A NEW ANTITUMOR ANTIBIOTIC, BACTOBOLIN PRODUCED BY *PSEUDOMONAS*

Sir:

A new chlorine-containing antibiotic, bactobolin, has been found in the culture broth of *Pseudomonas* BMG13-A7. Bactobolin inhibits the growth of Gram-positive and Gram-negative bacteria, and experimental animal tumors, and is structurally related to actinobolin produced by *Streptomyces griseoviridis* var. *atrofaciens*[1,2].

Pseudomonas BMG13-A7 was cultured at 27°C for 24 hours on a rotatory shaker (180 rpm) in a 500-ml Erlenmeyer flask which contained 110 ml of a medium consisting of maltose 1.5%, yeast extract 0.3%, NZ-amine (A type) 1.0% and NaCl 0.3% (pH 7.4). This culture (110 ml) was inoculated into 15 liters of the same medium in a 30-liter jar fermentor and the fermentation was carried out at 27°C under agitation at 250 rpm and aeration at 15 liters/minute. The 40-hour fermented broths in two fermentors were combined and centrifuged at 10,000 rpm to yield a supernatant (27 liters, 52 μg/ml). Concentrations of bactobolin were determined by the cylinder plate method against *Escherichia coli* K-12 using crystalline bactobolin (1,000 μg/ml) as an assay standard.

The antibiotic in the supernatant was adsorbed on a column of Amberlite XAD-2 resin (1,500 ml) and eluted with 50% aqueous methanol. The active eluate (4,270 ml) was concentrated to dryness and a crude powder (13.4 g, 71 μg/mg) was obtained. The crude powder in water (20 ml) was purified by column chromatography on Amberlite CG-50 (Type I, 70% NH_4^+, 600 ml) developed with water to give an yellowish powder (1.7 g, 489 μg/mg). The powder was further purified by repeated chromatography, yielding a colorless powder (940 mg, 719 μg/mg) of bactobolin. Crystallization of the powder from a mixture of water (1.5 ml) and ethanol (0.5 ml) gave colorless needles of bactobolin (604 mg, 1,000 μg/mg).

Bactobolin (**1**) shows mp 196~197°C (decomp.), $[\alpha]_D^{24}$ −26.7° (*c* 1.0, water), pKa′ 7.3 and 8.4. Anal. calcd. for $C_{14}H_{20}N_2O_6Cl_2$: C 43.88, H 5.26, N 7.31, O 25.05. Found: C 43.57, H 5.22, N 7.33, O 24.99. The molecular formula is derived by FD-MS of **1** (*m/e* 382, M^+) and by high-resolution MS of mono-N-acetylbactobolin (**2**) (*m/e* 424.0789, calcd. mol. wt. for $C_{16}H_{22}N_2O_7Cl_2$: 424.0801). UV max.: 276 nm (ε 10,000) in water, 262 nm (ε 11,200) in 0.1 N HCl and 287 nm (ε 17,000) in 0.1 N NaOH. The IR spectrum is shown in Fig. 1. The PMR chemical shifts are represented in Table 1. The CMR spectra (D_2O) shows signals of 14 carbons (184.7s, 173.9s, 167.6s, 85.3s, 84.3s, 75.9d, 72.6d, 70.1d, 50.0d, 47.7d, 42.0d, 40.3t, 19.1q and 19.0q). The antibiotic **1** gives positive ninhydrin, RYDON-SMITH and 2,4-dinitrophenylhydrazine reactions, and is soluble in water and methanol. By high-voltage paper electrophoresis with 3,500 V for 15 minutes in formic acid - acetic acid - water (1 : 3 : 36, v/v), the antibiotic **1** moves to the cathode with Rm (relative mobility to alanine) 0.68. Rf values of thin-layer chromatography on Silica gel G (Merck, Art. 5715) developed with ethyl acetate -

Fig. 1. The IR spectrum of bactobolin in KBr.

