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ANTITUMOR AGENTS, 110.[1,2] BRYOPHYLLIN B, A NOVEL POTENT CYTOTOXIC BUFADIENOLIDE FROM *BRYOPHYLLUM PINNATUM*

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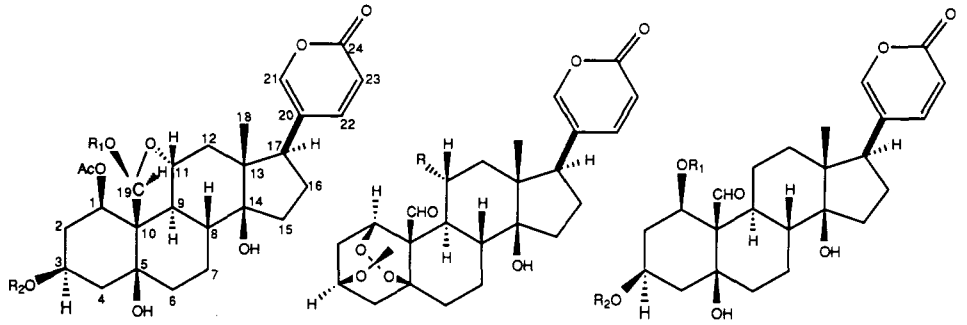
ABSTRACT.—Bryophyllin B [**1**], a potent cytotoxic bufadienolide, has been isolated from *Bryophyllum pinnatum* and its structure confirmed by the use of 2D-nmr techniques and difference nOe method. Transformation of bryotoxin C [**2**] to **1** with acid is also discussed.

We reported previously on the isolation of bryotoxin C (bryophyllin A) [**2**] (1), a bufadienolide 1,3,5-orthoacetate with potent cytotoxicity, and bersaldegenin-3-acetate [**3**] (3,4) from the $CHCl_3$ extract of *Bryophyllum pinnatum* (Crassulaceae). Bryotoxin C was first isolated by Capon *et al.* (2) from *Bryophyllum tubiflorum* without reporting any biological activity. Further investigation of a cytotoxic H_2O extract of this same plant has led to the isolation of bryophyllin B [**1**], which showed potent cytotoxity with ED_{50} <80 ng/ml against the in vitro growth of KB tissue culture cells (Table 1). We report herein the isolation and structural elucidation of bryophyllin B [**1**]. The transformation of **2** to **1** with acid is also discussed.

RESULTS AND DISCUSSION

The H_2O extract of the whole plant of *B. pinnatum* was concentrated and partitioned between H_2O and $CHCl_3$. Guided by the assay in KB cells as shown in Scheme 1, the active principles were concentrated in the $CHCl_3$ (Fractions A and B) and the H_2O extracts. Bryotoxin C (bryophyllin A) [**2**] and bersaldegenin-3-acetate [**3**] were isolated from the $CHCl_3$ extract. The H_2O-soluble part was extracted with *n*-BuOH, which in turn was chromatographed on Sephadex LH-20 Si gel and reversed-phase



1 $R_1=R_2=H$
4 $R_1=R_2=Ac$

2 R=OH
7 R=H

3 $R_1=H$, $R_2=Ac$
5 $R_1=R_3=Ac$
6 $R_1=Ac$, $R_2=H$

[1]For part 109 see D.J. Pan, Z.L. Li, C.Q. Hu, K. Chen, J.J. Chang, and K.H. Lee, submitted for publication in *Planta Med.*

[2]Presented, in part, at the 16th International Symposium on the Chemistry of Natural Products (IUPAC), Kyoto, Japan, May 29–June 3, 1988, "Abstracts," p. 232.

