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ISOLATION AND STRUCTURE DETERMINATION OF NEW MACROLIDE ANTIBIOTICS

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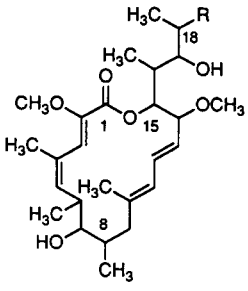
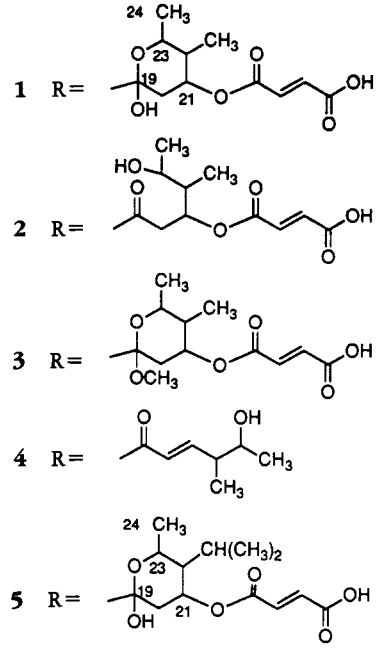
ABSTRACT.—Three new bafilomycin-like compounds PD 118,576-A1 [**1**], PD 118,576-A2 [**2**], and PD 118,576-A3 [**3**] were isolated from a new soil *Streptomyces* species (WP 3913). The structures of PD 118,576-A1, PD 118,576-A2, and PD 118,576-A3 were elucidated on the basis of spectroscopic studies including 2D nmr.

Recently, a new class of 16-membered macrolide antibiotics such as bafilomycins (1,2), hygrolidine (3), L-155,175 (4), and L-681,110 (5) has been reported. Compared to the usual 16-membered macrolides, these new macrolides do not contain sugar moieties connected by a glycosidic linkage; instead, they have a tetrahydropyran ring connected to the C-15 position through a three-carbon linker chain. All of these 16-membered macrolides share a common 16-membered macrocyclic nucleus but differ in substituents at the C-2, C-20, C-21, and C-23 position. These macrolides show a variety of biological activities such as antitumor, antifungal, and antiparasitic activity as well as being Na^+/K^+ ATPase inhibitors (1–5). It has been reported (6) that a new *Streptomyces* species (WP 3913), isolated from a soil sample, produced a new 16-membered macrolide compound, PD 118,576 [**4**], which is an open chain analogue of bafi-





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lomycin A_1 and showed antibacterial (Gram positive), antifungal (*Torulopsis abbida*), and antitumor (human colon adenocarcinoma) activities. The present report involves the isolation and structure determination of three additional new bafilomycin-like compounds, PD 118,576-A1 [**1**], PD 118,576-A2 [**2**], and PD 118,576-A3 [**3**], from the culture WP 3913.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The media ingredients for the fermentation of WP 3913 were as follows: Ambrex (Amber Lab), Cerelose (Adas Sugar), Maltrin (Rafal Spice Co.), N-Z case (Shefield Chemical Co.), kelp (Rafal Spice Co.), Pharmamedia (Trader's Protein), distiller's solubles (Grain Processing), Torula Yeast (Gallard Schlezinger Chemical Mfg.), Dow Corning Antifoam (Dow Chemical Co.). Other media ingredients were purchased from the Difco Laboratories. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, Si gel 60 (70–270 and 270–400 mesh) and tlc plate (Si gel 60F 254) were purchased from EM Scientific, and the hplc column (C18-μ Bondapak) was purchased from Waters. All other solvents and chemicals were analytical grade and used without further purification.

