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Two sulfur-containing ansamycin antibiotics from *Streptomyces albolongus*[1]

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Summary. Two sulfur-containing ansamycin antibiotics were isolated from the culture broth of *Streptomyces albolongus* C-46366; the major one was identical with awamycin and the minor one was a new ansamycin antibiotic, ansathiazin. Their structures were elucidated from their reactions and spectroscopic analyses. These antibiotics were active against gram-positive bacteria, acid-fast bacteria and a protozoan.
Key words. Ansamycin; ansathiazin; awamycin; *Streptomyces albolongus* C-46366; gram-positive bacteria.

In our search for ansamycin antibiotics using a rifampicin-resistant mutant of *Staphylococcus aureus* FDA 209P as an indicator, two sulfur-containing ansamycin antibiotics, awamycin[2] (**1**) and ansathiazin (**2**) (fig. 1), were isolated from the culture filtrate of *Streptomyces albolongus* C-46366, which was obtained from a soil sample collected in Okinawa, Japan.

Taxonomical studies showed that the strain C-46366 was assigned to chemotype I and to the white (W), rectiflexibiles (RF), chromogenic (C^+) and glabrous (SM) group within the genus *Streptomyces*. By other taxonomical characteristics, the strain C-46366 was judged to be related to *S. albolongus*[3] (from the Bergey's Manual of Determinative Bacteriology 8th edn). Strain C-46366 was subsequently compared with *S. albolongus* IFO 13465 (ISP 5570) under the same cultural conditions, and no marked differences were found. Thus, the strain was named *S. albolongus* C-46366. A scanning electron micrograph of the strain is shown in figure 2. The strain was clearly different from *Streptomyces* sp. No. 80–217, the awamycin-producing actinomycete[2], at the following points: spore chain morphology, spore surfaces and aerial mass color.

Table 1. Physicochemical properties of **1** and **2**

	1	**2**
m.p.	162–165 °C	170–175 °C (dec)
$[\alpha]_D$[a]	+949° (c 0.17)	−32° (c 0.1)
EI-MS	743 (M^+)	745 (M^+)
SI-MS	745 $(M+3)^+$	768 $(M+Na)^+$
Analysis found	C, 60.66; H, 6.69; N, 1.89; S, 4.11 (%)	C, 59.86; H, 6.48; N, 1.98; S, 3.52 (%)
Calculated	C, 60.62; H, 6.69; N, 1.86; S, 4,26 (%)	C, 59.58; H, 6.35; N, 1.88; S, 4.29 (%)
Formula	$C_{38}H_{49}No_{12}S.1/2H_2O$	$C_{37}H_{47}NO_{13}S$
UV: λ_{max}nm (ε) in MeOH	218 (40,200) 443 (4,890)	210(32,900) 273 (23,500) 420 (4,700)
IR: ν_{max} cm^{-1} in KBr	3450, 2980, 1730, 1670, 1630, 1470, 1410, 1380, 1300, 1260, 1210, 1170, 990	3450, 2980, 1720, 1670, 1640, 1600, 1460, 1410, 1380, 1290, 1150, 1100, 990
TLC(SiO_2)	Rf 0.57 ($CHCl_3$:MeOH = 9:1) Rf 0.25 (CH_2Cl_2:MeOH = 25:1)	Rf 0.37 (the same) Rf 0.10 (the same)
HPLC (ODS)	Rt 4.7 min (CH_3CN:H_2O = 7:3)	Rt 5.1 min (the same)

[a]The specific rotations of all samples herein were measured at 22–25 °CX in $CHCl_3$.

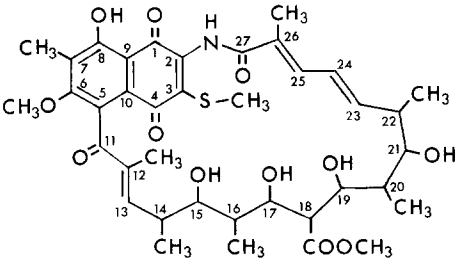
Table 2. ^{1}H NMR spectral data of **1** in $CDCl_3$ and **2** in DMSO-d_6 at 400 MHz (JEOL GX-400)