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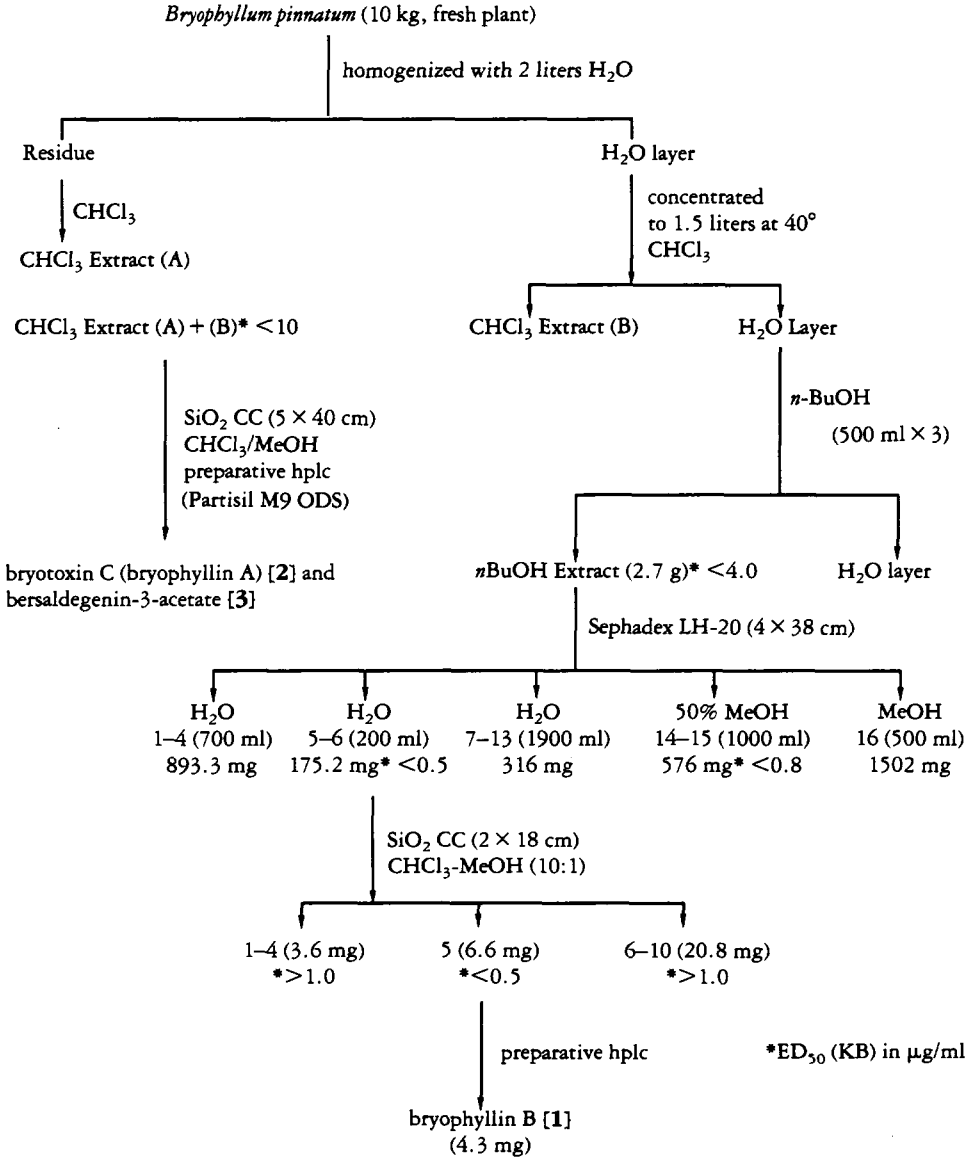
TABLE 1. Cytotoxicity of Compounds **1**, **2**, and **3** Against Various Tumor Cells.

Compound	KB (ng/ml)	A-549 (ng/ml)	HCT-8 (ng/ml)	P-388 (μg/ml)	L-1210 (μg/ml)
Bryophyllin B [**1**]	<80	—	—	—	—
Bryotoxin C [**2**]	14	10	30	>4	>4
Bersaldegenin-3-acetate [**3**] . .	<40	40	10	>4	>4

hplc, successively, to afford the active principle, bryophyllin B [**1**], in 0.000043% yield.

Bryophyllin B [**1**] was obtained as a colorless amorphous powder and analyzed for $C_{26}H_{34}O_9$. Its uv and ir spectra indicated the presence of a dienone system (λ max 298



SCHEME 1. Extraction and Isolation of Bryophyllin B [**1**].

nm) as well as hydroxyl (3400 cm^{-1}) and carbonyl (1695 cm^{-1}) groups as seen in **2**. The fabms of **1** showed the appearance of an $[M]^+$ peak at *m/z* 490 and fragment ion peaks at *m/z* 472 $[M-H_2O]^+$ and 432 $[M-HOAc]^+$.

Detailed analysis of the ^1H- and ^{13}C-nmr spectra (Table 2) of **1** in ^1H-^1H COSY (Correlation Spectroscopy) (Figure 1) and ^{13}C-^1H COSY (Figure 2) suggested the presence of a methyl (δ_H 0.84 and δ_C 20.57), a secondary acetoxyl (δ_H 2.07 and 4.69; δ_C 171.00, 21.20, and 73.38), a γ-substituted α,β,γ,δ-unsaturated-γ-lactone (α-pyrone) (δ_H 7.45, 7.87, and 6.92; δ_C 149.32, 147.32, 115.41, 122.66, and 162.07), a secondary hydroxyl (δ_H 3.79 and δ_C 65.01), two tertiary hydroxyl (δ_C 76.08 and 85.60), and lactol (δ_H 5.75, 3.99, and 1.30; δ_C 104.66, 79.99, and 49.53) groups. Treatment of **1** with Ac_2O in pyridine gave diacetate **4**, which was transformed to monoacetate **5** after standing in $CHCl_3$ for two days. These foregoing data and experiment indicate that **1** may be a compound closely related to **2** except that the orthoacetate and acetal groups differ from each other.

