A NOVEL MACROLACTAM-DISACCHARIDE ANTIFUNGAL ANTIBIOTIC

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES, STRUCTURE ELUCIDATION AND BIOLOGICAL ACTIVITY

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A novel natural product (**1**), with antifungal activity was isolated from the culture broth of an actinomadurae. The active compound was separated from broth by *n*-butanol extraction and purified by silica gel and multicoil counter current chromatography. Physico-chemical data suggested the structure of this compound to be a novel macrolactam disaccharide related to Sch 38518 (**3**). The structure was determined by spectroscopic studies on the acetate derivative. It was active against *Candida* spp. (MIC's, 4~64 μg/ml) but less active than the monosaccharide, Sch 38518 (MIC's, 1~16 μg/ml).

During our search for novel antifungal antibiotics using the galactose permeation assay[1], we isolated a series of eight novel macrolactams from the fermentations of three actinomadurae[2~6]. *Actinomadura* sp. SCC 1840, produced the macrolactam, Sch 38518 (**3**)[2] along with an unknown compound, **1**, identified as a novel macrolactam disaccharide.

In this report, we describe the taxonomy and fermentation of the producing culture, the isolation, physico-chemical properties, structure elucidation and biological activity of this novel antifungal compound.

Materials and Methods

The Microorganism

The producing culture was isolated from an Indian soil sample by suspending the soil in sterile distilled water and plating on water agar containing 10 μg/ml of everninomicin[7]. The pure culture was deposited in the Schering Central Culture Collection as SCC 1840 and in the American Type Culture Collection Rockville, Maryland, U.S.A., as ATCC 53714. Inoculum for morphological observation was prepared according to the procedure of HORAN and BRODSKY[8]. Morphological observations were made on plates of water agar or AV-agar[9] incubated at 30°C and observed for 4 to 6 weeks. Biomass for chemical analysis was prepared by growing the cells in yeast extract-dextrose broth[10] shaken at 250 rpm at 30°C for 5 to 7 days. Cell wall analysis was by the method of BECKER *et al.*[11]; whole cells were analyzed by the method of LECHEVALIER[12] and phospholipids by the method of LECHEVALIER *et al.*[13].

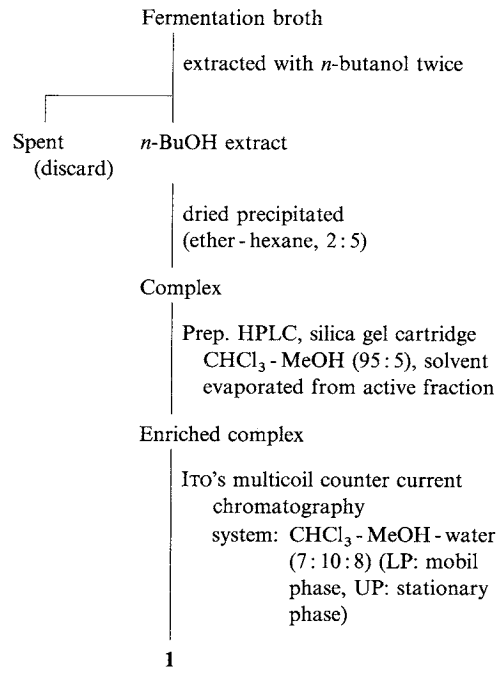
Fermentation

Fermentation studies were carried out in shake flasks, 14-liter and 100-liter fermentors. Stock cultures were maintained as frozen whole broths at −20°C in a final concentration of 10% glycerol. The inoculum medium for antifungal production contained lactose 2.5%, molasses 0.5%, ProFlo flour 1.0%, tryptone 0.5%, Edamins 0.1%, EDTA (0.01% solution) 1 ml, boric acid (0.0035% solution) 1 ml and 0.5 ml antifoam in one liter of tap water. The pH of the medium was adjusted to 7.8 prior to autoclaving. A 250-ml Erlenmeyer flask containing 70 ml of this medium was inoculated with 7.0 ml of the stock culture. The flasks was incubated at 30°C on a rotary shaker at 300 rpm for 48 hours. Fifty ml of this seed culture was

used to inoculate a 2-liter Erlenmeyer flask containing 350 ml of the same seed medium and the flask was incubated, as above, for 96 hours.

