MACROLACTAMS: TWO NOVEL HOMOLOGOUS SERIES OF COMPOUNDS PRODUCED BY *Actinomadura* sp. SCC 1778

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Eight antifungal compounds were identified from the fermentation of *Actinomadura* sp. SCC 1778. This culture produces four homologous compounds ($C_{22}H_{42}N_2O_5 \sim C_{25}H_{48}N_2O_5$) containing the sugar, mycosamine, and four homologous compounds ($C_{22}H_{42}N_2O_5 \sim C_{25}H_{48}N_2O_5$) containing the sugar, 3,6-dideoxy-3-amino-L-talopyranose. Five of the compounds identified were novel macrolactams. All these compounds exhibit antifungal activity against *Candida* spp. with geometric mean MICs ranging from approximately 1.0 μg/ml for the higher homologs to 30 μg/ml for the lower homologs.

The evaluation of fermentation products of rare actinomycetes led to the discovery of a complex of antifungal antibiotics active in an assay that detects inhibitors of fungal cell envelope integrity[1]. The producing culture, was identified as a member of the genus *Actinomadura*, but differed from *Actinomadura* sp., the macrolactam producers described elsewhere[2~4]. The complex, Sch 38510, was isolated from the fermentation broth of culture SCC 1778 by solvent extraction and consisted of eight macrolactam components. Five macrolactams are reported for the first time, three compounds, Sch 38516, Sch 38518 and Sch 39185, have been previously described[3,4].

This paper describes the isolation, characterization, structures and biological activity of this novel series of macrolactams.

Taxonomy

The producing culture, SCC 1778, was isolated from a soil sample collected in Uruguay. Soil was suspended in distilled water at a concentration of 1.0 g per 10 ml, serially diluted and 0.1 ml streaked onto the surface of plating medium containing soluble starch, 0.1%; yeast extract, 0.1%; agar, 1.5%; and neomycin, 1 μg/ml. Discreet colonies developed after 14~21 days at 28~30°C.

The culture is a Gram-positive, filamentous organism forming well developed, moderately branching, non-fragmenting substrate hyphae approximately 0.4 to 0.8 μm in diameter. Aerial hyphae are formed on some meida and when presented are approximately 0.6 to 1.0 μm in diameter and fragment into chains of 6 to 23 spores. Spores are round to ovoid, smooth walled and approximately 1.1 to 1.5 μm in diameter. Spore chains are arranged in tightly appressed spirals, forming pseudosporangia 1.5 to 5.5 μm in diameter. Motile elements were not observed. Purified cell wall preparations analyzed by the method of BECKER *et al.*[5] contain *meso*-2,6-diaminopimelic acid. Whole cells analyzed by the method of LECHEVALIER[6] contain madurose. The characteristic phospholipids are phosphatidyl ethanolamine,

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phosphatidyl methylethanolamine and phospholipids of unknown structure containing glucose (Type PIV of LECHEVALIER *et al.*[7]). The morphological and chemical characteristics of SCC 1778 identify this organism as a member of the genus *Actinomadura*.

The complete taxonomic analysis will be published elsewhere.