Each carbon signal, except the quarternary one, was assigned based on the ^{13}C-^1H COSY spectral data (Figure 2). In the ^1H-^1H COSY spectrum of **1**, six mutual relations from H-1, H-3 (twice), H-8, H-11, and H-17 to 2-CH_2, 4-CH_2, 7-CH_2, 12-CH_2, and 16-CH_2 methylene protons due to an ABX system were elucidated as shown in Figure 3. The configuration of each X-type proton of this ABX system is in an axial orientation, which has a large coupling constant (Table 1). Furthermore, the relationships be-

TABLE 2. ^1H and ^{13}C Chemical Shifts of Compound **1**.[a]

Position	^1H	^{13}C
1	4.69 (1H, dd, J = 4.03, 12.45 Hz)	73.38 (d)
2	2.11 (1H, ddd, J = 4.03, 4.64, 12.45 Hz)	39.51 (t)
	1.54 (1H, q, J = 12.45 Hz)	
3	3.79 (1H, m)	65.01 (d)
4	1.96–1.98 and 1.88 (each 1H, obscured signal)	47.79 (t)
5		76.08 (s)
6	1.98 and 1.58 (each 1H, obscured signal)	32.11 (t)
7	1.11 (1H, dddd, J = 3.78, 9.52, 12.94, 13.31 Hz)	20.78 (t)
	1.96–1.98 (1H, obscured signal)	
8	2.42 (1H, ddd, J = 2.93, 11.35, 13.31 Hz)	39.97 (d)
9	1.30 (1H, t, J = 11.35 Hz)	49.53 (d)
10		54.37 (s)
11	3.99 (1H, ddd, J = 3.30, 11.35, 11.85 Hz)	79.99 (d)
12	1.46 (1H, t, J = 11.85 Hz)	48.22 (t)
	2.03 (1H, dd, J = 3.30, 11.85 Hz)	
13		52.63 (s)
14		85.60 (s)
15	1.96–1.98 and 1.56 (each 1H, obscured signal)	34.92 (t)
16	2.21 and 1.83 (each 1H, m)	31.10 (t)
17	2.71 (1H, dd, J = 7.57, 8.79 Hz)	51.23 (d)
18	0.84 (3H, s)	20.57 (q)
19	5.75 (1H, s)	104.66 (d)
20		122.66 (d)
21	7.45 (1H, m)	149.32 (d)
22	7.87 (1H, dd, J = 2.57, 9.77 Hz)	147.32 (d)
23	6.29 (1H, dd, J = 0.85, 9.77 Hz)	115.41 (d)
24		162.07 (s)
-CO*Me*	2.07 (3H, s)	21.20 (q)
-*C*OMe		171.00 (s)

[a]The measurements were made on a JEOL GX-400 spectrometer in CD_3OD with TMS as an internal reference, and are expressed in terms of ppm.

