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J. Nat. Prod., **1993**, 56 (3), 341-344• DOI:
10.1021/np50093a005 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 3, pp. 341-344, March 1993

APPENOLIDES A–C: THREE NEW ANTIFUNGAL FURANONES FROM THE COPROPHILOUS FUNGUS *PODOSPORA APPENDICULATA*

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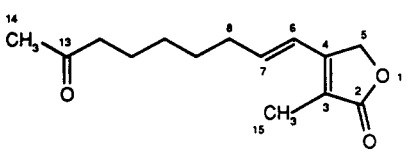
ABSTRACT.—Appenolides A [**1**], B [**2**], and C [**3**], three new antifungal 2(5*H*)-furanones, have been isolated from liquid cultures of the coprophilous fungus *Podospora appendiculata* (UAMH 7225) by repeated preparative tlc on Si gel. The structures were assigned on the basis of nmr and ms data.

Interspecies competition among coprophilous (dung-colonizing) fungi has been observed (1,2) and may play an important role in the pattern of colonization and successional changes within the corresponding fungal ecosystem. There is increasing evidence that chemical investigation of such species can lead to the discovery of novel antifungal compounds (3–7). During our continuing search for new antifungal agents from coprophilous fungi, we found that organic extracts of cultures of *Podospora appendiculata* (Awd. ex Niessl) Niessl (Sordariaceae) exhibited both antibacterial and antifungal effects. This report describes the isolation and structure determination of three new antifungal 2(5*H*)-furanones **1–3** from these extracts. Naturally occurring furanones are not uncommon, and members of this class have been reported to exhibit a variety of biological activities (8–13).

A subculture of an isolate of *P. appendiculata* (UAMH 7225) [**1**] originally obtained from deer dung was found to exhibit antifungal activity in assays for antagonism against competitor fungi. *P. appendiculata* was grown in liquid fermentation culture, and the EtOAc extract of the resulting culture filtrate displayed antifungal activity. The EtOAc extract was subjected to repeated preparative tlc on Si gel to afford appenolides A [**1**], B [**2**], and C [**3**] as the agents responsible for the activity. The structures of these three compounds were determined by analysis of nmr (Tables 1 and 2) and ms data. Hreims data for the most abundant compound, appenolide A [**1**], suggested the formula $C_{14}H_{20}O_3$. The ir spectrum indicated the presence of two carbonyl groups, and the absence of OH or carboxylic acid groups. ^{13}C-nmr and DEPT data for **1** confirmed that all 20 protons were attached to carbons and revealed the presence of ester and ketone groups, one trans-disubstituted ($^3J_{H,H}$ = 16 Hz) and one tetrasubstituted olefin, an oxygenated methylene unit, two Me groups, and five aliphatic methylene carbons. Selective INEPT (14) irradiation of either the oxymethylene signal at 4.94 ppm or the vinyl Me signal at 1.85 ppm resulted in correlations to both carbons of the tetrasubstituted olefin (121.7 and 156.6 ppm), and to the ester carbon (178.1 ppm). Irradiation of the vinylic proton doublet at 6.49 ppm resulted in polarization transfer to the same two



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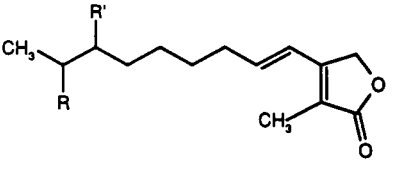

2 R=OH, R′=H
3 R=H, R′=OH

TABLE 1. ^{1}H-nmr Data for Appenolides A [**1**], B [**2**], and C [**3**] in CD_3OD.[a]

Proton	Compound		
	1	**2**	**3**
H-5	4.94 (m)[b]	4.88 (m)	4.87 (br s)
H-6	6.49 (br d, 16.1)	6.43 (d, 16.1)	6.43 (d, 16.1)
H-7	6.20 (dt, 16.1, 7.1)	6.14 (m)	6.13 (m)
H-8	2.26 (m)	2.20 (m)	2.20 (m)
H-9	1.49 (m)	1.42 (m)	1.41 (m)
H-10	1.33 (m)	1.33 (m)[c]	1.28 (m)[c]
H-11	1.57 (m)	1.33 (m)[c]	1.28 (m)[c]
H-12	2.48 (t, 8.1)	1.33 (m)[c]	3.35 (m)
H-13	—	3.64 (m)	1.28 (m)[c]
H-14	2.12 (s)	1.07 (d, 6.2)	0.85 (t, 7.4)
H-15	1.85 (t, 1.5)[b]	1.79 (br s)	1.77 (br s)

[a]The data for **1** were recorded at 600 MHz. Those listed for **2** and **3** were recorded at 300 MHz.

