Communications to the Editor

SIBANOMICIN, A NEW PYRROLO[1,4]-BENZODIAZEPINE ANTITUMOR ANTIBIOTIC PRODUCED BY A *MICROMONOSPORA* SP.

Sir:

A new member of the anthramycin group of antibiotics[1~11], sibanomicin has been found in the culture filtrate of *Micromonospora* sp. SF2364. The antibiotic exhibited a marked antitumor activity in mice bearing leukemia P388 cells and a weak activity against Gram-positive bacteria. In this communication, the production, isolation, characterization, structural elucidation and biological properties of the antibiotic are reported.

A slant culture of strain SF2364 was inoculated into a 100-ml Erlenmeyer flask which contained 20 ml of a seed medium consisting of starch 2.0%, glucose 1.0%, wheat germ 0.6%, Polypepton (Daigo Eiyo Kagaku) 0.5%, yeast extract 0.3%, soybean meal 0.2% and $CaCO_3$ 0.1% (pH 7.0). The inoculated flask was cultured on a rotary shaker (220 rpm) at 28°C for 4 days. The first seed culture (4 ml) was inoculated into 80 ml of the same medium in a 500-ml Erlenmeyer flask (shaking at 28°C for 2 days). The second seed culture (50 ml) was added to a 5-liter Erlenmeyer flask containing 1 liter of the same medium (shaking at 28°C for 2 days). The third seed culture (1 liter) was transferred into a 50-liter jar fermentor containing 35 liters of the same medium and fermentation maintained at 28°C for 2 days. The fourth seed culture (6 liters) was added to a 300-liter fermentor containing 200 liters of the production medium (sucrose 3.0%, cotton seed meal 1.0%, wheat germ 1.0%, soluble vegetable protein 0.6%, $CaCO_3$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, $FeSO_4 \cdot 7H_2O$ 0.0005% and $CoCl_2 \cdot 6H_2O$ 0.0005% in a tap water, pH 7.0 before sterilization) and fermentation was carried out at 28°C for 3 days with aeration of 100 liters/minute, and agitation of 100 rpm (0~41 hours) and 150 rpm (41~72 hours). The activity of the antibiotic was assayed by the paper-disc method using *Escherichia coli* (DNA repair deficient mutant) as the test organism.

The fermentation broth in two fermentors was filtered using Hyflo Super-Cel (Johns-Manville) as the filter aid to give 300 liters of the filtrate. The antibiotic in the filtrate was adsorbed on a column of Diaion HP-20 (15 liters) and eluted with 50% aq Me_2CO. The active eluate (45 liters) was concd to 20 liters and the antibiotic was extracted with 1-BuOH (20 liters). The extract was concd to a small volume and charged on a silica gel column (Wakogel C-200, 250 g). The column was eluted with $CHCl_3$ - MeOH (10:1) and the active fractions were combined and concd to dryness to afford a crude powder (3.7 g). The crude powder was dissolved in 0.01 N HCl (400 ml) and adjusted to pH 5.0 by 1 N NaOH and the antibiotic was adsorbed on a column of CM-Sephadex C-25 (Na^+, 150 ml). After washing with water, the column was eluted with 0.2 M NaCl. The antibiotic in the active eluate was adsorbed on a column of Diaion HP-20 (150 ml) and eluted with 50% aq Me_2CO. The active eluate was concd to dryness. The residue was further purified by Sephadex LH-20 (950 ml) chromatography developed with MeOH. After removal of MeOH, the residue was dried under reduced pressure at 40°C for 24 hours to give the pure sibanomicin hydrochloride (260 mg).

The hydrochloride was obtained as a water-soluble colorless amorphous powder melting at 185~195°C with decomposition and showed elemental analysis (*Anal* Calcd for $C_{23}H_{31}N_3O_5 \cdot HCl$: C 59.28, H 6.92, N 9.02, Cl 7.61. Found: C 59.45, H 7.10, N 8.94, Cl 7.56.); $[\alpha]_D^{22}$ +371° (*c* 0.2, DMSO), +59° (*c* 1.0, H_2O); high resolution (HR)-MS *m/z* 429.2289 (M^+, calcd for $C_{23}H_{31}N_3O_5$ 429.2262); UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$) 214 (760), 240 (sh, 290), 314 (78); $\lambda_{max}^{0.1N\ HCl}$ 206 (810), 240 (sh, 330), 310 (46); $\lambda_{max}^{0.1N\ NaOH}$ 217 (516), 240 (sh, 320), 314 (93); IR (KBr) cm^{-1} 3380, 2960, 2920, 1620, 1600, 1490, 1450, 1390, 1320, 1260, 1220, 1140, 1100, 1030, 1000, 840, 780 and positive Rydon-Smith and ninhydrin reactions.