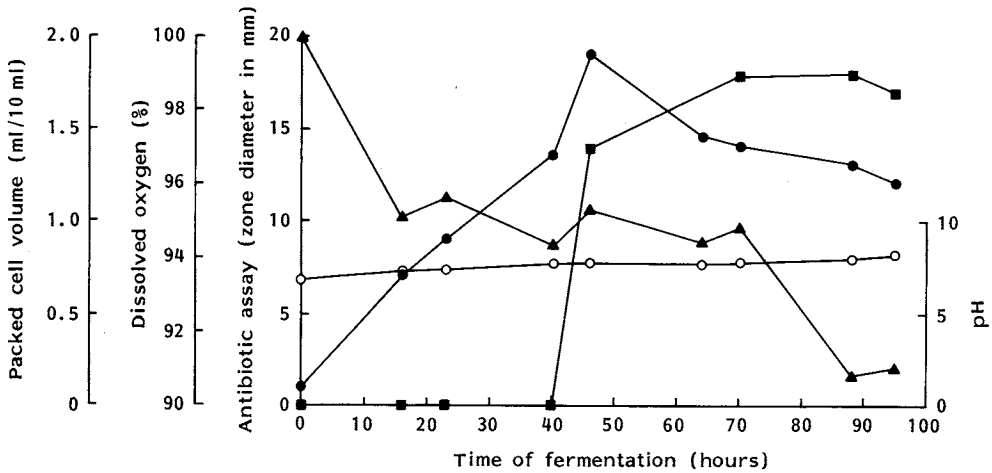
Fermentation

The initial stage inoculum for the fermentation of this culture was prepared by transferring 2.5 ml of a frozen whole broth (10% glycerol in seed medium stored at 30°C) to 50 ml of seed medium in a 250-ml Erlenmeyer flask. The medium consisted of (w/v): beef extract 0.3%, Tryptone 0.5%, yeast extract 0.5%, Cerelose 0.1%, potato starch 2.4% and $CaCO_3$ 0.2%. The pH of the medium was adjusted to 7.0 prior to sterilization with 0.1 N NaOH. The flasks were incubated at 30°C for 48 hours on a rotary shaker with a 5-cm throw at 300 rpm. For the second stage germination, 2-liter flasks containing 500 ml of the same medium were inoculated with 25 ml of the first stage seed. The conditions for incubation were the same as previously described. Production in flasks was initiated by transferring 25 ml of the second stage germination into 2-liter flasks containing 500 ml of production medium which consisted of (w/v): cotton seed flower 1.0%, peptone 0.1%, Cerelose 0.5%, corn steep liquor 0.5%, arabinose 0.5%, $MgCl_2 \cdot 6H_2O$ 0.5%, $ZnSO_4 \cdot 7H_2O$ 0.005%, $FeSO_4 \cdot 7H_2O$ 0.0014% and antifoam 0.1%. The pH of the solution was adjusted to 7.0 with 0.1 N NaOH prior to the addition of mineral salts. The fermentation was run for 90 hours at 30°C on a 300 rpm rotary shaker. Production and isolation of the antibiotic complex was monitored by agar diffusion against *Candida albicans*.

The second stage germination was also used to inoculate 100 liters of production medium (5% transfer) run in a 150-liter fermenter (Fermatron, New Brunswick Scientific, Edison, NJ). The fermentation was carried out at 30°C with aeration of 4 liters/minute and agitation of 400 rpm. The pH and dissolved oxygen levels were continuously monitored without adjustment and microbial growth was determined by packed cell volume at various intervals. Production and isolation of the antibiotic complex was followed as described above. A typical time course for the fermentation is shown in Fig. 1. Antibiotic production was measured by welling the broth against *Candida albicans*, approaching a maximum after 3 days before reaching a plateau.

Fig. 1. Fermentation profile of *Actinomadura fulva* subsp. *uruguayensis*.

○ pH, ● packed cell volume, ▲ dissolved oxygen, ■ antibiotic assay.