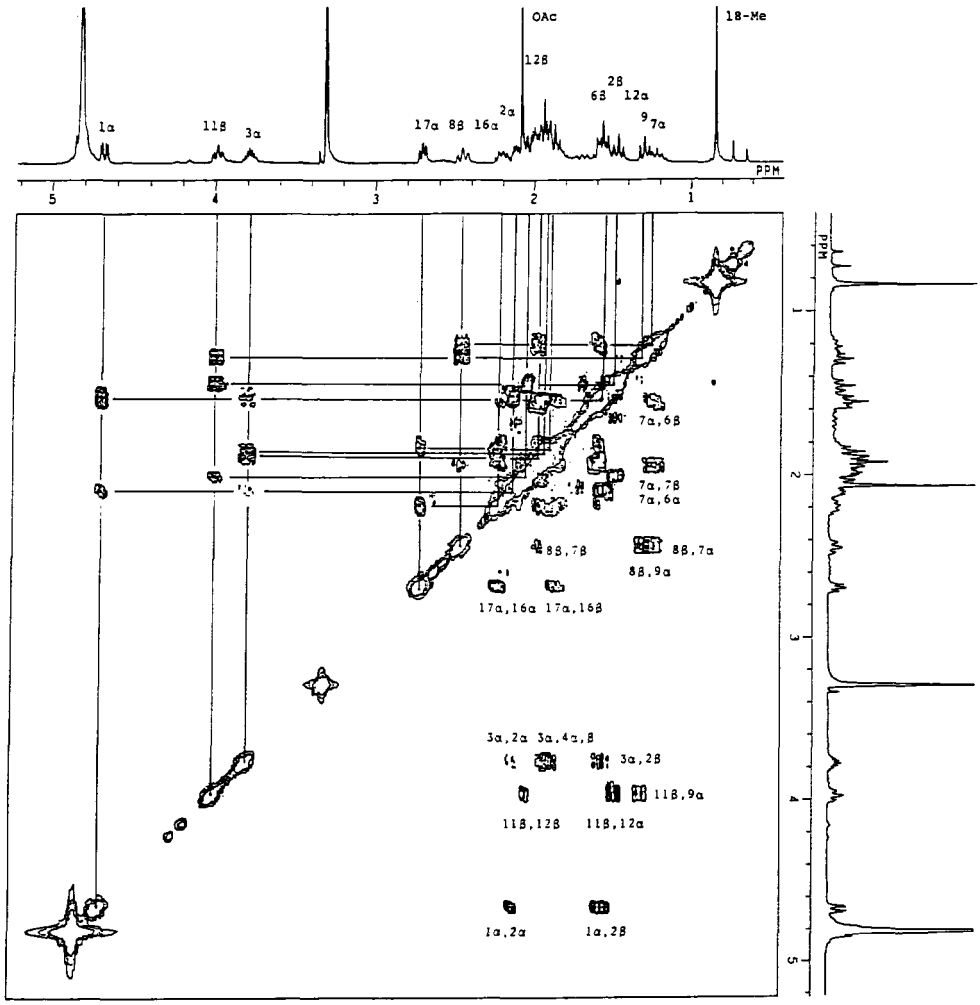


FIGURE 1. ^{1}H-^{1}H COSY Spectrum of Bryophyllin B [**1**].

tween H-8 and H-9 and between H-9 and H-11 were also trans diaxial ($J_{8,9}$ and $J_{9,11}$ = 11.35 Hz) to each other.

The ^{13}C-^{1}H long range COSY (5) of **1** was measured in order to confirm the partial structure discussed below. As shown in Figure 3, the proton signal at δ 0.84 (Me-18) is correlated with the carbons at δ 48.22 (C-12), 52.63 (C-13), 85.60 (C-14), and 51.23 (C-17), and the signal at δ 1.30 (H-9) is correlated with the carbons at δ 76.08 (C-5) and 54.37 (C-10). Also, the proton signals at δ 7.45 (H-21), 7.87 (H-22), and 6.29 (H-23) are correlated with the carbons at δ 122.66 (C-20), 147.32 (C-22) and 162.07 (C-24), and δ 162.07 (C-24) and δ 122.66 (C-20), respectively. Furthermore, the proton signal at δ 4.69 (H-1) is correlated with the carbon at δ 104.66 (C-19), suggesting that the position of the acetoxyl group is at C-1. Some of the other significant long-range correlations observed are shown by arrows in Figure 3.

The relative stereochemistry of **1** and connectivity between C-17 and C-20 were determined on the basis of coupling constants of each proton and the results of difference nOe experiments (Figure 4). Irradiation at the frequency of the Me-18 proton signal (δ 0.84) enhanced the signal intensity of two olefins (H-21 and H-22), one of the methylene (H-12), and two methine protons (H-8 and H-11). This suggests that the