Proton	**1** δ (ppm)	J (Hz)	**2** δ (ppm)	J (Hz)
H-25	6.445 d	10.7	5.397 d	15.0
H-24	6.948 dd	10.7, 15.6	5.348 dd	2.2, 15.0
H-23	5.795 dd	8.8, 15.6	4.592 bs	
H-22	2.302 m	< 0.1, 8.8	1.894 m	
H-21	3.449 bt	< 0.1, 7.3	3.777 m	
H-20	2.133 m	7.3, 7.6	1.650 m	
H-19	4.218 ddd	2.2, 7.6	3.707 m	
H-18	2.843 t	2.2, 2.2	2.806 t	10.4
H-17	4.150 ddd	2.2, 10.3	3.707 m	
H-16	2.028 m	2.0, 10.3	2.470 m	
H-15	3.523 ddd	9.3, 2.0	3.542 m	
H-14	2.676 ddq	10.0, 9.3	2.586 m	
H-13	5.731 dd	1.4, 10.0	6.122 dd	1.2, 8.1
3-S-CH_3	2.380s			
6-O-CH_3	3.856 s		3.668 s	
7-CH_3	2.283 s		2.213 s	
26-CH_3	2.194 bs		1.504 s	
22-CH_3	1.242 d	6.8	0.525 d	7.1
20-CH_3	0.892 d	6.6	1.002 d	7.1
18-$COOCH_3$	3.717 s		3.522 s	
16-CH_3	0.802 d	6.8	0.925 d	7.3
14-CH_3	0.748 d	6.8	0.652 d	6.8
12-CH_3	2.040 d	1.4	1.878 d	1.2
8-OH[a]	11.99 s		12.36 s	
-CONH-[a]	8.369 s		11.01 s	
23-OH[a]			5.045 bs	
21-OH[a]	4.402 d	7.6	5.197 d	5.6
19-OH[a]	4.640 d	8.8	5.226 d[b]	5.4
17-OH[a]	4.092 d	6.8	5.002 d[b]	7.8
15-OH[a]	2.173 d	4.2	4.848 d	4.2

[a]Exchangeable by addition of D_2O; [b]the assignments may be reversed.

Strain C-46366 produced only a small amount of antibiotics. Therefore we tried to improve the fermentation results. The amount of antibiotics was estimated by the bioassay method using *S. aureus* FDA 209P as the test organism and by the reverse-phase HPLC method. A high-producing mutant CM-11 derived by UV-mutagenesis produced 10 times more antibiotics than the parent strain. Optimal fermentation of these antibiotics with this mutant was conducted at 20 °C using a medium consisting of 2% maltose, 2% soluble starch, 1% peptone, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.01% $ZnSO_4$ and 0.5% $CaCO_3$ (pH 7). Production of the antibiotics in a large-scale fermentation at 20 °C using this medium started at about 24 h and reached a maximum at about 50 h (fig. 3), quickening with increasing temperature.

The antibiotics were extracted with ethyl acetate (1400 l) from the culture filtrate of strain CM-11 (4200 l). The extract was concentrated to an oily residue. This was washed with n-hexane and dissolved in ethyl acetate (9 l). The ethyl acetate solution was washed with dilute $NaHCO_3$ and concentrated to dryness. After washing with n-hexane, the residue was crystallized from ethanol, giving red prisms of **1** (38.0 g). The concentrate of the mother liquor was solidified with n-hexane, giving a powder (26.4 g). This powder (10.0 g) was chromatographed on a silica gel column using a mixture of dichloromethane and methanol and gave an orange powder of **2** (252 mg).



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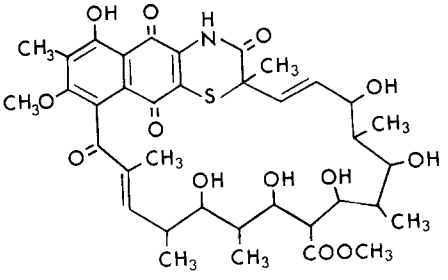

2

Figure 1. Structures of **1** and **2**.

Table 3. ^{13}C NMR spectra of **1** and **2** in DMSO-d_6 at 100 MHz (JEOL GX-400)

	1	**2**		**1**	**2**
C=O region:			sp^3 region:		
Ketone C	195.29 s	194.65 s	–CH–O–	80.43 d	78.18 d
Quinone C	182.18 s	179.73 s		72.59 d	75.31 d
	179.98 s	178.62 s		70.44 d	74.83 d
Ester C	171.78 s	172.94 s		69.11 d	73.39 d
Amide C	166.05 s	164.96 s			70.69 d
sp^2 region:			–CH–	51.25 d	54.54 d
Singlets	161.01 s	161.57 s		44.16 d	41.26 d
	160.91 s	161.49 s		42.36 d	a
	142.96 s	136.37 s		a	37.01 d
	137.35 s	136.02 s		33.56 d	33.58 d
	136.52 s	128.77 s	CH_3O–	62.44 q	61.64 q
	130.45 s	127.32 s		50.75 q	50.97 q
	129.75 s	126.97 s	CH_3–	20.45 q	22.85 q
	129.38 s	122.67 s		19.75 q	15.13 q
	126.46 s	108.85 s		19.24 q	14.20 q
	109.94 s			16.76 q	11.41 q
Doublets	144.64 d	150.24 d		16.16 q	10.81 q
	138.77 d	134.76 d		11.90 q	10.40 q
	133.19 d	125.74 d		11.51 q	9.30 q
	129.62 d			9.18 q	
			–C–S–		46.89 s

[a]One carbon signal was overlapped in the methyl signals of DMSO-d_6.

