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POLYTOLYPIN, A NEW ANTIFUNGAL TRITERPENOID FROM THE COPROPHILOUS FUNGUS *POLYTOLYPA HYSTRICIS*

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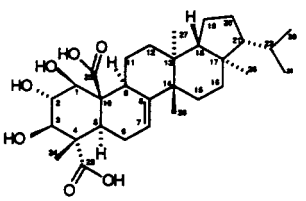
ABSTRACT.—Polytolypin [**1**], a new pentacyclic triterpenoid exhibiting antifungal and antibiotic activity, has been isolated from cultures of *Polytolypa hystricis* (JS189), a fungal colonist of porcupine dung. Two known compounds [**2** and **3**] were also isolated. Polytolypin was obtained by chromatographic fractionation of the EtOAc extract of *P. hystricis* liquid cultures, and the structure was assigned on the basis of 2D nmr and hrms data.

Observations of antagonistic interactions among coprophilous (dung-colonizing) fungi have led us to investigate the chemical causes of these effects as part of our search for new antifungal agents (1,2). Chemical studies of coprophilous fungi have been relatively limited, partly because these species are not found typically in soil samples commonly used as sources of microorganisms for screening purposes. During the course of our work a new fungal species was discovered. Sufficient differences from existing taxa were demonstrated to warrant the establishment of an entirely new genus in the Onygenales, and the new organism was subsequently assigned the name *Polytolypa hystricis* (JS 189=UAMH 7299) (3). Chemical investigation of *P. hystricis* cultures afforded a new antifungal triterpenoid [**1**], which we have named polytolypin, as well as two known compounds [**2** and **3**]. Herein, we report the details of the isolation, structure elucidation, and bioactivities of **1–3**.

The EtOAc extracts of the culture filtrate of *P. hystricis* were fractionated by Si gel vlc to yield polytolypin [**1**] as the most abundant antifungal metabolite. Although the hrfabms of **1** suggested a molecular formula of $C_{30}H_{46}O_7$, the ^{13}C-nmr spectrum (CD_3OD) contained only 29 signals. HMQC data, however, indicated that the signal at 31.9 ppm was attributable to overlapping methine and methylene carbon resonances, thus accounting for the 30 carbons indicated by the hrms data. The ^{13}C-nmr spectrum



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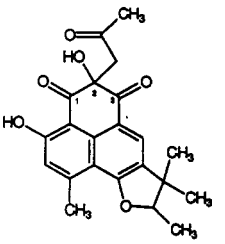

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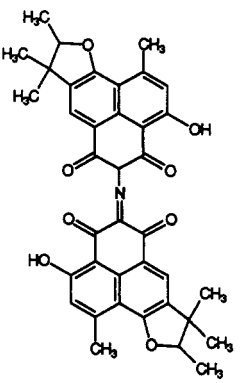

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included signals corresponding to two olefinic and two carbonyl carbons, along with six methyl groups, and three oxygenated sp^3 carbons. Based on this information, and the required number of unsaturations, a pentacyclic structure was indicated. In addition, the nmr data and molecular formula were suggestive of a triterpenoid (Table 1). The presence of five exchangeable protons (based on DEPT) and two carboxyl carbons required that the seven oxygens be present as three OH and two free-acid groups.

Correlations observed in the COSY spectrum indicated the presence of isolated spin-systems corresponding to five fragments in structure **1** (C-1–C-3; C-5–C-7; C-9–C-11–C-12; C-15–C-16; and C-18–C-22–C-29/30). These fragments were linked together primarily on the basis of HMBC data (Table 1). HMBC correlations of H-5 with C-1, C-3, C-4, and C-10, in addition to those of H-1 with C-9, C-10, and C-25, enabled the assignment of the A ring and secured the location of the C-25 carboxyl group. An additional correlation between H-5 and C-9 in the HMBC spectrum supported

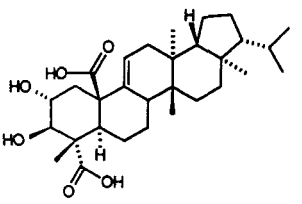
TABLE 1. Nmr Data for **1** in CD_3OD.