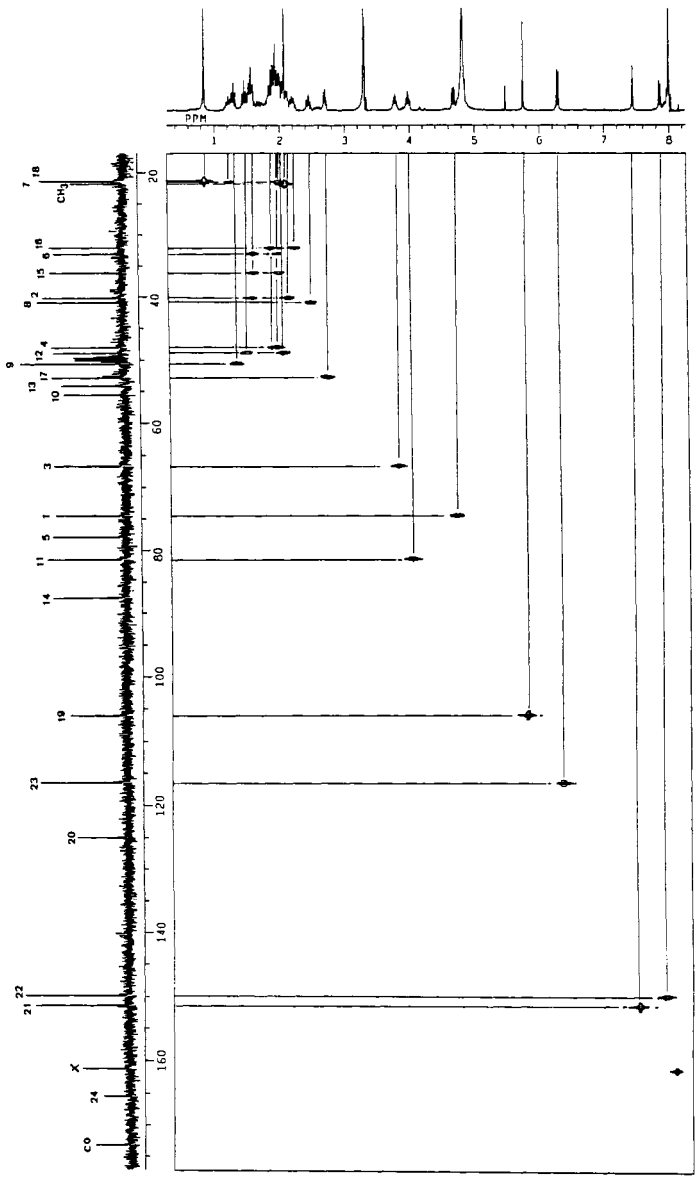


FIGURE 2. ^{13}C-^{1}H COSY Spectrum of Bryophyllin B [**1**].

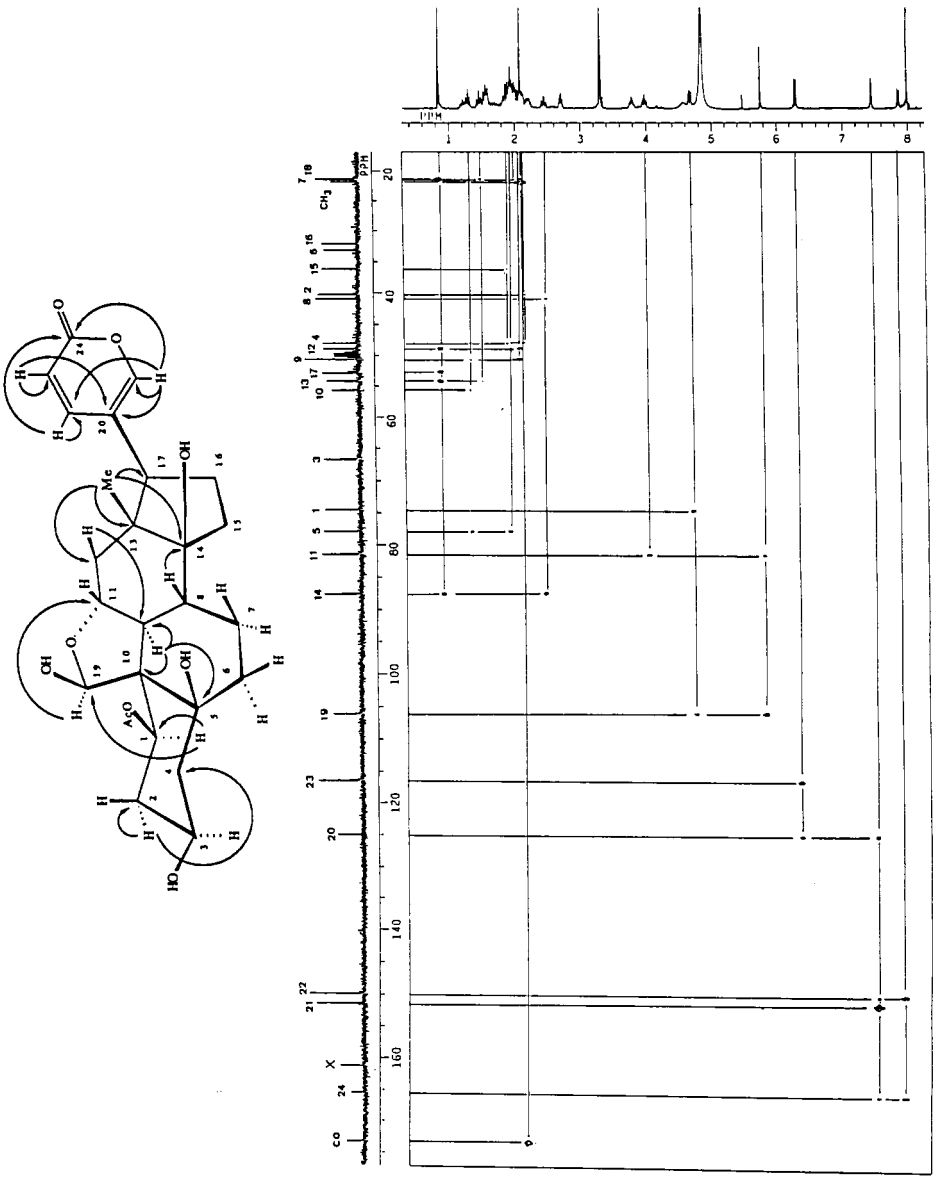


FIGURE 3. Long-range ^{13}C-^{1}H COSY of Bryophyllin B [1] (J = 10.0 Hz).