Tlc's were developed with the solvent system of EtOAc-EtOH-H_2O (8:1:0.5). The tlc plates were visualized under a uv light. An authentic sample of PD 118,576 [**4**] was a gift from Dr. J.H. Wilton (6). The R_f values of PD 118,576 [**4**], PD 118,576-A1 [**1**], PD 118,576-A2 [**2**], and PD 118,576-A3 [**3**] were 0.66, 0.40, 0.42, and 0.33, respectively. All hplc's were carried out on a reversed-phase column (Water's C18-μ Bondapak) with a Waters Model 660 (55% MeOH/0.05M NH_4OAc, pH 6.5, 2.5 ml/min, uv 254 nm). The retention times of compounds **4**, **1**, **2**, and **3** were 10.6, 12.8, 5.6, and 22.8 min, respectively.

The nmr spectra were recorded on an IBM-Bruker 270 MHz and GE 500 MHz FT instrument in $CDCl_3$. Samples dissolved in $CDCl_3$ are reported in ppm downfield from the internal reference TMS. The proton homonuclear-correlated spectra ($^1H,^1H$-COSY) were recorded on IBM 270 MHz spectrometer using the Bruker software program (COSY AU) (7) with the following parameters: spectral frequency SF = 270.133 MHz; spectral width SW1 = 0.5 × SW2 = 1077 Hz; pulse width P1 = P2 = 4.4 μsec (90°); relaxation delay D1 = 3 sec; data matrix SI1 = 0.5 × SI2 = 0.5 k; number of experiments NE = 128; number of scans NS = 32. The ^{13}C-nmr spectra were recorded on a GE 500 MHz nmr spectrometer with $CDCl_3$ as an internal standard. The $^1H,^{13}C$ heteronuclear-correlated spectra ($^1H,^{13}C$-COSY) were recorded on the GE 500 MHz nmr spectrometer with the following parameters: spectral frequency SF1 = 125.760 MHz and SF2 = 500.425 MHz; spectral window SW1 = 19607 Hz and SW2 = 3906 Hz; relaxation delay = 3 sec; number of experiments NE = 128; number of scans NS = 600.

FERMENTATION OF MICROORGANISM.—The microorganism (WP 3913) was maintained on a slant of yeast-malt extract agar at 23° and preserved at 4°. [For culture deposition, see Wilton *et al.* (6).] The seed medium contained Ambrex (0.5 g), Cerelose (0.1 g), Maltrin (2.4 g), N-Z case (0.5 g), Lexetin 152D (0.3 g), and $CaCO_3$ (0.2 g) in H_2O (100 ml). The antifoam (50% in H_2O, 2 ml) was added to the medium before autoclaving (121°, 15 lb, 20 min). To prepare the seed inoculum, one loop of the microorganism from the yeast malt extract agar slant was transferred aseptically to the seed medium (100 ml) in a 500-ml culture flask. The inoculated broth was incubated at 24° for 3 days on a Model GI rotary shaker at 130 rpm. The production medium contained sucrose (1.5 g), kelp (10 g), Pharmamedia (6.5 g), distiller's solubles (3.5 g), and Torula yeast (2.5 g) in distilled H_2O (1 liter). The pH of the medium was adjusted to 7 with 50% NaOH, and 50% antifoam in H_2O (10 ml) was added to the medium. The production medium was transferred to 250-ml culture flasks (50 ml each), and these culture flasks were autoclaved (121°, 15 lb, 20 min). The seed inoculum (2 ml each) was transferred aseptically to the production media culture flasks, and the culture flasks were incubated at 24° for 4 days at 300 rpm. A 10-liter fermentation was carried out under the same conditions described above and used for the isolation of macrolide antibiotics.

DETERMINATION OF OPTIMUM pH FOR EXTRACTION OF PD 118,576-A1 [**1**].—The production culture (50 ml), after fermentation, was equally divided into three separate flasks and the pH was adjusted to 3, 7, and 10 with 1 N HCl and 1 N NaOH. Each culture solution was extracted with EtOAc (2 × 25 ml). The organic layer was washed with H_2O (20 ml), dried over $NaSO_4$, and evaporated to dryness in vacuo. After the residue was partitioned between 90% MeOH (20 ml) and hexane (20 ml), the MeOH layer was evaporated to dryness in vacuo. The residue was dissolved in MeOH (2 ml) and assayed by both hplc and tlc.