Five percent of the second stage germination was used to inoculate the fermentation medium containing 2.5% soluble starch, 1% ProFlo flour, 0.5% Marcor Meat Peptones SB, 0.5% Cerelose, 0.1% $NaNO_3$, 0.1 ml $ZnSO_4 \cdot 7H_2O$ (0.05 g/ml), 0.05% $MgCl_2 \cdot 6H_2O$, 0.1 ml $FeSO_4 \cdot 7H_2O$ (0.014 g/ml), and 0.05 ml antifoam in 1 liter tap water. The pH was adjusted to 7.9 before sterilization. Fermentation was carried out at 300 rpm agitation with 3.5 liters/minute aeration at 30°C for 72 to 96 hours. The antibiotic production along with pH, packed cell volume and dissolved oxygen were monitored at regular intervals of fermentation. The production of antibiotic was measured by an agar diffusion disc assay against *Candida albicans* 406.

Fig. 1. Isolation and purification scheme for **1**.



Isolation

The culture, SCC 1840, produces one major compound, **1**, and several minor components consisting of Sch 38518[2] and other macrolactam monosaccharides. The procedure for the isolation of **1** is described in Fig. 1. A 5.5-liter portion of the culture broth was extracted twice with eight liters of *n*-butanol. The organic extract was concentrated to about 2 liters, washed with water and evaporated to dryness. The oily residue was dissolved in a minimum amount of methanol and precipitated by drop wise addition to a mixture of ether and hexane (2 : 5). The complex obtained was filtered, dried (4.08 g) and purified on a Waters Prep. 500 HPLC using a silica gel cartridge and eluting with a mixture of chloroform and methanol (95 : 5). Fractions containing **1** were combined and dried to yield 765 mg of solids enriched in **1**. Further purification was achieved by counter current chromatography on Ito's Multicoil CCC instrument. The lower phase of the mixture of chloroform, methanol and water (7 : 10 : 8) was used as the mobile phase while the upper phase served as the stationary phase. Purity of the fractions were monitored by TLC on silica gel using a chloroform - toluene (8 : 2) solvent mixture as developing solvent. **1** was visualized on TLC by bioautography against *C. albicans* 406 and by spraying with water and acid spray (sulfuric acid and methanol, (1 : 1)). The profile of elution of macrolactams obtained by CCC is shown in Fig. 3. **1** elutes as a broad band after Sch 38518 (**3**).

Acetylation of **1**

100 mg of **1** was acetylated with a mixture of 1 : 1.5 (v/v) acetic anhydride and pyridine at 0°C for 3 hours and then left overnight at room temperature. The reaction mixture was quenched in ice and extracted with 100 ml of ethyl acetate. The organic extract was washed with dilute HCl, brine (25 ml each), dried on anhydrous Na_2SO_4 and solvent evaporated to afford the crude solid. Chromatography on a silica gel column and elution with a mixture of ethyl acetate, hexane and methanol (50 : 50 : 15), gave 45.7 mg of pure acetyl derivative (**2**).

Hydrolysis with 6 N HCl

100 mg, of **1** was mixed with 5 ml 6 N HCl in a sealed tube under reflux for 4 hours, diluted with water and extracted with 200 ml ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated to obtain 28.5 mg white solid. This was compared by TLC (solvent system chloroform - methanol (95 : 5) with the aglycone of Sch 38518.

The aglycone (25 mg) was further converted to acetate by stirring with a mixture of acetic anhydride - pyridine (1 : 1.5, 5 ml) at room temperature overnight, quenched in ice and extracted with 100 ml

ethyl acetate. The solids obtained were purified by passing through a silica gel column and eluting with a mixture of ethyl acetate and hexane (3 : 7). The pure a glycone monoacetate obtained was compared by TLC (ethyl acetate - hexane - methanol, 50 : 50 : 1) with the aglycone monoacetate of Sch 38518[2] by TLC.

Mild Hydrolysis of **1**

100 mf of **1** was partially hydrolyzed by dissolving it in 30 ml mixture of 0.1 N HCl and methanol (2 : 1) and stirred overnight at room temperature, methanol was removed from the reaction mixture, filtered and the solids obtained were dried and compared by TLC (toluene - methanol, 8 : 2) and HPLC with authentic samples of Sch 38518 and Sch 39185.