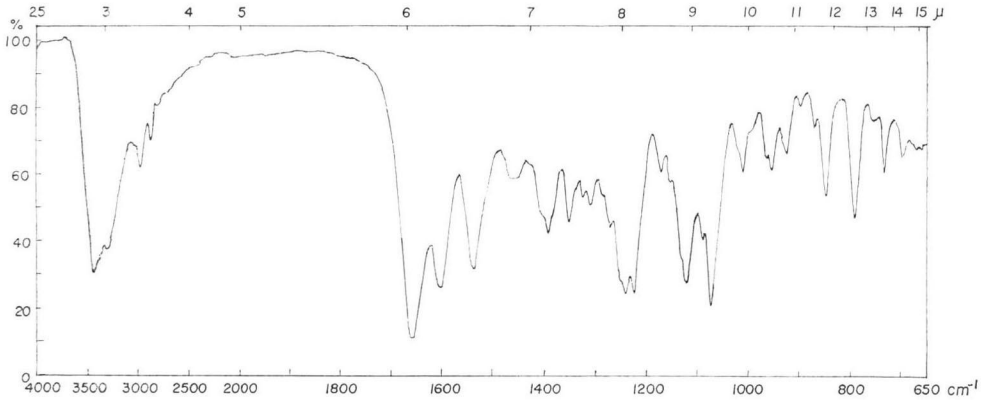


Table 1. PMR chemical shifts of bactobolin (**1**), mono-N-acetylbactobolin (**2**) and mono-N-acetylbactobolin methyl ether (**6**).

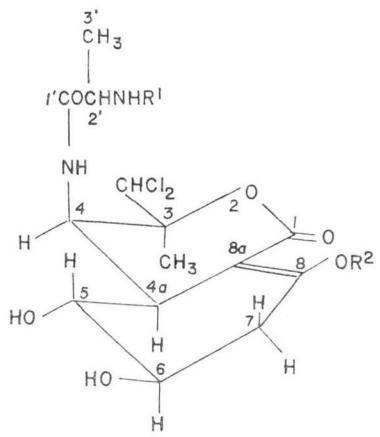
Proton	δ ppm (JHz)		
	1	**2**	**6**
3′-H_3	1.97 d (7)	1.85 d (7)	1.90 d (7)
3-CH_3	2.14 s	2.23 s	2.21 s
N-Ac		2.52 s	2.52 s
7-Hax.	2.73 dd (18, 10.5)	2.96 m (19, 10, 2.2)	2.96 m (18, 10, 2.5)
7-Heq.	3.23 dd (18, 6.5)	3.47 dd (19, 7)	3.70 dd (18, 6.5)
4a-H	~3.5 m	~3.5 m (3)	~3.6
5-H	3.63 t (9.5)	3.74 t (9)	3.71 t (9)
OCH_3			4.43 s
6-H	~4.4 m (10.5, 9.5, 6.5)	4.47 m (10, 9, 7)	~4.4
2′-H	4.50 q (7)	4.81 q (7)	4.80 q (7)
4-H	5.29 d (2.5)	5.38 d (3)	5.34 d (2.5)
3-$CHCl_2$	6.58 s	6.71 s	6.64 s

Chemical shifts, δ(ppm) were measured in D_2O using TMS as the external reference.

methanol - water (8: 5: 1, v/v) and with ethyl acetate - acetic acid - water (3: 1: 1, v/v) are Rf 0.26 and Rf 0.42, respectively.

Acid hydrolysis of **1** with 6 N HCl under reflux for 3 hours followed by column chromatography on Dowex 1-X2 (OH^-) eluted with 0.5 N HCl gave crystalline L-alanine hydrochloride [a little racemized: $[\alpha]_D^{24}$ +3.7° (*c* 1.0, 1 N HCl)], and hydrochlorides of a phenol derivative (**3**) and an alanyl-phenol derivative (**4**). The phenol derivative **3** was derived into the N,O-diacetyl derivative (**5**) by treatment with acetic anhydride in pyridine overnight at room temperature, mp 65~70°C, $[\alpha]_D^{24}$ −41° (*c* 0.5, methanol), MS: *m/e* 333 (M^+), PMR ($CDCl_3$): δ 1.19 (3H, s, C–CH_3), 1.98 (3H, s, N–Ac), 2.29 (3H, s, O–Ac), 3.29 (1H, broad, OH), 5.36 (1H, d, *J*=9.5 Hz, N–CH), 5.88 (1H, s, $CHCl_2$), 6.58 (1H, broad d, J=9.5 Hz, NH) and 7.0~7.3 (4H, 1,3-disubstituted benzene), and the structure of **5** was shown to be 3-(1-acetamido-2-dichloromethyl-2-hydroxypropyl)-1-acetoxybenzene.