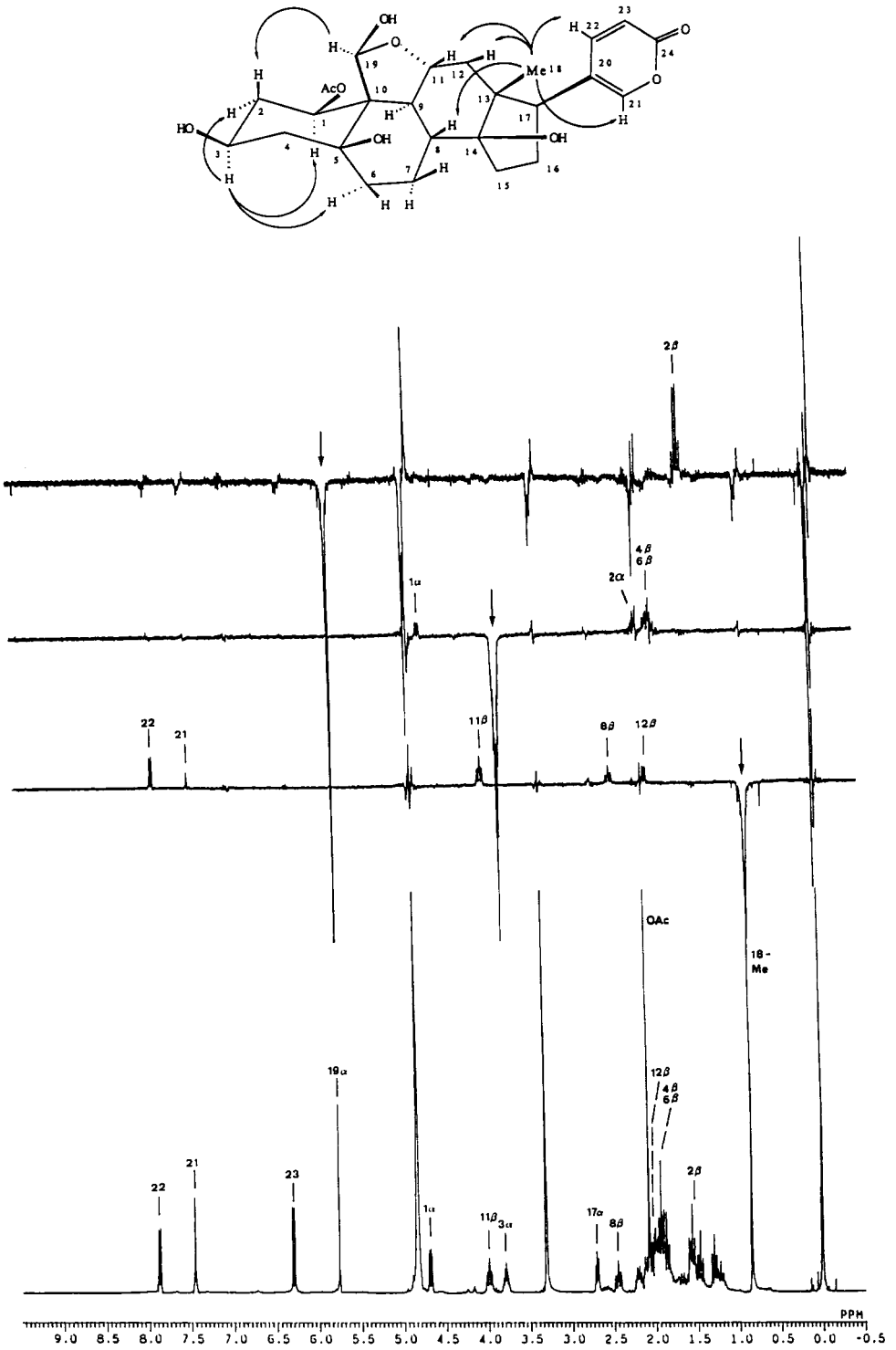


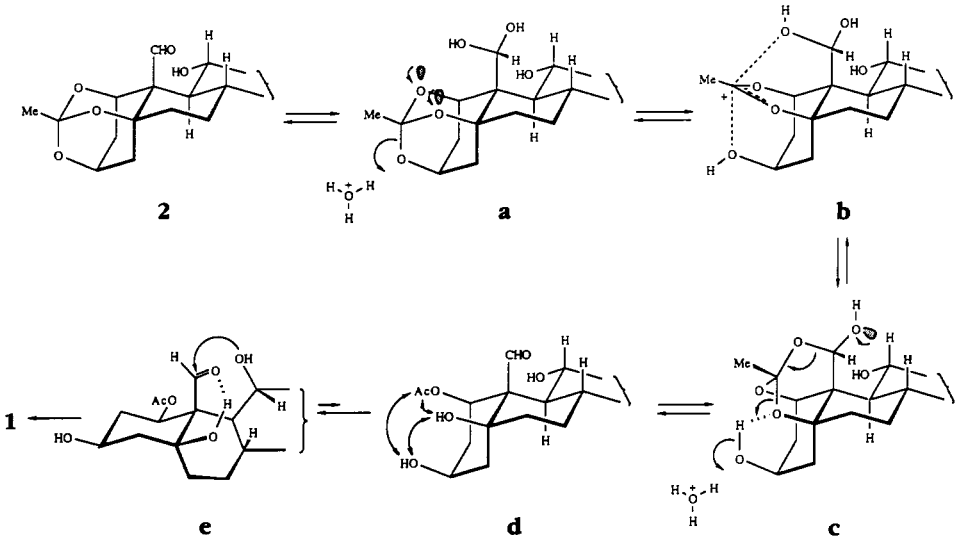
FIGURE 4. Difference nOe of Bryophyllin B [**1**]

Me-18, 8-, and 11-protons have a 1,3-diaxial relationship to each other and that the Me-18 and α-pyrone ring are in a cis orientation. Irradiation at the frequency of H-3 (δ 3.79) and H-19 (δ 5.75) enhanced the signal intensity of H-1 (δ 4.69), H-6 (δ 1.98), and the methylene proton (H-2β, δ 1.54).

Based on the above evidence and a biogenetic point of view regarding the co-occurrence of bryophyllin B with **2** and **3** from the same plant, the structure of bryophyllin B was determined to be **1**.

Added confirmation of the structure of **1** was achieved by the transformation of **2** to the 1β-acetate [**1**]. Compound **2** was treated with 10-camphor sulfonic acid in a mixture of CH_2Cl_2 and H_2O for 2 days at room temperature to afford a 2:1 mixture of **1** and **2**. This type of equilibrium was observed by Kupchan *et al.* (6) previously in their treatment of either bersaldegenin 1-acetate [**6**] or bersaldegenin 1,3,5-orthoacetate [**7**] with 80% HOAc at 90° to yield a 1:1 equilibrium mixture of **6** and **7**.

The formation of **1** from **2** under acidic conditions as described above may be controlled by stereoelectronic effects (7) as explained in Scheme 2.



