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TUBULOSINE: AN ANTITUMOR CONSTITUENT OF *POGONOPUS SPECIOSUS*

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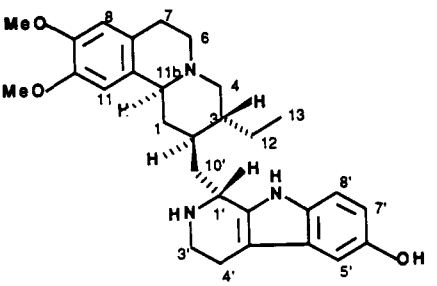
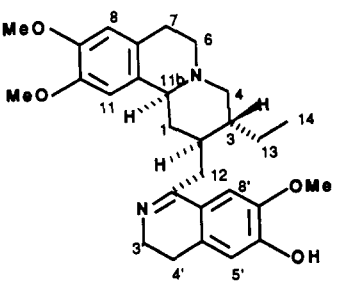
ABSTRACT.—From the antitumor-bioactive sap of *Pogonopus speciosus*, tubulosine [**1**] was isolated, by activity-directed fractionation using the brine shrimp lethality test, as the major antitumor constituent. ^{1}H-nmr assignments, obtained from HETCOR and COSY, and X-ray crystallographic results are reported for the first time. Psychotrine [**2**] was also isolated, and its spectral data are also reported.

The dried sap extract of *Pogonopus speciosus* (Jacq.) K. Schum. (Rubiaceae) was active in the 3PS (in vivo murine leukemia) antitumor screen at the National Cancer Institute and also inhibited the growth of crown gall tumors on potato discs in our laboratory (1). Directing fractionation with the brine shrimp lethality test (2), we have now isolated and characterized the major active antitumor component as the known alkaloid, tubulosine [**1**]. Compound **1** was previously isolated from *Pogonopus tubulosus* (DC.) Schumann (3) and *Alangium lamarckii* Thw. (Alangiaceae) (4) and reported as having antitumor (5) and amoebicidal activities (6). The structure of tubulosine was originally determined mainly by ms (7) and confirmed by total synthesis (8). However, only incomplete ^{1}H-nmr and ^{13}C-nmr data have been previously reported. In this paper, we report the complete ^{1}H-nmr and ^{13}C-nmr assignments of tubulosine, based on COSY and HETCOR, and its definitive relative stereochemistry based on X-ray crystallographic analysis (Figure 1).

The hrms spectra of tubulosine [**1**] showed its molecular formula as $C_{29}H_{37}N_3O_3$ (found 475.2830, calcd 475.2835) with characteristic peaks at 475 $[M]^+$, 288, 272, 246, 205, 201, 192, and 187. ^{13}C-nmr spectral assignments of **1** (Table 1) were made by comparison of the spectra with that of 9-demethyltubulosine (9) and were in agreement with the fully proton-coupled ^{13}C-nmr spectra. Because most of the peaks are well separated on the ^{13}C-nmr spectra, it was possible for the first time to assign the complete ^{1}H-nmr spectrum by HETCOR. The HETCOR spectrum showed the correlations of C-12 (22.85 ppm) with H-12 (1.10 and 1.57 ppm), C-7 (28.80 ppm) with H-7 (2.58 and 2.93 ppm), C-1 (36.47 ppm) with H-1 (1.04 and 2.61 ppm), C-10′