[b]At 600 MHz, the homoallylic coupling between H_3-15 and H_2-5 could be measured, and a very weak coupling between H_2-5 and H-6 ($\ll$1 Hz) could also be detected.

[c]Overlapping multiplets.

olefinic carbons, the oxymethylene carbon (71.2 ppm), and an aliphatic methylene carbon (34.2 ppm). Given the absence of any exchangeable protons, the number of unsaturations, and the number of available oxygen atoms, these results required the presence of a 3-methyl-2(5*H*)-furanone substituted at the 4 position with a trans-disubstituted olefin unit. The chemical shift of the other Me singlet (2.12 ppm) and its correlation with the ketone carbon (212.0 ppm) in a selective INEPT experiment indicated the presence of a methyl ketone. Moreover, the ms showed an abundant ion at *m/z* 58 characteristic of a McLafferty rearrangement for a methyl ketone with no α substituents. Only the aliphatic methylene carbons remained to be assigned, and these must be linked to form a five-carbon chain connecting the disubstituted olefin to the ketone carbon. This conclusion was supported by COSY data. These results, along with an

TABLE 2. ^{13}C-nmr Data (75 MHz) for Appenolides A [**1**], B [**2**], and C [**3**] in CD_3OD.

Carbon	Compound		
	1	**2**	**3**
C-2	178.1	178.1	178.4
C-3	121.7	121.6	121.9
C-4	156.6	156.6	156.9
C-5	71.2	71.2	71.5
C-6	121.6	121.5	121.8
C-7	141.5	141.7	141.9
C-8	34.2	34.4	34.8
C-9	29.5	30.4	30.2
C-10	29.7	26.7	26.7
C-11	24.6	29.8	37.9
C-12	44.1	40.1	74.1
C-13	212.0	68.5	31.4
C-14	29.8	23.5	10.6
C-15	8.4	8.5	8.7

HMQC experiment (15) conducted at 600 MHz, permitted assignment of all of the proton and carbon signals and verified the structure of appenolide A as **1**.

The structures of appenolides B and C were assigned by analysis of COSY and 1D nmr data and by spectral comparison with appenolide A. The only differences between these structures were found to reside in the side chain. DEPT, ^{13}C-nmr, and ^{1}H-nmr data for appenolide B [**2**] revealed the presence of a hydroxylated methine carbon in place of the ketone carbon of **1**. The ms of appenolide B is consistent with this assignment, as it showed a molecular ion two mass units higher ($[M]^+$ at *m/z* 238), and relatively intense $[M-18]^+$, $[M-15]^+$, and *m/z* 45 ions. The structure of the molecule was confirmed as **2** by analysis of COSY data. The ^{1}H and ^{13}C data for appenolide C [**3**] indicated that the only difference between **2** and **3** was the position of the OH group. The COSY data for appenolide C revealed that one of the methylene units associated with the complex multiplet at 1.28 ppm was apparently coupled to a Me triplet at 0.85 ppm and to the hydroxylated methine proton at 3.35 ppm. However, this observation alone did not permit conclusive assignment due to overlap of the aliphatic methylene signals. The location of the OH group at C-12 was confirmed by the ms of appenolide C, which contained major ions at $[M-18]^+$, $[M-29]^+$, and *m/z* 59. Thus, the structure of appenolide C was established as **3**. The considerable upfield shift of the terminal Me carbon (C-14, 10.6 ppm) is consistent with this assignment (16). The ^{13}C-nmr chemical shifts of **2** and **3** were assigned by comparison with those of appenolide A, 2-hexanol, and 3-hexanol (16). The absolute stereochemistry of appenolides B and C remains to be determined, although the sample of appenolide B obtained appears to be racemic ($[\alpha]_D$ 0°).

Appenolides A–C display activity in standard disc assays (17) against *Candida albicans* (ATCC 14053) with zones of inhibition ranging from 12 to 14 mm at 150 μg/disk. Appenolides B and C also show activity against *Bacillus subtilis* (ATCC 6051) with zones of inhibition of 8 mm at 150 μg/disc, although appenolide A is inactive at the same level. Appenolide A [**1**] displays activity in centerpoint inoculation disk assays against the coprophilous fungi *Sordaria fimicola* (NRRL 6459) and *Ascobolus furfuraceus* (NRRL 6460), causing 54 and 26% reductions in radial growth rates at 150 and 50 μg/disk, respectively. Compounds **2** and **3** exhibit similar antifungal effects.