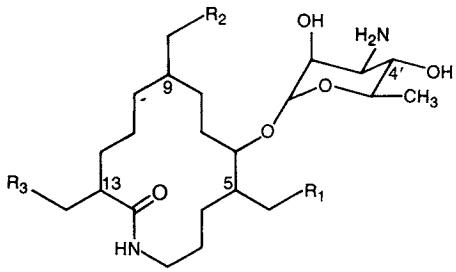


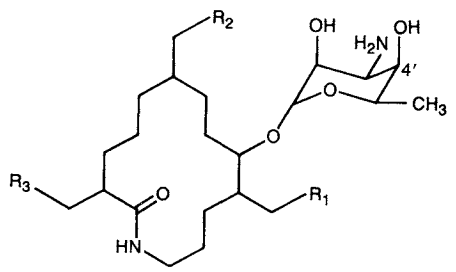
Isolation

The filtered broth (4 liters) from shake flask experiments was extracted with ethyl acetate, and the antifungal complex precipitated from ether-hexane (6:4). The enriched fraction was chromatographed on Sephadex LH-20 eluting with MeOH. Although TLC, followed by bioautography against *Candida albicans*, of the combined active fractions indicated one spot (Whatman, silica gel LK6F, $CHCl_3$-MeOH-petroleum ether-H_2O, 3:3:1:1, lower phase, Rf 0.3), liquid-liquid chromatography using droplet counter current chromatography (DCCC), resolved the mixture into 4 closely related components. A model DCCC-A-300 (Tokyo Rikakikai Co., Tokyo, Japan) instrument with 300 glass columns (2 mm (i.d.) × 40 cm) was used with a $CHCl_3$-MeOH-H_2O (7:13:8) lower phase mixture as the eluting solvent, in the ascending mode. Running at a flow rate of 6 ml/hour, Sch 38511 (**1**, 2.6 mg), Sch 38512 (**2**, 7.0 mg), Sch 38513 (**3**, 7.0 mg), and Sch 38518 (**4**, 0.5 mg) were obtained. Faster flow rates generally resulted in mixtures.

Large scale (300 liters) purification from culture broth and HPLC analysis[8] revealed a much

Fig. 2. Structures of macrolactams **1**~**8**.



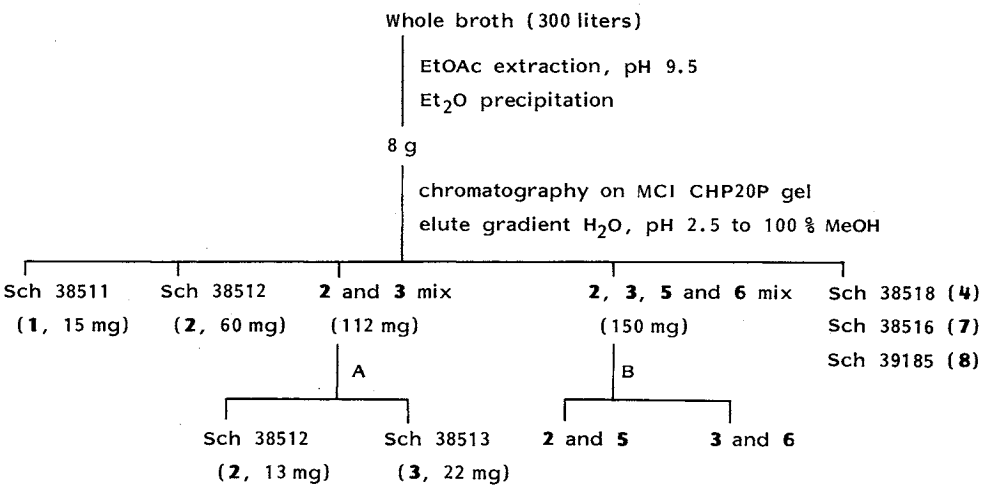


Sch 38511 (**1**) $R_1=R_2=R_3=H$
Sch 38512 (**2**) $R_1=R_2=H$ $R_3=CH_3$
Sch 38513 (**3**) $R_1=H$ $R_2=R_3=CH_3$
Sch 38518 (**4**) $R_1=R_2=R_3=CH_3$

5* $R_1=R_2=R_3=H$
6* $R_1=R_2=H$ $R_3=CH_3$
Sch 38516 (**7**) $R_1=H$ $R_2=R_3=CH_3$
Sch 39185 (**8**) $R_1=R_2=R_3=CH_3$

* Tentatively assigned based on HPLC and FAB-MS data.

Scheme 1. Isolation scheme for large scale isolation of the macrolactams **1**~**8**.



A: MCI CHP20P gel, elute gradient H_2O pH 2.5 to (30~90%) MeOH. B: Droplet counter current chromatography, $CHCl_3$-CH_3OH-H_2O (7:13:8, ascending).

more complex mixture, **1**~**8**, necessitating a modification of the original isolation scheme, described above. Thus as illustrated in Scheme 1, after ethyl acetate extraction and precipitation with ether, the crude complex was subjected to chromatography on a column of MCI CHP20P gel (5 × 60 cm) and eluted with a linear gradient consisting of dilute acetic acid (pH 2.5) to 100% MeOH. Components **1**~**3** are the major components from this broth and were chosen for further study. Components **4**, **7** and **8** have been reported elsewhere[2~4]. Components **5** and **6** were isolated as mixtures and were only characterized further by HPLC[8] and MS.