ISOLATION OF COMPOUNDS **1–3**.—The production culture (10 liters, pH = 7) was extracted with EtOAc (2 × 5 liters), and the EtOAc layer was washed with H_2O (2 liters). The volume of EtOAc solution was reduced to about 50 ml in vacuo at 30°. The concentrated EtOAc extract was partitioned between 90% MeOH (500 ml) and hexane (500 ml). The MeOH layer was taken to dryness to yield 2.6 g of solid. The

MeOH extract (2.6 g) was dissolved in 10 ml of EtOAc-EtOH-H_2O (8:1:0.5) and loaded on a Si gel column (200 g) pre-equilibrated with the same solvent system. The column was eluted with EtOAc-EtOH-H_2O (8:1:0.5), and 20-ml fractions were collected. Fractions 13–18 were combined and evaporated to dryness (510 mg). The combined fractions 13–18 (500 mg) were dissolved in EtOH and absorbed onto 5 g of Si gel. After removing the EtOH, the dry sample-laden Si gel was loaded onto a Si gel column (100 g) which was pre-equilibrated with hexanes-EtOAc (4:6). The column was eluted stepwise, using flash cc techniques (7), with the following solvent gradients (200 ml each): hexanes-EtOAc (4:6), hexanes-EtOAc (2:8), and EtOAc-EtOH-H_2O (9:0.7:0.3). Fractions (20 ml) were collected and separation monitored by tlc. Fractions 29–37 (140 mg) and 38–44 (60 mg) were combined separately and evaporated in vacuo. The combined fractions 29–37 (140 mg) redissolved in EtOH-$CHCl_3$ (1:99) were loaded on a Sephadex LH-20 column (50 g). The Sephadex LH-20 column was eluted at 6 ml/35 min with EtOH-$CHCl_3$ (1:99), and 10-ml fractions were collected. PD 118,576-A3 [**3**] (20 mg) and PD 118,576-A1 [**1**] (60 mg) were obtained from fractions 29–33 and 35–42 of the Sephadex LH-20 column, respectively, as white amorphous powders. Fractions 38–44 (54 mg) obtained from the previous flash column chromatography step were combined, evaporated to dryness, and chromatographed on a Sephadex LH-20 (10 g) as described above. PD 118,576-A2 [**2**] (20 mg) was isolated from fractions 16–20 as a white amorphous powder.

PD 118,576-A1 [**1**].—Mp 94–97°; negative fabms *m/z* $[M-1]^-$ 691.3, 115; positive fabms *m/z* 558, 557, 541, 527, 509, 389, 334, 209, 181, 109, 89; uv (MeOH) 204 (25,300), 246 (28,000), 283

TABLE 1. ^{1}H-nmr Chemical Shifts and Coupling Constants of PD 118,576-A1 [**1**] and PD 118,576-A2 [**2**].[a]