The filtrate was freeze dried and the sugar obtained was converted to pentaacetate by stirring with a mixture (5 ml) of acetic anhydride and pyridine (1 : 1.5) overnight. The reaction mixture was quenched in ice and the aqueous solution was extracted with 100 ml ethyl acetate. The organic extract was washed with dilute HCl, brine and dried over anhydrous Na_2SO_4 and the solvent removed. The sugar obtained was compared (by TLC) with pentaacetates of various hexoses.

General Procedure

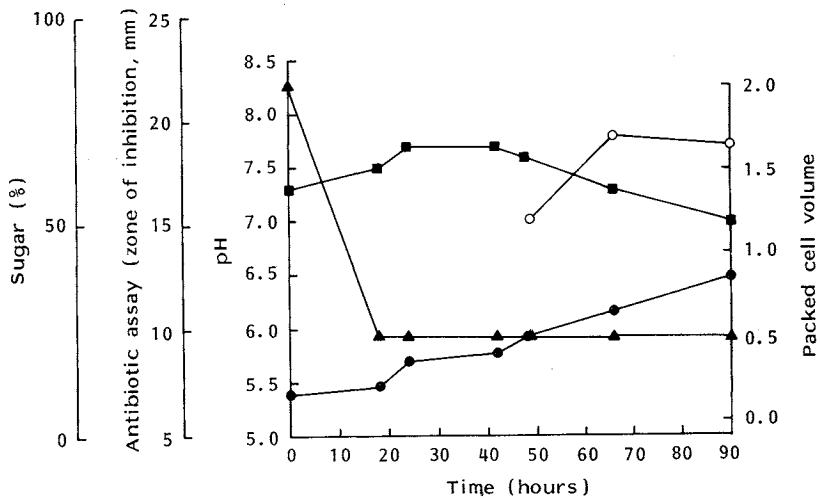
The melting points are uncorrected. IR spectra were determined on a Nicolet FTIR model 10-MX instrument. Ultraviolet spectra were obtained by using a Hewlett Packard '8450 A' UV-vis spectrophotometer equipped with HP-9872B plotter. All FAB mass spectra were obtained by using a Finnigan MAT-312 mass spectrometer in a glycerol-thioglycerol matrix. NMR spectra were measured on a Varian XL-300 instrument operating at 300 and 75 MHz for ^1H and ^{13}C NMR, respectively. ^1H and ^{13}C NMR spectra were recorded relative to TMS as an internal standard.

Results

The culture, SCC 1840 is a gram positive, filamentous organism that forms a well-developed substrate mycelium with non-fragmenting, moderately branching hyphae which are approximately 0.4 to 0.8 μm in diameter. Spores are not present on the substrate mycelium. The aerial hyphae, approximately 0.4 to 0.9 μm in diameter, bear chains of smooth-walled, round to ovoid, 0.9 to 1.5 μm in diameter, spores. The spore chains contain from 6 to 33 spores which are arranged in tightly appressed spirals forming pseudosporangia 2 to 7 μm in diameter. Motile elements were not observed in either the substrate or aerial mycelium. Purified cell walls contain *meso* 2-6-diaminopimelic acid, alanine, glutamic acid, glucosamine, muramic

Fig. 2. Fermentation profile of SCC 1840.

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



acid and traces of mannose. Whole-cell hydrolysates contain glucose, mannose, madurose, ribose, and traces of galactose and rhamnose. The phospholipids present are diphosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine and unknown glucosamine-containing phospholipids. Therefore, SCC 1840 has a type III cell wall with a type B whole-cell sugar pattern and type PIV phospholipid composition. On the basis of chemical and morphological characteristics, strain SCC 1840 was identified as a member of the genus *Actinomadura*.

A typical time course study profile of a 100-liter fermentation is shown in Fig. 2. The sugar level in the fermentation medium depletes as the cells grow and then levels off as the antibiotic production starts. The packed cell volume which indicates cell growth continues to increase until the end of the fermentation. The antifungal production starts at about 50 hours and peaks at 70~80 hours of fermentation.

It is basic in nature, shows a positive ninhydrin test, and end absorption in the UV spectrum. The IR spectrum shows the presence of an amide

Table 1. Physico-chemical properties of the acetate derivative of **1**.