Position	^{1}H nmr ppm (mult., J=Hz)	^{13}C nmr (ppm)	HMBC correlations (Carbon)	NOESY correlations (Proton)
1ax	3.23 (d, 10)	83.5	2,3,9,10,25	3,5,9
2ax	3.68 (t, 10)	75.0	1,3	24
3ax	3.82 (d, 10)	77.9	1,2,4,5,23,24	1,5
4		53.7		
5	2.08 (dd, 6.0, 12)	44.6	1,3,4,6,9,10,24,25	1,3,9
6ax	2.49 (dddd, 2.9,2.9,12,18)	27.2	5,7,8	24
6eq	1.87 (m)		7,8,10	
7	5.53 (m)	119.3	9,14	
8	—	143.9		
9	3.00 (m)	48.3		1,5,27
10	—	51.9		
11	2.74 (m)	21.5	8,9,12,13	
	1.48 (m)			
12eq	1.47 (m)	34.1		
12ax	1.37 (m)			
13	—	36.8		
14	—	43.5		
15ax	1.65 (ddd, 3.2,13,13)	31.9		
15eq	1.54 (m)			
16eq	1.75 (ddd, 3.2,3.2,13)	37.6	14,15,18,28	28,30
16ax	1.52 (m)			
17	—	43.9		
18	1.53 (m)	55.5		26
19	1.46 (m)	20.9		
	1.30 (m)			
20	1.85 (m)	29.3	17,21	
	1.27 (m)		19,22	
21	0.97 (m)	60.9		
22	1.45 (m)	31.9		
23	—	178.9		
24	1.08 (s)	10.7	3,4,5,23	2ax
25	—	178.2		
26	1.06 (s)	25.1	8,13,14,15	12ax, 18
27	0.98 (s)	22.2	12,13,14,18	9, 15ax, 28
28	0.78 (s)	14.8	16,17,18,21	15ax, 16eq, 27, 30
29	0.84 (d, 6.5)	23.4	21,22,30	21
30	0.92 (d, 6.5)	22.6	21,22,29	16eq, 28

the connection of C-9 to C-10, and a correlation between H-7 and C-9 allowed closure of the B ring. The six well-dispersed methyl groups, four of which are bound to different quaternary carbons, showed all of the possible HMBC correlations, and this proved to be especially useful in determining the structure of polytolypin [**1**]. The signal for H_3-24 showed HMBC correlations to C-3, C-4, C-5, and C-23, requiring the connection of the second carboxyl group (C-23) to C-4 of the A ring. Correlations observed for the signals of the two adjacent methyl groups (H_3-26 and H_3-27) permitted closure of the C ring. Additional correlations between H_3-28 and C-16, C-17, C-18, and C-21 allowed connection of the remaining spin-systems, and required linkage of the D and E rings as shown in **1**.

The protons at positions C-1, C-2, C-3, and C-5 were assigned axial orientations based on ^{1}H-^{1}H coupling constants ($J_{H1-H2}=J_{H2-H3}=10$ Hz; $J_{H5-H6ax}=12$ Hz), and these assignments were consistent with NOESY data (Table 1). NOESY correlations between H-1 and H-9, and between H-3_{ax} and H-5_{ax}, led to the assignment of a trans ring fusion between rings A and B, and indicated that H-9 must be pseudo-axial. A NOESY correlation between H_3-24 and H-2 established the relative stereochemistry at C-4. The relative stereochemistry at C-13 was indicated by the presence of a NOESY correlation between H-9 and H_3-27, implying that they must be on the same face of the molecule. This also requires that the C ring adopt a boat-like conformation. Further correlations of H_3-27 with H_3-28 and H-15_{ax} in the NOESY spectrum allowed the relative stereochemistry at C-17 and C-21 to be assigned. Assignments of the C-14 and C-18 stereocenters were based on correlations of H_3-26 with both H-12_{ax} and H-18.

The structure of **1** is most closely related to retigeric acid B [**4**], a metabolite previously reported from the lichen *Lobaria retigera* (4). Polytolypin differs



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from **4** in the location of the double bond and the addition of an OH group at C-1. Although complete nmr data for **4** were not available, comparison of the nmr data for **1** with those of other model compounds (5) supported the structural assignment. Pentacyclic triterpenoids are widely distributed among plants and lichens (5,6), but are less common among fungi (6,7).

Fractionation of the EtOAc extract (2.0 g) obtained from a larger-scale fermentation of *P. hystricis* afforded two additional compounds [**2** and **3**]. Hrfabms data for **2** suggested the molecular formula $C_{22}H_{22}O_7$. The ^{1}H- and ^{13}C-nmr spectra exhibited a doubling of most of the signals. When each doubled signal was counted as a single ^{13}C-nmr resonance, the number of carbons was in agreement with the ms data. Based on these data, analysis of 2D nmr spectra (HMBC, HMQC, and NOESY), and comparison with literature data, this compound was identified as the tetracyclic triketone **2**. Compound **2** has been identified previously as an artifact formed by Me_2CO addition to atrovenetinone, a metabolite isolated from *Gremmeniella abietina* (8). Although atrovenetinone itself was not isolated from *P. hystricis*, the crude extracts had been exposed to Me_2CO before fractionation. Compound **2** was found as a mixture of inseparable C-2 epimers, as evidenced by the doubled nmr signals.