Experimental

All FAB mass spectra were obtained by using a Finnigan MAT-312 mass spectrometer in a glycerol-thioglycerol matrix. NMR, spectra were measured on a Varian XL-200 instrument operating at 200 and 50 MHz for ^{1}H and ^{13}C NMR, respectively. ^{1}H and ^{13}C NMR spectra recorded relative to TMS as an internal standard.

Physico-chemical Properties and Structures

The compounds **1**~**4** show only end absorption in the UV spectrum and exhibit bands at 3500 (br), 1650 amide and 1550 (br) cm^{-1} in the IR (KBr). The compounds test positively using ninhydrin and Rydon reagents. Mild acid hydrolysis of each compound (2 N HCl, 100°C, 2 hours) liberated mycosamine, determined by TLC comparison with an authentic standard (obtained by hydrolysis of amphotericin B).

Molecular weights were determined by high resolution FAB-MS data (Table 1). Fragmentation of the protonated molecular ion for each compound proceeded in the same manner. Loss of the amino sugar from the molecule was observed, indicated by the ion fragments observed at *m/z* 164 and 146. Upon cleavage of this sugar, the corresponding ion fragment for the aglycone portion for **1** to **4** was observed differing by 14 mass units, indicating an homologous series in the macrolactam ring. In the FAB mass spectrum, we observed protonated molecular ions $(M+H)^+$ at *m/z* 415 and *m/z* 429 for **5** and **6**, respectively. Since the HPLC retention times differentiated them from **1** and **2**, they are assigned as the corresponding isomers[8].

^{1}H NMR spectra of **1**, **2** and **3** revealed a complex pattern. Multiplet signals in the region δ 1~1.2 pertaining to methyl groups were observed. The complex of signals at δ 2.8~3.8 and δ 4.7~5.0 were not further resolved (full details and the ^{1}H NMR analysis of Sch 38518 have been reported[4]). The ^{13}C NMR spectra were much more informative and the data are presented in Table 2. The chemical shift assignments for **1**~**3** are based on fully decoupled and APT spectra. These data are compared to the data reported for Sch 38518, **4**, possessing mycosamine, and to Sch 38516, **7**, containing 3,6-dideoxy-3-amino-L-talopyranose. The chemical shifts for the sugar moiety agree with published data for mycosamine. The chemical shifts of mycosamine and 3,6-dideoxy-3-amino-L-talopyranose differ due to the inversion of the hydroxyl group at C-4′. Thus in the ^{13}C NMR spectra of compounds **1** to **4**, we observed a 6~8 ppm shift difference at C-3′ compared to the C-3′ shift in **7**. The other carbon chemical shifts of the sugar portion are comparable. These data reflect that **1**, **2**, **3** and **4** all possess the same sugar, mycosamine. We also noted that the chemical shift

Table 1. High resolution FAB mass spectral data.

Sch compound $(M+H)^+$	Found	$(M+H)^+$ calculated	*m/z*
38511 (**1**) $C_{22}H_{43}N_2O_5$	415.3162	415.3172	252, 164, 146
38512 (**2**) $C_{23}H_{45}N_2O_5$	429.3333	429.3328	266, 164, 146
38513 (**3**) $C_{24}H_{47}N_2O_5$	443.3353	443.3485	280, 164, 146
38518 (**4**) $C_{25}H_{49}N_2O_5$	457.3674	457.3641	294, 164, 146

Table 2. ^{13}C NMR chemical shifts[a].