Treatment of **1** with acetic anhydride in methanol overnight at room temperature afforded crystalline mono-N-acetylbactobolin **2**, mp 242~243°C (decomp.), $[\alpha]_D^{24}$ −32° (*c* 1.0, water). By treatment with diazomethane, **2** was easily converted into the methyl ether (**6**), mp 151~158°C (decomp.), $[\alpha]_D^{28}$ −31° (*c* 1.0, water), MS: *m/e* 438 (M^+). The presence of an enolic hydroxyl group in **2** was confirmed by formation of the methyl ether.

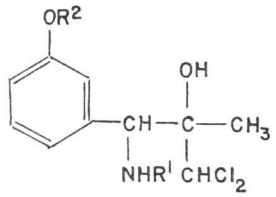


1 : R^1 , R^2 = H

2 : R^1 = $COCH_3$, R^2 = H

6 : R^1 = $COCH_3$, R^2 = CH_3

The PMR chemical shifts of **2** and **6** are represented in Table 1. A partial structure CH_2-CHCHCHCH and its configurations (axial or equatorial assignment of ring protons) were shown by the PMR spectra of **1**, **2** and **6**. The PMR spectra of **2** and **6** are very similar to those of N-acetylactinobolin and its methyl ether[2]. The long-range homoallylic coupling between 4a-H and 7-Hax. was also observed in the PMR spectra of **2** and **6** (2.2 and 2.5 Hz, respectively). The NOE experiments of **2** in D_2O irradiating 3–CH_3 (δ 2.23) resulted in enhance-



3: $R^1, R^2 = H$

4: $R^1 = Ala, R^2 = H$

5: $R^1, R^2 = COCH_3$

ment of 10.5% of the integrated area of 4a-H (δ 3.5), and the saturation of 4a-H showed enhancements of 8% and 12% of the integrated areas of 3-CH_3 and 6-H (δ 4.47), respectively. The results indicate that (6-H)-(4a-H) and (4a-H)-(3-CH_3) must be all in 1,3-diaxial arrangements. By application of the CuAm method reported by S. UMEZAWA *et. al.*[3], **2** and **6** showed positive contributions ($\Delta[M]_{CuAm}$ +1260° and +1300°, respectively) and thus the absolute configurations of **2** and **6** were determined to be 5*R* and 6*R*.

From the foregoing results, the absolute structure of bactobolin (**1**) can be proposed to be (3*S*, 4*R*,4a*R*,5*R*,6*R*)-4-(L-alanylamino)-3-(dichloromethyl)-3,4,4a,5,6,7-hexahydro-5,6,8-trihydroxy-3-methyl-1*H*-2-oxa-1-naphthalenone.

Bactobolin shows a strong antibacterial activity, as shown in Table 2. A marked prolongation effect in the survival period of mice implanted with the mouse leukemia L-1210 cells has been observed after treatment with bactobolin. The antitumor effects of bactobolin will be reported elsewhere. Acute LD_{50} of bactobolin in mice by the intravenous injection was 6.25~12.5 mg/kg.

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Table 2. The antimicrobial spectrum of bactobolin

Test organisms	Minimum inhibitory concentrations (μg/ml)
Staphylococcus aureus FDA209P	0.39
Staphylococcus aureus Smith	0.39
Micrococcus flavus FDA16	<0.20
Sarcina lutea PCI1001	<0.20
Bacillus anthracis	12.5
Bacillus subtilis PCI219	0.78
Bacillus subtilis NRRL B-558	0.39
Bacillus cereus ATCC10702	6.25
Corynebacterium bovis 1810	0.20
Escherichia coli NIHJ	0.78
Escherichia coli K-12	3.13
Escherichia coli K-12 ML1629	6.25
Escherichia coli K-12 ML1630	3.13
Klebsiella pneumoniae PCI602	6.25
Shigella dysenteriae JS11910	<0.20
Shigella flexneri 4b JS11811	1.56
Shigella sonnei JS11746	3.13
Salmonella typhi T-63	6.25
Salmonella enteritidis 1891	3.13
Proteus vulgaris OX19	0.39
Serratia marcescens	>100
Pseudomonas aeruginosa A3	100
Pseudomonas aeruginosa No. 12	>100

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