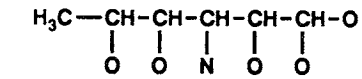
UV (MeOH) λ_{max} nm	End absorption
IR (KBr) ν_{max} cm^{-1}	3320, 2930, 1750, 1655, 1540, 1375, 1230, 1050
FAB mass spec.	871 $(M+H)^+$, 541
^1H NMR ($CDCl_3$)	5.5 (bd d, J=4, 4 Hz, 1 H), 5.1 (t, J=8 Hz, 1H), 4.93 (t, J=8 Hz, 1H), 4.7 (m, 4H), 4.15 (dd, J=8, 4 Hz, 1H), 4.05 (d, J=8 Hz, 1H), 3.92 (dd, J=8, 2 Hz, 1H), 3.75 (dt, J=6, 4 Hz, 1H), 3.5 (m, 4H), 3.1 (br t, J=12 Hz, 1H), 3.0 (br d, J=12 Hz, 1H), 2.05 (s, 3H), 2.00 (s, 6H), 1.96 (s, 3H), 1.94 (s, 3H), 1.92 (s, 3H), 0.9~1.6 (several CH and CH_2), 1.08 (d, J=7 Hz, 3H), 0.82 (t, J=7 Hz, 3H), 0.77 (t, J=7 Hz, 3H), 7.4 (t, J=7 Hz, 3H)

Table 2. ^{13}C chemical shift of acetate derivatives of **1**, Sch 38518 and Sch 39185.

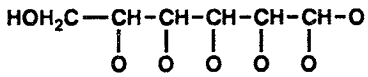
	Acetates of				Acetates of		
	1	Sch 38518	Sch 39185		**1**	Sch 38518	Sch 39185
$-CH_3$	9.10	8.91	8.83	Sugar	94.06	94.28	95.02
	12.46	12.31	12.30	I	73.25	73.14	70.33
	12.66	12.41	12.43		55.15	48.50	45.06
$-CH_2$	20.79	20.90	20.85		72.43	72.35	70.12
	22.49	22.69	22.24		67.00	66.66	65.50
	23.12	23.09	22.32		17.76	17.52	16.64
	25.06	24.92	24.99	$COCH_3$	20.79	21.06	21.18
	25.27	25.09	25.04		20.79	21.64	21.40
	26.77	26.59	26.55		20.79	23.31	22.89
	27.25	27.11	27.06		20.79		
	28.23	28.36	28.16		21.20		
	31.78	31.69	31.72		21.66		
	33.73	33.52	33.53	$COCH_3$	169.36	169.76	169.52
	38.66	38.69	38.69		169.69	170.03	170.80
-CH	38.85	38.53	38.48		169.99	171.59	170.80
	40.89	40.91	40.70		170.15		
	50.89	50.74	50.77		170.20		
	77.93	78.75	78.35		170.61		
-C=O	175.85	176.02	176.02	Sugar	89.97		
				II	74.35		
					72.89		
					71.09		
					68.63		
					61.96		

functionality (1655, 1550 cm^{-1}). The Physico-chemical properties and lipid nature showed a striking resemblance to other macrolactams[2~6]. **1**, like other macrolactams, behaves as a lipid and can be detected on a silica gel plate by water spray. The FAB mass spectrum showed a molecular ion peak at 619 $(M+H)^+$, 162 mass units higher than Sch 38518 and a fragment ion peak at 457, which is due to the Sch 38518 moiety. High resolution mass measurements revealed the molecular formula to be $C_{31}H_{59}N_2O_{10}$. This is $C_6H_{10}O_5$ higher than Sch 38518. These data suggested that this compound contains one more sugar, a hexose. However because of the unstable nature of the compound, further structural studies were accomplihsed by evaluation of the spectral data of the stable acetyl derivative.

It was acetylated with a mixture of acetic anhydride and pyridine to obgain a hexaacetyl derivative (**2**). The spectroscopic properties of **2** are shown in Table 1. The FAB mass spectrum of this compound showed a molecular ion peak at 871 $(M+H)^+$, revealing it to be the hexaacetyl derivative. ^{13}C NMR of **2** along with triacetate of Sch 38518 and Sch 39185 are shown in Table 2. ^1H NMR showed three primary methyls at δ 0.8~0.9, and a secondary methyl at δ 1.08 and suggested the pattern of the macrolactam aglycone moiety, as that of Sch 38518 and an additional sugar. 2D (^1H-^1H) correlation spectrum showed not only that the partial structures A and B were those of two pyranosyl hexoses, but also that the sugar B is attached to sugar A at C-2 and that the sugar A is attached to the aglycone at C-9. Comparison of the ^{13}C chemical shift data of the acetate of **1**, Sch 38518 and Sch 39185 confirmed the presence of macrolactam ring and two sugars in the molecule.