Carbon[b]	Sch 38511 (1)	Sch 38512 (2)	Sch 38513 (3)	Sch 38518 (4)	Sch 38516 (7)
Aglycone:					
2	38.9	39.1	39.8	39.2	38.2
3	28.5	28.8	28.3	28.1	27.0
4	29.9	29.6	22.4	25.5	24.5
5	36.1	36.4	42.0	41.2	40.3
5′-CH_2	—	—	22.0	21.5	19.8
5″-CH_3	17.3	17.4	9.2	8.9	8.3
6	79.5	79.9	78.3	76.9	76.0
7	20.4	20.6	22.0	21.7	20.7
8	25.8	26.0	26.2	22.7	24.5
9	31.1	29.9	28.6	39.0	30.5
9′-CH_2	—	—	—	27.6	—
9″-CH_3	18.5	18.6	18.9	12.6	19.8
10	35.4	35.8	36.0	32.5	33.5
11	24.5	23.2	23.5	25.5	24.3
12	34.5	32.7	33.2	33.9	32.8
13	42.6	51.7	49.8	50.7	49.8
13′-CH_2	—	27.6	26.2	26.9	26.0
13″-CH_3	13.8	12.6	12.9	12.3	11.5
14	178.6	178.9	179.0	178.2	176.8
Sugar:					
1′	96.1	96.4	98.0	97.5	97.1
2′	70.7	70.5	70.4	72.9	72.6
3′	54.3	54.3	55.2	53.8	47.6
4′	69.3	69.6	70.7	71.0	71.4
5′	68.4	69.6	69.9	69.8	67.2
6′	17.2	17.3	17.7	17.7	16.0

[a] Chemical shifts in δ ppm relative to dioxane 67.6 ppm in $CDCl_3$-CD_3OD (1:1) solution.
[b] Assignments based on data obtained for **4** and **7** and on INEPT data for **1**.

Table 3. *In vitro* antifungal data for the macrolactams.

Organism (No. strains)	Medium	Geometric means MIC (μg/ml)					
		Sch 38511	Sch 38512	Sch 38513	Sch 38518	Sch 38516	Sch 39185
Candida (7)[c]	SDB[a]	28.98	17.67	4.42	1.81	0.91	2.0
Dermatophytes[d] (6)	SDB	≥114	≥128	≥57	≥40.3	≥80.6	≥101.6
Candida (7)	EM EM[b]	≥105	≥70.66	23.78	5.94	3.62	6.56

[a] SABOURAUD-dextrose broth, pH 5.7.
[b] EAGLE's minimum essential medium, pH 7.
[c] 48 hours.
[d] 72 hours.

Strains of fungi:

Candida		Dermatophytes	
Candida albicans	C-43	*Trichophyton mentagrophytes*	D-23
C. albicans	C-40	*T. mentagrophytes*	D-24
C. albicans	C-41	*T. mentagrophytes*	D-30
C. albicans	C-42	*T. rubrum*	D-61
C. albicans	C-60	*Microsporum canis*	D-18
C. tropicalis	C-44	*M. gypseum*	D-16
C. stellatoidea	C-45		

of the anomeric carbon for the macrolactam compounds is found at 96~98 ppm and would indicate that the sugar is α-linked to the aglycone[9~11].

Examination of the chemical shift values in the aglycone portion of compounds **1**, **2**, **3**, **4** and **7** indicated that differences occur due to the introduction of an extra methylene group at the substitution

sites at C-5, C-9 or C-13. All other chemical shift assignments for **1**~**3** are in good agreement with those for both Sch 38518 and Sch 39185.

Biologial Properties

The *in vitro* antifungal activity of the various macrolactams is shown in Table 3. For the homologous series of compounds containing mycosamine (Sch 38511, Sch 38512, Sch 38513 and Sch 38518), Sch 38518 was the most active compound against yeasts in both media. The decrease in antifungal activity observed for this series, corresponds to a decrease of the substitution around the macrolactam ring. For Sch 38518 and Sch 39185, which differ only in the sugar, comparable activity is observed.

Discussion

In this paper we have described the isolation of two series of novel macrolactams. The aglycone in these compounds has a 14 membered macrolactam having C-1 or C-2 alkyl side chains at positions 5, 9 and 13. These compounds form two homologous series containing mycosamine or 3,6-dideoxy-3-amino-L-talopyranoside. The antifungal potency increases for the higher homologs (Table 3, see Sch 38511 through Sch 38518). These compounds represent a novel structural class which present a unique opportunity to prepare potent semi-synthetic analogs. Compounds **4** and **8** have been recently reported as antivirals[12,13].

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