Proton	Compound	
	1	**2**
2-OMe	3.65, s	3.66, s
H-3	6.61, brs	6.64, brs
4-Me	1.97, s	1.97, s
H-5	5.75, brd (9.5)	5.76, brd (9.5)
H-6	2.51, dqd (9.5, 7.3, 2.6)	2.52, dqd (9.5, 5.6, 1.5)
6-Me	1.05, d (7.3)	1.06, d (5.6)
H-7	3.29, dd (2.6, 5.6)	3.29, dd (5.6, 1.5)
H-8	ca. 2.00 (obsc.)[b]	ca. 2.00 (obsc.)[b]
8-Me	0.92, d (6.0)	0.91, d (6.9)
H_a-9	ca. 2.00 (obsc.)[b]	ca. 2.00 (obsc.)[b]
H_b-9	ca. 2.00 (obsc.)[b]	ca. 2.00 (obsc.)[b]
10-Me	1.92, brs	1.91, brs
H-11	5.79, brd (10.6)	5.80, brd (10.6)
H-12	6.50, dd (15.0, 10.6)	6.49, dd (15.0, 10.6)
H-13	5.15, dd (15.0, 8.7)	5.14, dd (15.0, 9.1)
H-14	3.89, dd (8.7, 8.5)	3.75, dd (9.1, 7.7)
14-OMe	3.24, brs	3.22, brs
H-15	4.79, dd (8.7, <1)	4.97, dd (8.8, <1)
H-16	ca. 2.00 (obsc.)[b]	ca. 2.00 (obsc.)[b]
16-Me	0.82, d (6.8)	0.92, d (4.4)
H-17	4.09, brd (11.9)	3.80, brd (8.8, 3.7)
H-18	1.72, dd (5.4, 1.6)	2.68, dd (7.0, 3.7)
18-Me	1.01, d (6.8)	1.17, d (7.0)
H_a-20	ca. 2.00, m (obsc.)[b]	2.76, dd (AB sp, 2.9)
H_b-20	1.44, dd (12.0, 11.3)	2.67, dd (AB sp, 2.7)
H-21	5.40, ddd (12.0, 4.9, 4.9)	3.84, ddd (8.8, 3.8, 2.9)
H-22	ca. 2.00, m (obsc.)[b]	1.66, dqd (8.8, 6.9, 2.2)
22-Me	0.81, d (6.8)	0.91, d (6.9)
H-23	4.28, qd (6.5, 1.9)	5.44, dd (6.6, 2.2)
24-Me	0.99, d (6.5)	1.27, d (6.6)

[a]The ^{1}H-nmr spectra of PD 118,576-A1 and PD 118,576-A2 in $CDCl_3$ were recorded at 270 MHz. Chemical shifts are given in ppm downfield from TMS. *J* values, in Hz, are in parentheses.

[b]obsc. = obscured (overlapping signals from 1.95 to 2.20 ppm).

(11,400) nm; ir (KBr) 3433, 2974, 2931, 1756, 1686, 1647, 1457, 1248, 1103 cm^{-1}; ^1H nmr see Table 1; ^{13}C nmr see Table 2.

PD 118,576-A1 STABILITY STUDIES IN VARIOUS SOLVENTS.—Compound **1** (2 mg) was dissolved in 3 ml of $CHCl_3$, and 0.5 ml portions of this solution were transferred to four separate small round-bottom flasks. The solvent in each flask was evaporated to dryness in vacuo. $CHCl_3$, MeOH, 95% EtOH, and H_2O (2 ml each) were separately added to each flask. Each sample was allowed to stand at room temperature (24°), and the solution was assayed by hplc after 2, 4, and 14 days.

ASSAYS FOR BIOLOGICAL ACTIVITIES.—The biological results are provided by Warner-Lambert/ Parke-Davis Pharmaceutical Research.

TABLE 2. ^{13}C-nmr Chemical Shifts (ppm) and Coupling Constants (Hz) of PD 118,576-A1 [**1**].[a]

Carbon	δc, multiplicity (J_{CH})	Carbon	δc, multiplicity (J_{CH})
C-1	168.6, s	14-OMe	56.2, q(141.6)
C-2	143.9 s	C-15	77.3, d (obsc.)[b]
2-OMe	60.4, q(144.3)	C-16	38.1, d(156.9)
C-3	133.8, d(150.7)	16-Me	10.3, q(126.9)
C-4	141.9, s	C-17	71.0, d(145.0)
4-Me	14.7, q(127.8)	C-18	42.2, d(176.9)
C-5	143.5, d(147.7)	18-Me	7.6, q(126.5)
C-6	37.3, d(152.9)	C-19	99.6, s
6-Me	17.9, q(127.0)	C-20	34.5, t(128.8)
C-7	81.8, d(139.8)	C-21	73.7, d(149.6)
C-8	40.8, d(181.4)	C-22	36.8, d(159.2)
8-Me	22.4, q(125.7)	22-Me	5.2, q(125.9)
C-9	41.9, t(126.6)	C-23	66.2, d(143.6)
C-10	133.7, s	23-Me	18.5, q(126.2)
10-Me	20.8, q(125.4)	C-1′	167.1, s
C-11	125.9, d(149.2)	C-2′	133.5, d(169.7)
C-12	133.8, d(150.7)	C-3′	136.1, d(147.7)
C-13	127.7, d(154.2)	C-4′	163.8, s
C-14	82.7, d(139.9)		

[a]The ^{13}C-nmr spectra of PD 118,576-A1 were recorded in $CDCl_3$ at 125.4422 MHz. Chemical shifts are given in ppm downfield from TMS. The J values (Hz) are shown in parentheses.