EXPERIMENTAL

GENERAL PROCEDURES.—Nmr spectra were recorded in CD_3OD, and chemical shifts were referenced relative to the corresponding solvent signals (3.30 ppm/49.0 ppm). ^{1}H-nmr, ^{13}C-nmr, COSY, and selective INEPT experiments were performed on a Bruker AC-300 spectrometer operating at 300 and 75 MHz, respectively. The HMQC data was obtained on a Bruker AMX-600 operating at 600 MHz (^{1}H dimension). All carbon assignments (Table 2) are consistent with multiplicities established by DEPT experiments. Selective INEPT experiments were optimized for a $^{n}J_{CH}$ value of 7 Hz. Procedures for the antifungal assays have been described previously (5).

CULTIVATION OF *P. APPENDICULATA*.—The culture of *P. appendiculata* employed in this work (UAMH 7225) was a subculture of an isolate originally obtained from a sample of deer dung collected by DM in Little Lepreau, Charlotte County, New Brunswick, Canada. This subculture of *P. appendiculata* is deposited with the University of Alberta Mycological Herbarium Collection (UAMH). Twenty 2-liter flasks, each containing 400 ml of potato dextrose broth (Difco), were individually inoculated with one-cm^2 agar plugs taken from stock cultures of *P. appendiculata* (Difco potato dextrose agar). Flask cultures were incubated at 25–28° and aerated by agitation on a New Brunswick Model G10 orbital shaker at 160 rpm for 30 days. Efforts to produce the appenolides on soy-flour- or corn-meal-based media were unsuccessful.

ISOLATION AND CHARACTERIZATION OF APPENOLIDES A–C.—The filtered broth (8 liters) was extracted with EtOAc (1.2 liters), and the organic phase was dried ($MgSO_4$) and concentrated to afford 1.2 g of brown oil which showed antibacterial and antifungal activity. Portions of the crude extract (2 × 220 mg) were separated into four distinct bands by preparative tlc on Analtech Si gel plates (20 × 20 × 0.1 cm) using $CHCl_3$-MeOH (9:1) as the developing solvent. The antifungal activity resided in the two least polar

fractions [R_f 0.79 (23 mg) and R_f 0.63 (45 mg)]. The first band was separated by preparative tlc with $CHCl_3$-Me_2CO (9.5:0.5) to afford appenolide A (6.6 mg, R_f 0.89). The second fraction was further purified by developing four times on preparative tlc with $CHCl_3$-Me_2CO (8.5:1.5), affording appenolides B (5.9 mg, R_f 0.51) and C (6.0 mg, R_f 0.65).

Appenolide A [**1**].—Colorless oil: uv (MeOH) 223 (ε 3300), 262 nm (6900); ir (neat) 3025, 2934, 1747, 1712, 1228, 1204, 795, 731, 726 cm^{-1}; ^1H nmr see Table 1, ^{13}C nmr see Table 2; eims *m/z* $[M]^+$ 236 (55%), 193 (4.3), 160 (16), 151 (100), 147 (18), 138 (10), 137 (12), 133 (43), 125 (30), 112 (30), 105 (33), 95 (21), 93 (28), 91 (35), 79 (38), 77 (37), 58 (14), 55 (26); hreims *m/z* 236.1421 (calcd for $C_{14}H_{20}O_3$, 236.1412).

Appenolide B [**2**].—Colorless oil: uv (MeOH) 219 (ε 2700), 262 nm (6400); $[\alpha]_D$ 0° (*c* = 0.12, MeOH); ^1H nmr see Table 1; ^{13}C nmr see Table 2; eims *m/z* $[M]^+$ 238 (9.9), 223 (7.1), 220 (1.0), 181 (3.0), 150 (50), 125 (66), 121 (10), 112 (30), 107 (14), 105 (15), 96 (26), 95 (24), 91 (51), 81 (40), 79 (45), 77 (41), 57 (21), 55 (59), 45 (100), 43 (47), 41 (55).

Appenolide C [**3**].—Colorless oil: uv (MeOH) 219 (ε 3400), 262 nm (7400); $[\alpha]_D$ +5.3° (*c* = 0.13, MeOH); ^1H nmr see Table 1; ^{13}C nmr see Table 2; eims *m/z* $[M]^+$ 238 (8.6%), 220 (2.8), 209 (15), 178 (2.2), 163 (4.1), 150 (100), 125 (93), 112 (22), 107 (20), 106 (23), 105 (23), 96 (41), 91 (94), 81 (36), 79 (63), 77 (57), 59 (53), 55 (46), 43 (35), 41 (64).

ACKNOWLEDGMENTS

We thank the National Institutes of Health (R01 AI27436 and K04 CA01571) and the Alfred P. Sloan Foundation for financial support of this research. Some mass spectral data were obtained at the Midwest Center for Mass Spectrometry, which is partially supported by the National Science Foundation (DIR 9017262).

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Received 30 June 1992