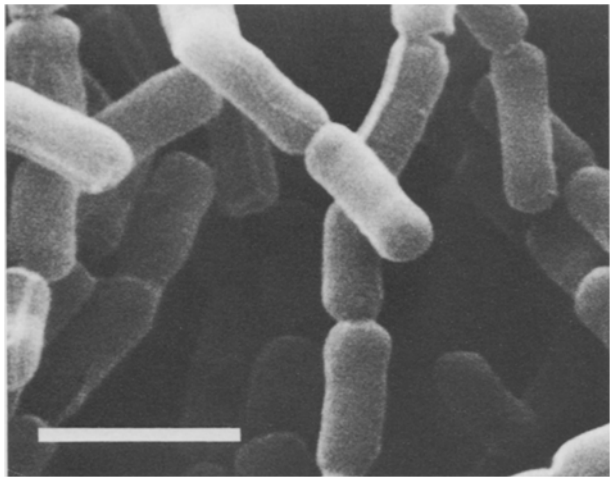

Figure 2. A scanning electron micrograph of matured aerial mycelia of *S. albolongus* C-46366 on yeast extract-mal extract agar after 3 days of cultivation at 28 °C. Bar 1 μm.

The physicochemical properties of **1** and **2** are shown in table 1. The molecular formulae of these antibiotics were determined by mass spectral data and elemental analyses to be $C_{38}H_{49}NO_{12}S$ and $C_{37}H_{47}NO_{13}S$, respectively. They were assumed to belong to the group of ansamycin antibiotics, especially, streptovaricins[4] or protostreptovaricins[5], from their NMR spectra, but they had a sulfur atom in each molecule. As the IR and UV spectra and the molecular formula of the crystalline compound, **1**, were similar to those of awamycin reported very recently[2], we compared them directly and found the two antibiotics to be identical. The amorphous compound, **2**, is apparently a new antibiotic and was named ansathiazin from its skeleton.

We tried to determine the structure of the aliphatic moiety in **1** by ^{13}C and ^{1}H NMR spectroscopy, including the proton spin-decoupling method. The results, summarized in table 2, suggested a structure for the aromatic moiety which was confirmed by the following reactions; **1** gave: 1) a dihydro derivative, hydroquinone, m.p. 158–160 °C, by treatment with 3% sodium hydrosulfite-ethyl acetate, 2) two epimeric sulfoxides, A) m.p. 161–164 °C, $[\alpha]_D$ +726° (c 0.18), SI-MS, m/z 760 $(M+1)^+$, B) m.p. 186–189 °C, $[\alpha]_D$ +779° (c 0.18), SI-MS, m/z 760$(M+1)^+$, and a sulfonyl derivative, m.p. 165–168 °C, $[\alpha]_D$ +719° (c 0.18), SI-MS, m/z 778 $(M+3)^+$, by the oxidation of the 3-methylthio group with m-chloro-perbenzoic acid in dichloromethane, 3) phenylthio, m.p. 151–153 °C, $[\alpha]_D$ +1640° (c 0.04), SI-MS, m/z 807$(M+2)^+$, and n-propylthio derivatives, m.p. 148–150 °C, $[\alpha]_D$ + 992° (c 0.10), SI-MS, m/z 773$(M+2)^+$, by exchange reaction of the methyl sulfoxide group with thiophenol in triethylamine and 1-propanethiol in triethylamine-dichloromethane, and 4) 6-demethyl derivatives[6].

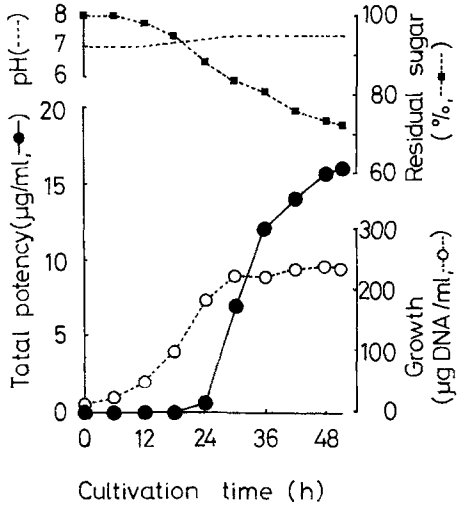

Figure 3. Time course of a large scale fermentation using a high-producing mutant CM-11. The fermentation was carried out with a 6000-l fermentor containing 4000 l of the fermentation medium.