Sibanomicin (**1**) was placed in the anthramycin group by its UV spectrum. The ^1H and ^{13}C NMR spectra in DMSO-d_6 of **1** (Tables 1 and 2) indicated the presence of an azomethine group

Table 1. ^{1}H NMR data of sibanomicin.

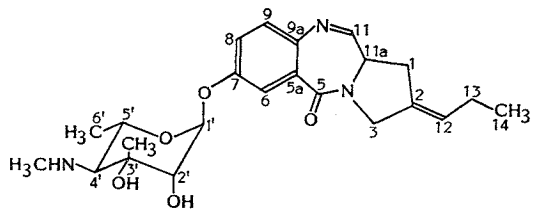
Proton No.	1[a]		1a[b]		1b[b]	
	ppm m	J (Hz)	ppm m	J (Hz)	ppm m	J (Hz)
1-H	2.92 br dd	16.5, 9.0	2.66 m		2.70 m	
	3.04 br d	16.5				
3-H	4.09 br d	15.5	4.07 br d	15.9	4.23 br s	
	4.11 br d	15.5	4.19 m			
6-H	7.46 t	1.7*	7.25 d	2.8	7.28 d	2.8
8-H	7.28 d	1.7*	7.16 dd	8.7, 2.8	7.06 dd	9.0, 2.8
9-H	7.28 d	1.7*	7.01 d	8.7	6.75 d	9.0
11-H	7.81 d	4.5	4.74 d	9.2	5.14 br s	
11a-H	3.92 br dd	9.0, 4.5	3.50 ddd	9.2, 8.2, 3.1	3.73 br dd	9.2, 3.3
12-H	5.51 br t	7.4	5.59 br t	7.6	5.44 br t	7.6
13-H	2.09 m		2.02 m		2.07 m	
14-H	0.99 t	7.4	0.96 t	7.6	0.95 t	7.6
1′-H	5.52 d	1.3	5.48 d	1.3	5.38 d	1.3
2′-H	3.59 dd	4.4, 1.3	3.86 d	1.3	3.84 d	1.3
3′-CH_3	1.40 s		1.56 s		1.55 s	
4′-H	2.92 br d	9.0	3.14 d	10.3	3.13 d	10.3
4′-NCH_3	2.67 s		2.91 s		2.91 s	
5′-H	3.94 m		4.19 m		4.19 m	
6′-H	1.28 d	6.2	1.40 d	6.2	1.40 d	6.2

Solvents: [a] DMSO-d_6, [b] D_2O.
* Virtual coupling.
m: Multiplicity.

Table 2. ^{13}C NMR data of sibanomicin and its derivatives.

Carbon No.	1[a] (ppm m)	1a[b] (ppm)	1b[b] (ppm)	2[a] (ppm m)	3a[c] (ppm)	3b[c] (ppm)
C-1	30.5 t	31.3	31.5	122.4 d		
C-2	132.0 s	131.5	132.5	130.2 s		
C-3	51.2 t	52.1	53.9	121.7 d		
C-5	162.7 s	168.9	168.3	160.3 s		
C-5a	127.9 s	128.3	120.2	122.0 s		
C-6	116.0 d	117.6	119.0	116.9 d		
C-7	153.1 s	151.5	148.5	156.2 s		
C-8	119.6 d	122.9	124.3	122.5 d		
C-9	128.2 d	124.8	121.0	135.4 d		
C-9a	140.2 s	138.2	140.6	139.2 s		
C-11	165.0 d	87.8	85.4	143.5 d		
C-11a	53.4 d	61.1	60.2	128.0 s		
C-12	124.5 d	127.6	125.2	28.1 t		
C-13	22.0 t	23.0	23.1	22.9 t		
C-14	14.0 q	14.4	14.2	13.7 q		
C-1′	98.5 d	99.9	100.3		101.2	99.3
C-2′	72.8 d	74.0	74.0		74.1	75.0
C-3′	70.2 s	71.7	71.7		72.6	73.3
3′-CH_3	19.5 q	19.4	19.4		19.8	18.2
C-4′	65.7 d	66.6	66.6		66.5	67.3
4′-NCH_3	35.6 q	36.4	36.4		38.0	38.5
C-5′	65.6 d	66.2	66.1		68.7	72.8
C-6′	19.1 q	19.0	19.0		19.2	19.3
1′-OCH_3	q				55.1	56.7

Solvents: [a] DMSO-d_6, [b] D_2O, [c] $CDCl_3$.
m: Multiplicity.