[b]obsc. = obscured (overlapping with solvent peaks).

RESULTS AND DISCUSSION

An optimum pH value for the extraction of culture media was determined. The hplc analyses of MeOH extracts obtained from the production culture at different pH's showed the highest yield for PD 118,576-A1 [**1**] at pH 7. At pH 10, the amount of [**1**] was reduced while the amount of PD 118,576 [**4**] increased, and at pH 3, the amount of PD 118,576-A3 [**3**] increased while the amount of **1** again decreased. Therefore, the culture (10 liters) was extracted with EtOAc at pH 7. A series of chromatographic purifications of the EtOAc extract afforded the major compound **1**, with two minor compounds, **2** and **3**. Stability studies in several solvents of **1**, at room temperature, for a period of 2 weeks showed that the compound was stable in $CHCl_3$, but unstable in MeOH, 95% EtOH, and H_2O. The tlc and hplc analyses indicated that **1** decomposed to either **2** or **4** in 95% EtOH and H_2O. In an MeOH solution, **1** was mainly converted to **3**. Thus it may be that compounds **4**, **2**, and **3** are artifacts of **1** formed during the isolation procedure. The negative fab mass spectra of **1** showed a nominal M-1 peak at *m/z* 691.3 ($C_{37}H_{56}O_{12}$, calcd 692.377) and an intense mass peak due to the fumaryl group. Its strong uv absorptions at 283 and 246 nm are in agreement with those chromophores of an α-methoxy-β-methyl-α,γ-dienone and an isolated diene, respec-

tively (1,3,4). Along with the above evidence, the structure of **1** was determined by various nmr experiments such as proton selective decoupling experiments, ^{1}H,^{1}H-homonuclear COSY (8), proton decoupled and gated decoupled ^{13}C nmr, DEPT (9), and ^{1}H,^{13}C-heteronuclear COSY (10). The characteristic ^{1}H-nmr spectral properties of **1** (Figure 1) included a typical AB-type quartet (δ 6.79, δ 6.89, $J = 15.8$ Hz) due to the two hydrogens of the fumaryl groups and ten methyl peaks due to two methoxy groups, two methyl groups attached to the quaternary carbon (C-4 and C-10), and six methyl groups attached to the various methine carbons. The proton-decoupled ^{13}C-nmr spectrum showed a total of 37 carbon peaks, including three carbonyl peaks (δ 163.8, δ 167.1 and δ 168.6) and a ketal carbon at δ 99.6 (1, 3–5). In the DEPT spectrum, with a final proton pulse of 135°, only two inversed methylene peaks were observed. A series of proton homonuclear selective decoupling experiments and a ^{1}H,^{1}H-homonuclear COSY spectrum of **1** showed the connectivity for C-5–C-6–C-7–C-8–C-9, C-11–C-12–C-13–C-14–C-15–C-16–C-17–C-18, and C-20–C-21–C-22–C-23–C-24. The difficulty in assigning the ^{1}H-nmr chemical shift values due to the overlapping signal density around 2 ppm was solved by the ^{1}H,^{13}C-COSY spectrum, which resolved all the peaks in both dimensions. Comparison of the ^{1}H- and ^{13}C-nmr data of **1** with those of other related macrolides such as **4**, bafilomycin C1 [**5**], and L-681,110 indicated that **1** has the same 16-membered macrolide lactone moiety. The ^{1}H- and ^{13}C-nmr data of the six-membered ring side chain portion of **1** were compared to those of bafilomycin C1 [**5**] (1) and hygrolidine (3), which also have a six-membered ring side chain portion but contain an isopropyl group and an ethyl group, respectively, on the C-22 carbon instead of a methyl group on the C-22 carbon as does **1**. From the above experimental results the chemical structure of **1** was proposed.