The methoxycarbonyl moiety was confirmed by mild alkaline hydrolysis; **1** afforded a free carboxyl derivative, m.p. 176–178 °C, $[\alpha]_D$ +956° (c 0.16), SI-MS, m/z 731$(M+2)^+$, with N-sodium hydroxide in ethanol and two δ-lactone derivatives, A and B, which were condensed at the 21- and 15-positions, respectively, with N-sodium hydroxide in tetrahydrofuran, A) m.p. 172–175 °C, $[\alpha]_D$ +858° (c 0.14), SI-MS, m/z 714 $(M+3)^+$, B) m.p. 259–263 °C, $[\alpha]_D$ +921° (c 0.12), SI-MS, m/z 714$(M+3)^+$. The free craboxyl derivative gave **1** by methylation with diazomethane in diethyl ether. All these findings are in accord with structure **1** (fig. 1).

The structure of **2** was proposed by the ^{1}H and ^{13}C NMR spectra in comparison with those of **1** (tables 2 and 3). One double bond and one methyl group were absent while a methine group and a quarternary carbon were present in **2**. Long range couplings of the quarternary carbon (δ 46.89) with the amide proton (10.99), the methyl protons (1.56, 26-CH_3) and the vinyl proton (5.40, H-25) were observed, which confirmed the partial structure of $-NHCO-C(CH_3)-CH=CH-$.

The structure of the aliphatic moiety was assigned as that of **1** from proton spin-decoupling studies of **2** (table 2).

The antimicrobial activities of **2** and **1** are shown in table 4. Ansathiazin was active against gram-positive bacteria, acid-fast bacteria and a protozoan but was not active against gram-negative bacteria and yeasts. Rifampicin resistant mutants of *S. aureus* FDA 209P, *Micrococcus luteus* IFO 12708 and *M. flavus* IFO 3242 showed cross-resistance to **1**, but only *M. flavus* MfR

Table 4. Antimicrobial activities[a] of **2** and **1**

Test organism	MIC (μg/ml) **2**	**1**
Escherichia coli K12	> 100	> 100
Escherichia coli NIHJ JC-2	> 100	> 100
Proteus milabilis IFO 3849	> 100	> 100
Pseudomonas aeruginosa IFO 3080	> 100	> 100
Klebsiella pneumoniae IFO 3317	> 100	> 100
Serratia marcescens IFO 3046	> 100	> 100
Alcaligenes faecalis IFO 13111	> 100	> 100
Salmonella typhimurium IFO 12529	> 100	> 100
Bacillus subtilis PCI 219	> 50	> 2.5
Bacillus cereus IFO 3514	> 50	> 1.0
Bacillus pumilus IFO 3813	25–50	> 10
Bacillus megaterium IFO 12108	50–100	> 0.25
Staphylococcus aureus FDA 209P	> 50	> 0.5
Staphylococcus aureus SR[b]	> 50	> 50
Micrococcus luteus IFO 12708	> 25	> 0.1
Micrococcus luteus MR[b]	> 25	> 100
Micrococcus flavus IFO 3242	2.5–5.0	> 0.1
Micrococcus flavus MfR[b]	> 25	> 100
Mycobacterium smegmatis ATCC 607	> 50	> 10
Mycobacterium avium IFO 3154	> 25	> 5.0
Candida albicans IFO 0583	> 100	> 100
Saccaromyces cerevisiae IFO 0209	> 100	> 100
Tetrahymena pyriformis W	> 100	50–100

[a]The activity against bacteria and fungi were determined by an agar dilution method. Trypticase soy agar (BBL) was used as the assay medium for common bacteria. The medium was supplemented with 3% glycerol for acid-fast bacteria and 1% glucose for yeasts. The activity against *Tetrahymena pyriformis* W was assayed by a broth dilution method[7]. Bacteria were grown for 18 h at 37 °C, whereas yeasts and the protozoan were grown for 2 days at 28 °C. [b]A rifampicin-resistant strain.

displayed partial cross-resistance to **2**. We assume that this antibacterial activity of **2** is caused mainly by a mechanism different from that of inhibition of bacterial DNA-dependent RNA polymerase.