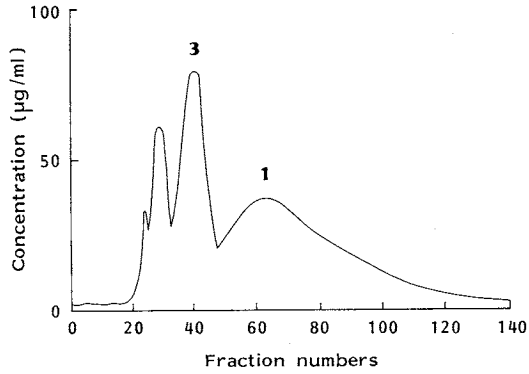


A



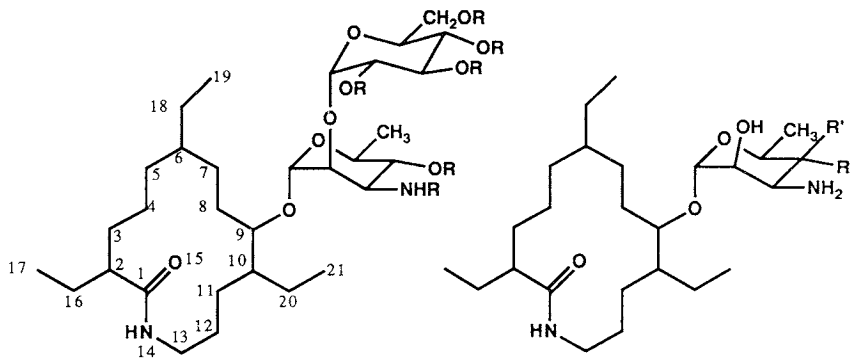
B

Fig. 3. Elution profile of macrolactams from multicoil counter current instrument.



ITO's multicoil counter current chromatography. System: $CHCl_3$-MeOH-water (7:10:8) (LP: mobil phase, UP: stationary phase). Flow rate: 4 ml/minute.

Fig. 4. Structure of **1**, hexaacetate of **1** (**2**), Sch 38518 (**3**) and Sch 39185 (**4**).



1 R=H
2 R=Ac

3 R=OH, R'=H
4 R=H, R'=OH

This compound (**1**) was hydrolyzed with 6 N HCl and the aglycone obtained on TLC comparison with the aglycones of **3** and **4** indicated it to be identical with that of **3**. The aglycone was further acetylated with acetic anhydride-pyridine to yield a monoacetate which on further comparison (TLC and spectal data[2]) with Sch 38518 aglycone monosaccharide confirmed its structure.

Mild hydrolysis of **1** by 0.1 N HCl and methanol (2:1) yielded a monosaccharide and a sugar. The monosaccharide was found to be identical with Sch 38518, indicating the amino sugar attached to be mycosamine. The sugar was converted to its pentaacetate and was identified to be glucose pentaacetate on comparison with an authentic sample. The sugar residues were determined to be α-mycosamine and α-D-glucopyranose, respectively, from their ^{1}H and ^{13}C chemical shifts and ^{1}H-^{1}H spin coupling constants. Thus the structure of **1** is shown in Fig. 3.

Table 3. *In vitro* activities of **1** and Sch 38518 against various fungi.

Organism (No. of strains)	Medium	Geometric mean MIC's (μg/ml)	
		1	Sch 38518
Candida (12)	SDB[a]	≥10.7	3.8
	EMEM[b]	32.0	17.3
Dermatophytes (7)	SDB[c]	≥64.0	≥64.0
Aspergillus (2)	SDB[c]	≥128.0	≥512.0

[a] Sabouraud dextrose broth, pH 5.7, 48 hours.
[b] Eagles minimum essential medium, pH 7.0, 48 hours.
[c] Sabouraud dextrose broth, pH 5.7, 72 hours.

Biological Activity

The *in vitro* antifungal activity of **1** and **3** against various yeasts and dermatophytes in Sabouraud dextrose broth (SDB) and Eagles minimum essential media (EMEM) are shown in Table 3. Both compounds were active against *Candida* spp. and activity in SDB was better (3~4-fold) than in EMEM. In general, **1** was less active than its parent macrolactam monosaccharide Sch 38518.

Acknowledgment

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