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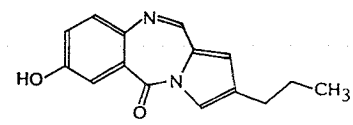

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(7.81 ppm (11-H) and 165.0 ppm (C-11)) similar to that of neothramycin[5]. The azomethine group of **1** easily formed carbinolamine (NHCH(OH)) by addition of H_2O, as an inseparable epimeric mixture (**1a** and **1b**) at C-11. The NMR spectra of **1a** and **1b** are shown in Tables 1 and 2.

Acid hydrolysis of **1** with 6 N HCl at 80°C for 30 minutes gave a compound **2** with an extended chromophore. The structure of **2** was determined by HR-MS (*m/z* 254.1017, M^+, calcd for $C_{15}H_{14}N_2O_2$ 254.1054), ^1H NMR (δ 7.16 (br d, J=2.0 Hz, 1-H), 8.00 (br s, 3-H), 7.94 (d, J=2.9 Hz, 6-H), 7.24 (dd, J=8.6 and 2.9 Hz, 8-H), 7.67 (d, J=8.6 Hz, 9-H), 8.38 (s, 11-H), 2.56 (br t, J=7.4 Hz, 12-H), 1.63 (tq, J=7.4 and 7.4 Hz, 13-H), 0.93 (t, J=7.4 Hz, 14-H), 10.30 (s, 7-OH)) and ^{13}C NMR spectra (Table 2). By a long range ^1H-^{13}C shift correlation spectroscopy (long range ^1H-^{13}C COSY) and long range selective proton decoupling (LSPD) experiments, the assignments of all carbons of **2** were established. Methanolysis of **1** with 1 N HCl in MeOH under reflux for 15 hours gave an anomeric mixture of methyl glycosides and methyl 2-amino-5-hydroxybenzoate (field desorption (FD)-MS *m/z* 167 (M^+)). The mixture was separated into methyl 4,6-dideoxy-3-*C*-methyl-4-methylamino-α-L-mannopyranoside hydrochloride (**3a**: FD-MS *m/z* 205, $[\alpha]_D^{21}$ −43° (*c* 0.5, H_2O)) and its β-anomer (**3b**: FD-MS *m/z* 205, $[\alpha]_D^{21}$ +68° (*c* 0.5, H_2O)) by preparative TLC of their *N-tert*-butoxycarbonyl derivatives followed by deprotection with TFA. In the nuclear Overhauser effect (NOE) difference spectra irradiated at 3-CH_3 protons of **3a** and **3b**, NOEs were observed for 2-H and 5-H protons, and 1-H, 2-H and 5-H protons, respectively. These results indicate that **3a** and **3b** are in the α and β forms, respectively. These methyl glycosides were identical with methyl sibirosaminides[12,13] obtained by methanolysis of sibiromycin. Furthermore, in the NOE difference spectrum irradiated at 3′-CH_3 protons of **1** in D_2O, NOEs were observed for 2′-H and 5′-H but not for 1′-H. Therefore, the mode of sugar linkage of **1** was deduced to be α.

Table 3. Antitumor activity of sibanomicin against P388 leukemia.

Dose (mg/kg)	MST (days±SD)	T/C (%)
2.1	16.3±1.3	196
1.0	14.0±0	169
0.5	13.0±2.0	157
0.25	11.5±1.7	139
0.125	10.0±0.5	121
Control	8.3±0.5	100

Mice: CDF_1, male, *ca.* 22 g, n=5.
Tumor: 1×10^6 cells/0.2 ml Hanks solution/mouse, ip.
Administration: Day 1, 0.2 ml/20 g body weight, ip.
MST: Mean survival time.

By LSPD experiments of **1** in D_2O, it was clarified that 1′-H was coupled to C-7 which was further coupled to 6-H, 8-H and 9-H. Accordingly, it was deduced that the sugar attached at C-7 position. The 6-H was also coupled to an amide carbonyl carbon C-5 which was weakly coupled to 3-H. An olefinic proton 12-H was long range coupled to 1-H and 3-H, and 11-H was coupled to C-9a which was further coupled to 6-H and 8-H. NOE difference spectra irradiated at 1-H, 12-H and 13-H indicated enhancement of the signals for 13-H, 3-H and 1-H, respectively. Therefore, the branched olefin at C-2 and C-12 positions was confirmed to have *E* configuration as in tomaymycin. From these results, the structure of **1** was deduced.