SCHEME 2. Transformation of **2** to **1**.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All melting points were taken on a Fisher-Johns melting-point apparatus and are uncorrected. Ir spectra were recorded on a Perkin-Elmer 1320 spectrophotometer. ^1H- and ^{13}C-nmr spectra were measured on JEOL GX-400 and Varian XL-400 spectrometers with TMS as an internal standard. Mass spectra were taken on a Shimazu DF 2000 spectrometer by the fab method. Si gel (Kieselgel 60, 230–400 mesh, Merck) was used for cc, and pre-coated Si gel plates (Kieselgel 60 F_{254}, 0.25 mm, Merck) were used for analytical tlc. Detection of bufadienolides was made by spraying with 10% H_2SO_4 solution containing 1% $Ce(SO_4)_2$ followed by heating. Hplc was carried out on a Waters Associates Model ALC/GPC 244 liquid chromatograph with a 450 variable wavelength detector. The column used in this system was Nucleosil $7C_{18}$ (Macherey-Nagel) 10 × 250 mm. MeOH-H_2O-HOAc (60:40:0.2) and MeCN-H_2O-HOAc (40:60:0.2) were used as the mobile phase, and the flow rate was 2–4 ml/min.

PLANT MATERIAL.—*B. pinnatum* (8) was collected in the spring of 1987 in Taipei, Taiwan. A voucher specimen of this plant is kept at the Institute of Botany, Academia Sinica, Taiwan.

