-10), 6.149 (1H, *dd*, *J* = 9.6, 14.8 Hz, H-11), 6.073 (1H, *dd*, *J* = 10, 15.2 Hz, H-12), 7.273 (1H, *dd*, *J* = 10, 15.6 Hz, H-13), 5.796 (1H, *d*, *J* = 15.2 Hz, H-14), 0.792 (3H, *d*, *J* = 6.4 Hz, H-16), 0.912 (3H, *d*, *J* = 6.4 Hz, H-17), 0.96 (3H, *d*, *J* = 7.2 Hz, H-18), 3.753 (3H, s, $OCH_3$). *S*-ester (δ, $CDCl_3$): 1.639 (1H, *m*, H-1), 4.61 (1H, *t*, *J* = 10 Hz, H-2), 1.746 (1H, *m*, H-3), 1.814 (1H, *dt*, *J* = 4, 13.2 Hz, H-$4_{ax}$), 1.070 (1H, *q*, *J* = 12.4 Hz, H-$4_{eq}$), 1.89 (1H, *m*, H-5), 5.47 (1H, *dt*, *J* = 2.4, 9.6 Hz, H-6), 5.619 (1H, *ddd*, *J* = 2.8, 4.4, 9.6 Hz), 2.23 (1H, *m*, H-8), 2.467 (1H, *dt*, *J* = 5.6, 9.2 Hz, H-9), 1.173 (1H, *q*, *J* = 10.4 Hz, H-10), 6.171 (1H, *dd*, *J* = 9.6, 15.2 Hz, H-11), 6.10 (1H, *dd*, *J* = 10, 14.8 Hz, H-12), 7.29 (1H, *dd*, *J* = 10, 15.6 Hz, H-13), 5.816 (1H, *d*, *J* = 15.6 Hz, H-14), 0.882 (3H, *d*, *J* = 6.4 Hz, H-16), 0.819 (3H, *d*, *J* = 6.4 Hz, H-17), 0.968 (3H, *d*, *J* = 7.2 Hz, H-18), 3.761 (3H, s, $OCH_3$).
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