1



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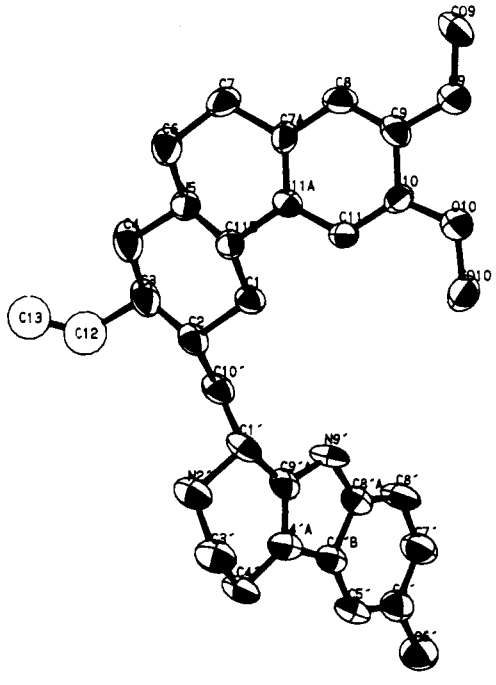

FIGURE 1. ORTEP plot of tubulosine [**1**].

(37.72 ppm) with H-10′ (1.54 and 1.84 ppm), C-3′ (41.32 ppm) with H-3′ (2.90 and 3.11 ppm), C-6 (51.81 ppm) with H-6 (2.37 and 2.90 ppm), and C-4 (60.89 ppm) with H-4 (1.99 and 2.96 ppm). The protons attached to each of the above carbons showed AB systems due to their unusual environments; initially, this caused confusing complications in the aliphatic range of the ^{1}H-nmr spectra. All of the proton assignments were further confirmed by COSY spectra. The two H-12 protons (1.10 and 1.57 ppm) correlated with each other and also correlated with H-13 (0.86 ppm) and H-3 (1.25 ppm), which was related to H-4 (1.99 and 2.96 ppm) and H-2 (1.66 ppm). H-2 was related to H-10′ (1.54 and 1.84 ppm) and H-1 (1.04 and 2.61 ppm), which correlated to each other and to H-11$_b$ (2.99 ppm). The two H-10′ protons correlated to each other and related to H-1′ (4.11 ppm). Two H-3′ protons (2.90 and 3.11 ppm) correlated to each other and to H-4′ (2.52 ppm). Two H-6 protons (2.37 and 2.90 ppm) correlated to each other and to the two protons at H-7 (2.58 and 2.93 ppm), which also correlated to each other. The assignments of the aromatic protons were straightforward. H-7′ showed a doublet of doublets at 6.77 ppm (J = 8.6, 2.4 Hz). H-8′ gave a doublet at 7.01 ppm (J = 8.6 Hz). A peak at 6.66 ppm (J = 2.4 Hz) indicated H-5′. The peaks at 6.60 and 7.01 ppm were assigned to H-8 and H-8′, respectively.

The relative stereochemistry of **1** was determined by X-ray crystallographic analysis (Table 3), and the results confirmed the previously established structure (10). However, one small portion of the X-ray data was inconclusive, suggesting some randomness in the crystal lattice for the ethyl group.

Compound **1** exhibited excellent activities in brine shrimp, potato disc, 9PS, 9KB, 3PS, human lung, breast, and colon cancer cytotoxicity tests (Table 2). The entire isolation and separation process was directed by the brine

TABLE 1. ^{13}C- and ^{1}H-nmr Chemical Shifts of **1** in DMSO-d_6.