The structure of PD 118,576-A2 [**2**] was elucidated by extensive proton-selective decoupling experiments. A typical AB type quartet of a fumaryl group also appeared in the ^{1}H-nmr spectrum of **2** (Figure 1), as had been seen for PD 118,576-A1 [**1**]. The proton chemical shift and coupling constant values of **2** for the macrocyclic ring portion were essentially identical to those of **1**. All the hydrogens except those on the C-8 and C-9 carbons were correlated and assigned. However, the ^{1}H-nmr data of the side chain portion of **2** showed a major difference. Irradiation of the H-16 peak (δ 2.06) collapsed the resonances of H-15 (δ 4.97), 16-Me (δ 0.92), and H-17 (δ 3.80). When H-18 (δ 2.68) was irradiated, the coupling to the H-17 and to the 18-Me disappeared. Thus the connectivity of C-15–C-16–C-17–C-18 was established. Comparing the ^{1}H-nmr data to that of **1** the chemical shift values of the three hydrogens on C-18 (methine) and C-20 (methylene) of **2** exhibited downfield shifts (δ 2.68, δ 2.67, and δ 2.76 from δ 1.72, δ 1.44, and δ 2.04, respectively), which suggested the presence of a ketone at the C-19 position rather than a hemiketal. When H-17 (δ 3.80) and H-21 (δ 3.84) were irradiated at the same time the two geminal hydrogens on C-20 appeared as an AB-type quartet and the hydrogen on C-18 appeared as a quartet ($J = 6.8$ Hz) due to the remaining coupling to the adjacent methyl group (Figure 1). Irradiation of the H-22 (δ 1.66) of **2** decoupled H-21 (δ 3.84), 22-Me (δ 0.91) and H-23 (δ 5.44). Irradiation of the H-23 of **2** collapsed the doublet of H-24 (δ 1.27). Therefore, the correlation among C-20–C-21–C-22–C-23–C-24 was proven. From the above results, the structure of **2** was proposed.

In MeOH solution, PD 118,576-A1 [**1**] was readily converted to PD 118,576-A3 [**3**] as determined by comparing its tlc and hplc to that of **3** isolated directly from the fermentation broth. During the isolation of bafilomycin, which involved MeOH partitioning, Werner and co-workers (1,2) reported the isolation of the major bafilomycins A1, B1, and C1 as well as their minor methylated derivatives, A2, B2, and C2, respectively. They demonstrated that bafilomycin C1, a hemiketal, could be converted to