A marked prolongation of life was observed when mice bearing leukemia P388 cells were treated with sibanomicin, as shown in Table 3,

Table 4. Antimicrobial activity of sibanomicin.

Test organisms	MIC (μg/ml)
Staphylococcus aureus 209-P JC-1	50
S. aureus Smith S-424	12.5
S. aureus No. 26	50
S. epidermidis ATCC 14990	100
S. epidermidis 109	50
Enterococcus faecalis ATCC 8043	25
Bacillus anthracis No. 119	25
Escherichia coli JC-2	>100
E. coli No. 29	>100
E. coli W3630 RGN823	>100
E. coli JR66/W677	>100
Citrobacter freundii GN346	>100
Salmonella typhi 0-901-W	50
S. enteritidis No. 11	50
S. typhimurium LT-2	>100
Salmonella sp. D-0001	>100
Shigella sonnei EW33 Type 1	>100
Klebsiella pneumoniae PCI 602	>100
K. pneumoniae 22#3038	>100
Proteus vulgaris OX 19	>100
P. mirabilis GN310	>100
Providencia rettgeri J-0026	>100
Morganella morganii Kono	>100
Serratia marcescens MB-3848	>100
Pseudomonas aeruginosa MB-3829	>100
P. cepacia M-0527	>100
Xanthomonas maltophilia M-0627	>100

however, the antimicrobial activity against all organisms tested was very weak (Table 4). The acute LD_{50} values of the antibiotic in mice were 1.7~2.5 mg/kg and 1.1~1.6 mg/kg by iv and ip injections, respectively.

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References

1) LEIMGRUBER, W.; A. D. BATCHO & F. SCHENKER: The structure of anthramycin. J. Am. Chem. Soc. 87: 5793~5795, 1965
2) AOKI, H.; N. MIYAIRI, M. AJISAKA & H. SAKAI: Dextrochrysin, a new antibiotic. J. Antibiotics 22: 201~206, 1969
3) KARIYONE, K.; H. YAZAWA & M. KOHSAKA: The structures of tomaymycin and oxotomaymycin. Chem. Pharm. Bull. 19: 2289~2293, 1971
4) MESENTSEV, A. S.; V. V. KULJAEVA & L. M. RUBASHEVA: Structure of sibiromycin. J. Antibiotics 27: 866~873, 1974
5) MIYAMOTO, M.; S. KONDO, H. NAGANAWA, K. MAEDA, M. OHNO & H. UMEZAWA: Structure and synthesis of neothramycin. J. Antibiotics 30: 340~343, 1977
6) KUNIMOTO, S.; T. MASUDA, N. KANBAYASHI, M. HAMADA, H. NAGANAWA, M. MIYAMOTO, T. TAKEUCHI & H. UMEZAWA: Mazethramycin, a new member of anthramycin group antibiotics. J. Antibiotics 33: 665~667, 1980
7) SHIMIZU, K.; I. KAWAMOTO, F. TOMITA, M. MORIMOTO & K. FUJIMOTO: Prothracarcin, a novel antitumor antibiotic. J. Antibiotics 35: 972~978, 1982
8) TOMITA, F.; I. KAWAMOTO, T. TAMAOKI, K. ASANO, M. MORIMOTO, R. IMAI & K. FUJIMOTO (Kyowa Hakko): DC-81. Jpn. Kokai 180487 ('83), Oct. 21, 1983
9) KONISHI, M.; H. OHKUMA, N. NARUSE & H. KAWAGUCHI: Chicamycin, a new antitumor antibiotic. II. Structure determination of chicamycins A and B. J. Antibiotics 37: 200~206, 1984
10) MORI, M.; Y. UOZUMI & Y. BAN: Structure and syntheses of SEN-215 and oxotomaymycin. Heterocycles 24: 1257~1260, 1986
11) HOCHLOWSKI, J. E.; W. W. ANDRES, R. J. THERIAULT, M. JACKSON & J. B. MCALPINE: Abbeymycin, a new anthramycin-type antibiotic produced by a streptomycete. J. Antibiotics 40: 145~148, 1987
12) PARKER, K. A. & R. E. BABINE: Revision of assignment of structure to the pyrrolodiazepinone antitumor antibiotic sibiromycin. J. Am. Chem. Soc. 104: 7330~7331, 1982
13) MESENTSEV, A. S. & V. V. KULJAEVA: Methyl sibirosaminide, a novel branched-chain aminohexopyranoside from the antibiotic sibiromycin. Tetrahedron Lett. 1973: 2225~2228, 1973