Atom	δC	$J_{H,C}$(Hz)	δH	$J_{H,H}$(Hz)
1	36.47	t, 126.95	1.04	$J_{gem}=12.2;J_{1,2}=12.2;J_{1,11b}=12.2$
1			2.61	m
2	36.00	d, 126.29	1.66	m
3	41.63	d, 124.64	1.25	m
4	60.89	t, 132.23	1.99	$J_{gem}=11.2;J_{4,3}=11.2$
4			2.96	m
6	51.81	t, 133.88	2.37	$J_{gem}=11.2;J_{6,7}=10.6;J_{6,7}=6.1$
6			2.90	m
7	22.85	t, 126.62	2.58	m
7			2.93	m
7a	126.64	s		
8	111.76	d, 156.46	6.60	s
9	146.83	s		
10	147.11	s		
11	109.10	d, 154.98	6.27	s
11a	130.40	s		
11b	62.14	d, 130.57	2.99	m
12	22.85	t, 120.35	1.10	$J_{gem}=14.2;J_{12,3}=7.6;J_{12,13}=7.6$
12			1.57	m
13	10.98	q, 124.81	0.86	$J_{12,13}=7.6$
1′	48.52	d, 138.82	4.11	$J_{1',10'}=12.0$
3′	41.32	t, 124.64	2.90	m
3′			3.11	m
4′	22.54	t, 126.62	2.52	m
4′a	106.29	s		
5′	101.74	d, 156.79	6.66	$J_{5',7'}=2.4$
5′a	127.85	s		
6′	150.06	s		
7′	109.26	d, 156.62	6.77	$J_{7',5'}=2.4;J_{7',8'}=8.6$
8′	110.82	d, 157.94	7.01	$J_{5',7'}=8.6$
8′a	129.97	s		
9′a	138.74	s		
10′	37.72	t, 125.32	1.54	$J_{gem}=12.0;J_{10',1'}=12.0$
10′			1.53	m
-OMe	55.57	q, 143.77	3.70	

shrimp and potato disc tests, and the results showed once again that these simple bioassays can lead to in vivo active plant antitumor components.

Psychotrine [**2**] was also isolated during the separation and was identified based on its physical and spectral data. Its ^{1}H- and ^{13}C-nmr spectra confirmed that the double bond is endocyclic (11) not exocyclic (12). This compound was previously isolated from *Psychotria ipecacuanha* Stokes (*Cephaelis ipecacuanha* Rich.) (Rubiaceae) (13) and *A. lamarckii* (Alangiaceae) (14). Gupta *et al.* (15) have proposed that the activity of tubulosine is due to its structural feature that presents two aromatic rings (in the same plane) and the nitrogen atom with its free electron at a certain distance from the aromatic rings. Although the overall shape of the molecule is not changed compared with the structural determinants, the double bond in the C ring of **2** causes the electronic properties to be changed. Therefore, psychotrine [**2**], as we have observed, is not biologically active.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—

TABLE 2. Bioactivities[a] of Compounds **1** and **2** and Sap Extract of *Pogonopus speciosus*.

Sample	BST LC_{50} ppm	PD % inhibition	9PS ED_{50} μg/ml	9KB ED_{50} μg/ml	A-549 ED_{50} μg/ml	MCF-7 ED_{50} μg/ml	HT-29 ED_{50} μg/ml	3PS % T/C
Sap extract	50	25	3.9×10^{-1}	4.9×10^{-2}	NT	NT	NT	181(1) 25 mg/kg
1	0.02	81	3.8×10^{-4}	1.0×10^{-2}	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	186[b] 0.25 mg/kg
2	>1000	NT	1.0	NT	>10	>10	>10	NT

[a]BST = brine shrimp lethality test; PD = % inhibition of crown gall tumors on potato discs. Cytotoxicities: 9PS = murine lymphocytic leukemia; 9KB = human nasopharyngeal carcinoma; A-549 = human lung carcinoma; MCF-7 = human breast carcinoma; HT-29 = human colon adenocarcinoma; 3PS = in vivo murine leukemia; NT = not tested.

[b]Information supplied by Dr. Matthew Suffness, NCI, National Institutes of Health.

TABLE 3. Atomic Coordinates of Tubulosine [**1**].