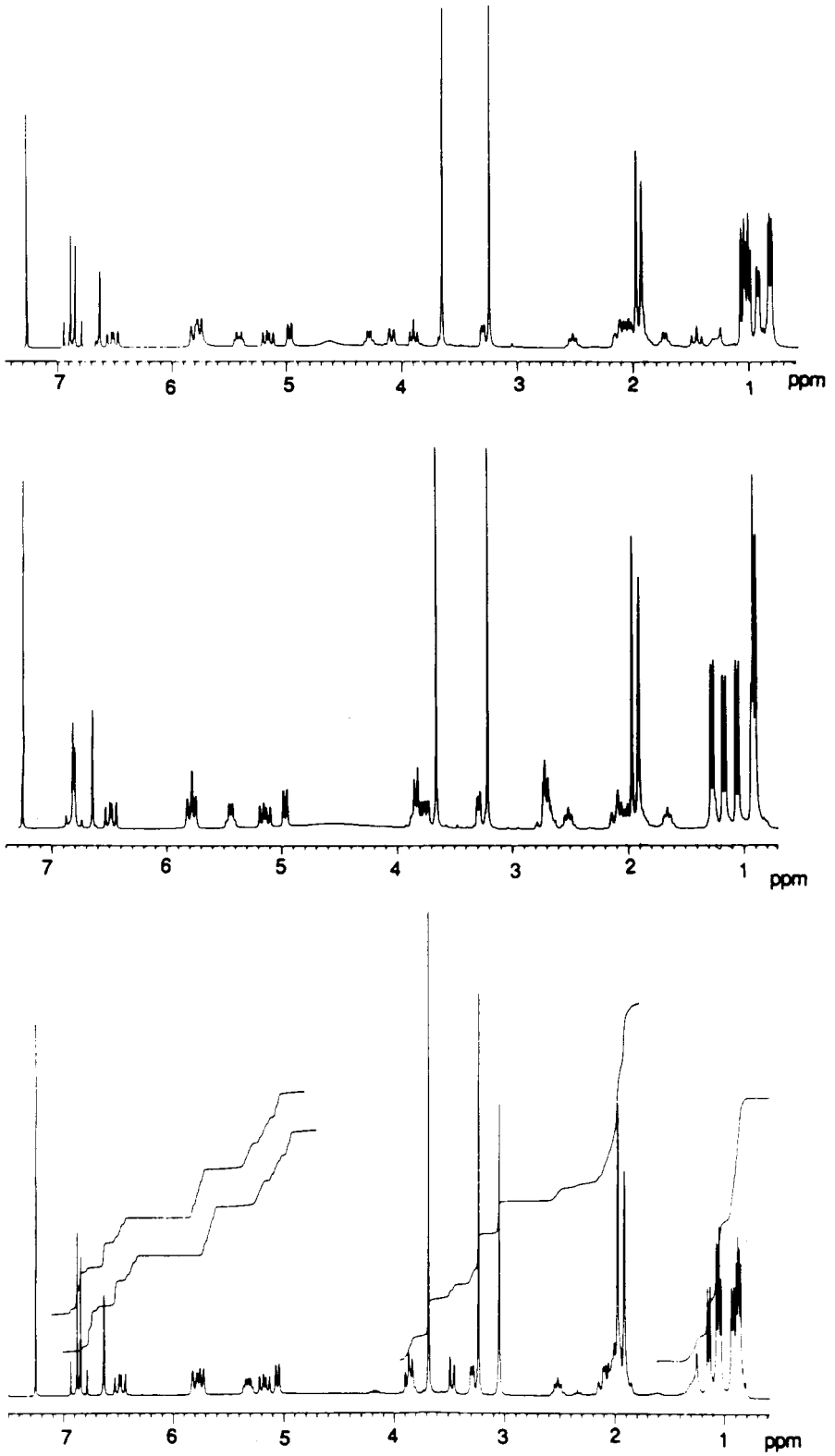

FIGURE 1. The ^{1}H-nmr spectra of PD 118,576-A1 [**1**] (upper), PD 118,576-A2 [**2**] (middle), and PD 118,576-A3 [**3**] (lower).

bafilomycin C2, a methyl ketal, in a MeOH solution. The ^{1}H-nmr spectrum of **3** (Figure 1) was similar to that of **1** but showed an additional OMe group at δ 3.02. Based on the above literature, decomposition experiments in various solvents, and ^{1}H-nmr data, we believe **3** may be a methylated derivative of **1**, at the 19-OH position, generated during the isolation procedure.

It is tempting to speculate, based on the various solvent and pH stability studies, that PD 118,576 [**4**] and PD 118,576-A3 [**3**] are both potential isolation artifacts. Compound **4** could have been formed by the base-catalyzed removal of one of the acidic C-20 methylene hydrogens, adjacent to the ketone function, followed by the elimination of the C-21 fumaryl group. The original isolation of **4** was carried out at an elevated pH (6). Compounds **1** and **2** are tautomers of each other. Compound **3**, formed only after the presence of MeOH solutions, is the methylated ketal derivative of **2**.

Of the three compounds which were isolated, only PD 118,576-A1 [**1**] was tested for biological activity and showed moderate activity against *Streptococcus pneumoniae*, *Bacillus cereus*, *Schizosaccharomyces pombe*, and *Rhodotorula aurantiaca*. Weak activities against L-1210 lymphocytic leukemia and HCT-8 human colon adenocarcinoma cell lines were also noted.

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