Protozoa have been used as preliminary test organisms for screening cytotoxic antitumor agents, because these eukaryotic microorganisms and mammalian cells resemble each other morphologically and metabolically. Ansathiazin as well as **1** had inhibitory activities against *Tetrahymena pyriformis* W (table 4). This suggests that **2** may have antitumor activity. In fact, like **1**, **2** had strong cytocidal activity against murine leukemic cells (data not shown).

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Growth and the average duration of larval life in the southern hemisphere lamprey, *Geotria australis* Gray

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Summary. The average duration of larval life in the anadromous lamprey, *Geotria australis* (the sole representative of the Geotriidae) is estimated as 4¼ years. Compared with other lampreys, the ammocoetes of *G. australis* have a slow growth rate, increase in length during the year preceding metamorphosis and typically enter metamorphosis at a small mean length (< 100 mm) and weight (< 1.2 g).

Key words. Lampreys; *Geotria australis;* ammocoetes; larval life; growth; metamorphosis.

The extant lampreys, which have an antitropical distribution, are separated into three families[1]. All holarctic species are placed in the Petromyzontidae, while those of the southern hemisphere are divided into either the Geotriidae or Mordaciidae. Larval lampreys (ammocoetes), which are sometimes the most abundant vertebrate in temperate rivers and streams[2,3], are burrowing and relatively sedentary animals. They feed by filtering algae, detritus and microorganisms from the water overlying the substrate surface[4-8].

Since ammocoetes do not possess bony structures suitable for the examination of annual growth rings, estimates of growth and the duration of larval life have had to be derived from the analysis of length-frequency data[7,9]. These have shown that ammocoetes grow slowly and that the protracted larval phase in both the Petromyzontidae and Mordaciidae typically ranges from 3¼ to 6¼ years[7]. There have been no comparable studies on *Geotria australis,* the sole representative of the Geotriidae[1], although a tentative estimate of 3¼ years was given for the duration of larval life in this species in a review of ammocoete ecology[7].

The present study was undertaken to examine the trends exhibited by the modes in length-frequency curves for samples of larval *G. australis* collected over several years from south-western Australian streams. The results, when compared with the pattern of growth exhibited by groups of tagged ammocoetes, provide data on larval growth and a more accurate estimate of the average duration of larval life than that given by Potter[7]. The data are compared with those obtained for representatives of the two other lamprey families.

Materials and methods. Larval and metamorphosing lampreys were collected with an electric fish shocker in Southwestern Australia from a number of sites in Carey Brook (34°24′S, 115°50′E), which is a tributary of the Donnelly River, and Dombakup Brook (34°35′S, 116°04′E) and Big Hill Brook (34°32′S, 116°15′E), which enter the Warren River. Since lampreys show a tendency to occur in slightly different habitats just prior to and during metamorphosis[7], electrofishing was extended to areas surrounding those where large numbers of larvae were found. Sampling was carried out monthly between January 1977 and July 1982, whenever prevailing turbidity and stream flow permitted. All animals were measured to the nearest 1 mm. In addition, groups of 65 and 85 larvae from Carey Brook, with lengths of 50–70 and 65–80 mm respectively, were tagged in March 1980 with a small s.c. injection of cadmium dye[10,11] so that their growth could be followed in subsequent months. Those tagged animals which were recaptured at intervals were lightly anaesthetized with benzocaine[12], measured, allowed to recover and then returned to the site. All length data were smoothed by a moving average of 5 mm.

Results and discussion. The smallest group of larval *G. australis,* which came from Carey Brook, ranged in length from 10–13 mm. Although the precise time of spawning in *G. australis* is not known, the age of these very young larvae can be estimated by assuming that embryological development and early growth in this species is similar to that of holarctic *Petromyzon marinus.* Since this latter species has a similar egg size at maturity[13,14] and begins burrowing at lengths of 7.5–9 mm after 17–33 days and reaches 9–10 mm after 33–40 days[15], the 10–13 mm larval *G. australis* were probably 1.5 months old. Since they had been collected in mid-December 1978, they were apparently the product of an early November spawning. The capture of larvae in January and February 1977 with modal lengths of 17–19 mm from single sites in the upper reaches of Dombakup Brook and Big Hill Brook (fig. 1) would be consistent with this proposed spawning time.

The small size, narrow length range and sharply defined peak in the length-frequency curves for samples of larval *G. australis* taken from a single site in both Dombakup and Big Hill Brook in January and February 1977, provide strong evidence that these samples contained only 0+ recruits resulting from the 1976 spawning season (fig. 1). Since the same age group clearly predominated at both sites throughout the following 13–16 months,