Atom	*x*	*y*	*z*
C-1′	.3075 (10)	.5942 (0)	−.3219 (6)
N-2′	.2100 (8)	.566 (2)	−.3998 (5)
C-3′	.2236 (11)	.396 (2)	−.4448 (7)
C-4′	.3331 (10)	.417 (2)	−.4841 (6)
C-4′a	.4409 (10)	.480 (2)	−.4185 (6)
C-4′b	.5714 (9)	.478 (2)	−.4173 (6)
C-5′	.6430 (10)	.416 (2)	−.4696 (6)
C-6′	.7674 (10)	.433 (2)	−.4480 (6)
O-6′	.8443 (12)	.379 (2)	−.4965 (7)
C-7′	.8250 (11)	.526 (2)	−.3742 (7)
C-8′	.7568 (11)	.586 (2)	−.3195 (6)
C-8′a	.6293 (9)	.562 (2)	−.3404 (6)
N-9′	.5412 (8)	.609 (2)	−.2991 (5)
C-9′a	.4267 (10)	.563 (2)	−.3484 (6)
C-10′	.2975 (10)	.779 (2)	−.2854 (6)
C-1	.2530 (9)	.7161 (0)	−.1460 (6)
C-2	.1957 (9)	.790 (2)	−.2334 (6)
C-3	.1501 (10)	.979 (2)	−.2291 (7)
C-4	.0667 (10)	.994 (2)	−.1663 (7)
N-5	.1333 (8)	.931 (2)	−.0847 (5)
C-6	.0562 (10)	.964 (2)	−.0240 (7)
C-7	.1370 (11)	.943 (2)	.0616 (7)
C-7a	.2109 (9)	.765 (2)	.0683 (6)
C-8	.2702 (11)	.702 (2)	.1481 (6)
C-9	.3309 (10)	.535 (2)	.1560 (6)
O-9	.3946 (8)	.464 (1)	.2313 (4)
C-09	.4041 (13)	.580 (2)	.3043 (7)
C-10	.3396 (9)	.432 (2)	.0870 (6)
O-10	.4003 (7)	.267 (1)	.1028 (4)
C-010	.4192 (10)	.164 (2)	.0347 (8)
C-11	.2836 (9)	.497 (2)	.0086 (6)
C-11a	.2230 (9)	.670 (2)	−.0004 (6)
C-11b	.1646 (9)	.737 (2)	−.0881 (6)
C-12	.078 (2)	1.049 (3)	−.3133 (12)
C-13	−.007 (2)	1.166 (3)	−.3355 (12)

^{1}H- and ^{13}C-nmr, COSY, and HETCOR spectra were taken on a Varian VXR-500S spectrometer in DMSO-d_6. Cims and eims were measured on the Finnigan 4000. Hrms were determined on the Kratos MS-50. Ir spectra were recorded on a Perkin-Elmer 1600 series FTIR in KBr. Uv spectra were obtained on a Beckman DU-7 spectrometer in MeOH. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Mp's were determined in capillaries on a Mel-temp apparatus and were uncorrected.

CRYSTAL DATA.[1]—$C_{29}H_{37}N_3O_3$, FW = 475, monoclinic, $a = 11.121$ (5) Å, $b = 7.292$ (2) Å, $c = 16.61$ (1) Å, $\beta = 103.16$ (5)°, V = 1312 (1) Å^3, Z = 2, ρ calcd = 1.20 g/cm^3, F(000) = 512, μ(CuKα) = 5.43, space group $P2_1$ from systematic absences. Data were collected using CuKα X-rays, and a monochromator on a Nicolet P3 four-circle diffractometer, with the θ–2θ scan technique out to a 2θ of 116.0°. A variable scan rate was used with a maximum of 29.30°/min, and a minimum of 7.23°/min. The scan range was from $1.2° < K\alpha_1$ to $1.2° > K\alpha_2$; the time backgrounds at both ends of the scan range were counted as equivalent to the scan time. Two standard reflections were measured every 50 reflections and showed no decay during the data collection. Of the 2023 reflections collected, 1940 were unique, and 1669, which met the condition of $F_o > 5\ \sigma\ (F_o)$, were considered observed. The atoms were located independently both with MULTAN80 and SHELXS86, and each solution

[1]Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK.

was then refined with SHELX76. In both cases there was so much disorder in the ethyl region that C-12 and C-13 could not be allowed to refine anisotropically: C-12 was kept isotropic, and the isotropic temperature factor of C-13 was fixed at 0.1200 (approximately equal to that of C-12). The C-13–C-12 bond and C-13–C-12–C-3 angle are unusual as might be expected due to the disorder. All hydrogens were fixed in calculated positions except H-6′ which was located on a difference map. The final difference map showed two peaks near C-12 at 0.80 and 0.43ē/Å^3; the next peak was at 0.26ē/Å^3. The final R was 0.0942, which is high due to the disorder of the ethyl group. The atomic coordinates are listed in Table 3.