While the nmr spectra of **3** were similar to those of **2**, the ^{13}C-nmr data contained one less ketone carbonyl signal, and the ms data suggested a formula of $C_{38}H_{33}NO_{10}$. This highly colored com-

pound was identified as scleroderris blue [**3**], another *Gremmeniella abietina* metabolite, after comparison of spectral data with those published previously (8).

Compounds **1** and **2** demonstrated moderate activity against the early-successional coprophilous fungal colonist *Ascobolus furfuraceus* (NRRL 6460) (71 and 47% inhibition of growth, respectively) at 100 μg/disk (9). Compound **3** showed no activity in these assays at the same level. Polytolypin [**1**] was also found to be active against *Candida albicans* (ATCC 14053), producing an 11-mm zone of inhibition in a standard disk assay at 80 μg/disk. Compounds **1–3** are the first metabolites to be reported from this new fungal genus, and compounds **1** and **2** account for the antifungal activity of the *P. hystricis* extract.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Stock cultures were maintained on Difco potato dextrose agar slants and stored at 4°. ^{1}H- and ^{13}C-nmr spectra were obtained in CD_3OD on a Bruker AC300 spectrometer operating at 300 and 75 MHz, respectively. All 2D nmr spectra were recorded on a Bruker AMX600 spectrometer, operating at 600 MHz (^{1}H dimension). Chemical shifts were referenced to residual solvent signals (3.30 and 49.9 ppm). Fabms data were obtained on a VG ZAB-HF instrument, and cims data were recorded on a Nermag R1010C instrument.

CULTIVATION OF *P. HYSTRICIS*.—The culture of *P. hystricis* (JS 189) was isolated from porcupine dung collected by J.A.S. near Stoneleigh, Ontario, Canada (3). A subculture of this isolate has been deposited at the University of Alberta Mycological Herbarium Collection, and assigned the accession number UAMH 7299. Two 2-liter Erlenmeyer flasks, each containing 400 ml of potato dextrose broth, were inoculated with 1-cm^2 agar plugs from *P. hystricis* stock cultures. The flask cultures were then aerated by agitation on an orbital shaker at 150 rpm for 30 days at room temperature.

EXTRACTION AND ISOLATION.—The combined, filtered culture broth (800 ml) was extracted with EtOAc (3×500 ml). After evaporation of the solvent, the residual material (840 mg) was subjected to vlc on Si gel using a step gradient from 100% hexane to 100% $CHCl_3$, and finally to 50% MeOH in $CHCl_3$. The fraction eluting at 20% MeOH in $CHCl_3$ (102 mg) consisted of pure polytolypin [**1**].

Polytolypin [**1**].—White powder; mp 288–291°; $[\alpha]_D$ +34° (c=1.1 mg/ml, MeOH); ir (KBr) ν max 3600–2400 (br, OH, COOH), 3438 (br), 2955, 1700 (br), 1394, 1230, 1212, 1123 cm^{-1}; ^{1}H-, ^{13}C-nmr and HMBC nmr and NOESY data, see Table 1; lrcims *m/z* 536 $[M+H+NH_3]^+$ (17) 519 $[M+H]^+$ (100), 501 (7), 483 (10), 378 (18), 360 (6), 197 (3.2), 136 (17), 109 (13), 84 (23), 74 (27), 60 (67); hrfabms (3-NBA) observed $[M+Na]^+$ 541.3123, calcd for $C_{30}H_{46}O_7Na$ 541.3141.

ISOLATION AND IDENTIFICATION OF COMPOUNDS **2** AND **3**.—A 2.0-g portion of the EtOAc extract (2.5 g) obtained from a larger fermentation of *P. hystricis* (6×400 ml PDB) was subjected to Sephadex LH-20 cc (3 cm×27 cm), eluting with a solvent system of hexane-toluene-MeOH (3:2:1). The first active fraction to elute (fraction 2; 93 mg) was rechromatographed on Sephadex LH-20 (1×10 cm), using the same solvent system, to yield **3** (2.0 mg) as the first subfraction to elute. The second subfraction (44 mg) was further purified by reversed-phase hplc (C_{18}, 5 μm, 10×25 mm, 215 nm, 2 ml/min), eluting with 70% MeCN/H_2O, to yield 10 mg of **2**. Si gel cc of the major Sephadex LH-20 fraction (fraction 3; 643 mg) afforded 143 mg of **1**. The ^{1}H-nmr, ^{13}C-nmr, and ms data obtained for compounds **2** and **3** were in agreement with published values (4).

ACKNOWLEDGMENTS

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