EXTRACTION AND ISOLATION OF BRYOPHYLLIN B.—As shown in Scheme 1, the whole fresh plant of *B. pinnatum* (10 kg), was homogenized with H_2O (2 liters). This H_2O extract was filtered and concentrated in vacuo to 1.5 liters. After having been shaken with $CHCl_3$, it was extracted with *n*-BuOH (500

ml × 3). The *n*-BuOH extract (2.7 g) was subjected to cc on Sephadex LH-20 (4 × 38 cm) and eluted with a gradient of H_2O, 50% MeOH, and MeOH to give 16 fractions. Fractions 5, 6, 14, and 15 were found to show significant cytotoxity in KB cells. Fraction 5 (119.6 mg) was further chromatographed on Si gel (2 × 18 cm) and eluted with $CHCl_3$-MeOH (10:1, each fraction 30 ml) to afford 10 fractions. Purification of the active fraction 5 (6.6 mg) by reversed-phase hplc furnished bryophyllin B [**1**] (4.3 mg).

BRYOPHYLLIN B [**1**].—Bryophyllin B was isolated as a colorless amorphous powder: $C_{26}H_{34}O_9$; mp 178–180°, $[\alpha]^{20}D$ +20° (*c* = 0.1, $CHCl_3$); uv λ max (MeOH) 298 (ε 5800) nm; ir ($CHCl_3$) 3400 (OH), 1695 (C=O), 1120 (C-O) cm^{-1}; fabms *m/z* $[M]^+$ 490, $[M-H_2O]^+$ 472, $[M-HOAc]^+$ 432.

ACETYLATION OF BRYOPHYLLIN B [**1**].—A solution of **1** (2.0 mg) in a mixture of pyridine (0.2 ml) and Ac_2O (0.1 ml) was allowed to stand at room temperature overnight, then diluted with H_2O and extracted with EtOAc. The EtOAc extract was washed with saturated NaCl solution, dried over Na_2SO_4, and evaporated. The residue was purified by preparative tlc to give a diacetate **4**: ^1H nmr ($CDCl_3$) δ 7.67 (1H, dd, *J* = 2.57, 9.71 Hz, H-22), 7.23 (1H, br s, H-21), 6.74 (1H, s, H-19α), 6.29 (1H, dd, *J* = 0.73, 9.71 Hz, H-23), 4.89 (1H, m, H-3α), 4.74 (1H, dd, *J* = 4.22, 12.46 Hz, H-1α), 3.96 (1H, ddd, *J* = 3.30, 11.17, 11.54 Hz, H-11β), 2.60 (1H, dd, *J* = 8.14, 8.80 Hz, H-17α), 2.43 (1H, br t, *J* = 11.73 Hz, H-8β), 2.22 (3H, s, 19β-OAc), 2.12 (3H, s, 3β-OAc), 2.03 (3H, s, 1β-OAc), 0.85 (3H, s, Me-18).

When compound **4** was dissolved in $CDCl_3$ and allowed to stand for two days, it yielded a monoacetate **5**: ^1H nmr ($CDCl_3$) δ 7.67 (1H, dd, *J* = 2.57, 9.71 Hz, H-22), 7.23 (1H, br s, H-21), 6.29 (1H, dd, *J* = 0.73, 9.71 Hz, H-23), 5.84 (1H, s, H-19α), 4.89 (1H, m, H-3α), 4.74 (1H, dd, *J* = 4.22, 12.46 Hz, H-1α), 4.05 (1H, ddd, *J* = 3.30, 11.17, 11.54 Hz, H-11β), 2.60 (1H, dd, *J* = 8.14 and 8.80 Hz, H-17α), 2.43 (1H, br t, *J* = 11.73 Hz, H-8β), 2.09 (3H, s, 3β-OAc), 2.03 (3H, s, 1β-OAc), 0.85 (3H, s, Me-18).

TRANSFORMATION OF BRYOTOXIN C [**2**] TO BRYOPHYLLIN B [**1**].—A solution of **2** (1.0 mg) in a mixture of CH_2Cl_2 (1.0 ml) and H_2O (1 drop) was treated with a catalytic amount of (±)-10-camphor sulfonic acid for 2 days at room temperature. The reaction mixture was diluted with H_2O (3.0 ml) and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with saturated NaCl and evaporated to yield a residue. This residue showed two peaks in a ratio of 1:2, corresponding to **1** and **2**, respectively, on reversed-phase hplc [$7C_{18}$, 10 × 250 mm, MeOH-H_2O-HOAc (50:50:0.1), 3 ml/min, 298 nm].

CYTOTOXICITY ASSAY.—The in vitro KB cytotoxicity assay was carried out according to procedures described in Geran *et al.* (9) and Ferguson *et al.* (10). The assay against A-549, HCT-8, P-388, and L-1210 tumor cells was based on a method reported in Lee *et al.* (11).

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