PLANT MATERIAL AND ISOLATION.—The sap of *P. speciosus* was collected in Costa Rica between 1977 and 1980 for the National Cancer Institute (voucher numbers B-850370, B-850371, B-850372, B-850373, B-850374, and 850375). The freeze-dried sap (108 g) was partitioned between CH_2Cl_2 and H_2O; the H_2O layer was then partitioned with *n*-BuOH, and the latter yielded 50 g of residue. The residue (48 g) was subjected to Al_2O_3 (neutral) cc using hexane, $CHCl_3$, and MeOH gradient mixtures of increasing polarity, and the single pool, which showed activity to brine shrimp (17.2 ppm) was further separated over Si gel on a Chromatotron eluted with a $CHCl_3$ and MeOH gradient. Tubulosine (61 mg) and psychotrine (11 mg) were obtained as free bases.

TUBULOSINE [**1**].—Colorless crystals from MeOH; mp 255° [lit. (3) 259–261°]; $[\alpha]_D$ −58.8° ($c=0.2$, pyridine) [lit. (3) $[\alpha]^{24}_D$ −65.9°]; uv λ max (MeOH) 228 (log ε 3.13), 285 (log ε 3.14); ir ν max (KBr) 3390, 1637, 1594; eims (70 eV) *m/z* 475 (14.09), 288 (8.30), 272 (33.78), 246 (42.48), 201 (34.35), 192 (37.99), 187 (81.05); cims (NH_3) 476; hrms found 475.2830, calcd 475.2835; ^{1}H and ^{13}C nmr see Table 1.

PSYCHOTRINE [**2**].—Yellow crystals; mp 122° [lit (12) 117–120°]; $[\alpha]_D$ +70° ($c=0.1$, MeOH); uv λ max (MeOH) 240 (3.2), 280 (3.6), 360 (4.2); ir ν max (KBr) 3430, 1385, 1340; eims 464 (29.23), 274 (18.91), 273 (45.46), 272 (48.86), 244 (100), 191 (41.42), 190 (46.86); eims (NH_3) 465; ^{1}H nmr (500 MHz, DMSO-d_6) δ 7.25 (1H, s, H-11), 6.76 (1H, s, H-8′), 6.66 (1H, s, H-5′), 6.51 (1H, s, H-8), 3.81, 3.70, 3.62 (-OMe), 1.80 (1H, m, H-13), 1.22 (1H, m, H-13), 0.93 (3H, t, $J=7.5$, H-14), 3.62 (s, -OMe), 0.93 (t, -Me); ^{13}C nmr (50 MHz, DMSO-d_6) δ 170.78 (C-1′), 152.56 (C-6′), 147.34 (C-9, C-7′), 146.88 (C-10), 133.25 (C-4′a), 128.32 (C-11a), 125.73 (C-7a), 117.24 (C-8′a), 115.21 (C-5′), 111.92 (C-8′) and 111.82 (C-8) (may be reversed), 108.40 (C-11), 61.43 (C-11b) and 59.42 (C-4) (may be reversed), 55.98, 55.42, 55.35 (-OMe), 51.00 (C-6), 42.75 (C-3) and 39.92 (C-3′) and 39.76 (C-12) (may be interchanged), 37.06 (C-2), 36.23 (C-1), 27.93 (C-7), 22.78 (C-13), 10.79 (C-14).

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