MACROLACTAMS: A NOVEL CLASS OF ANTIFUNGAL ANTIBIOTICS PRODUCED BY *Actinomadura* spp. SCC 1776 AND SCC 1777

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(Received for publication September 28, 1991)

Three novel antifungal antibiotics, Sch 38518, Sch 39185 and Sch 38516 were isolated from the fermentation broths of two actinomycetes identified by chemical, morphological and physiological analysis as a new species of *Actinomadura*. The compounds were isolated from broth by solvent extraction and purified by silica gel chromatography. Physico-chemical properties, mass spectral analysis, IR and UV suggested the compounds were similar. Sch 38518 and Sch 39185 have a molecular formula of $C_{25}H_{48}N_2O_5$. ^1H NMR, ^{13}C NMR and hydrolysis indicated the aglycones were identical, however the compounds differed in containing isomeric sugar moieties. Sch 38518 contains mycosamine while Sch 39185 contains 3,6-dideoxy-3-amino-L-talopyranose. Sch 38516 has a molecular formula of $C_{24}H_{46}N_2O_5$ and is a lower homolog of Sch 39185. The three compounds, Sch 38518 (**1**), Sch 39185 (**2**), and Sch 38516 (**3**) exhibit similar activity against *Candida* spp. with geometric mean MICs of 1.81, 2.00 and 0.91 μg/ml, respectively.

A directed search for rare actinomycetes capable of producing novel secondary metabolites resulted in the isolation of a group of filamentous organisms producing antifungal antibiotics active in a mechanistic assay that detects inhibitors of fungal cell envelope integrity.[1] Two of the producing strains, SCC 1776 and SCC 1777, were identified as members of the genus *Actinomadura*. The isolated and purified compounds were found to be members of a new class of antifungal antibiotics characterized by the presence of a 14 membered macrocyclic lactam.[2~8]

In this report, the taxonomy and fermentation of both cultures, and the isolation, physico-chemical properties, and biological activities of these compounds are described.

Taxonomy

The producing strains, SCC 1776 and SCC 1777, were isolated from deciduous forest soil samples. Soils were suspended in distilled water and aliquots steaked onto the surface of plating medium containing glycerol, 0.1%; yeast extract, 0.1%; agar, 1.5%; and rosaramicin[9] 10 μg/ml. Discreet colonies developed after 14~21 days at 28~30°C.

The isolates are Gram-positive, filamentous organisms forming yellow-brown vegetative mycelial pigments and aerial mycelia approximately 0.5 to 1.0 μm in diameter that fragment into chains of 5 to 29 spores. Spore chains are straight, hooked, irregularly curved or arranged in spirals of 2 to 5 turns. The spores are round to ovoid, 0.8 to 1.1 μm in diameter. By electron microscopy the spore surface appears

folded. Purified cell wall preparations analyzed by the method of BECKER *et al.*[10] contain *meso*-2,6-diaminopimelic acid; whole cells analyzed by the method of LECHEVALIER[11] contain madurose. The characteristic phospholipids are phospholipids of unknown structure containing glucosamine (Type PIV of LECHEVALIER *et al.*[12]). The above chemical characteristics are consistent with organisms classified as *Actinomadura*.

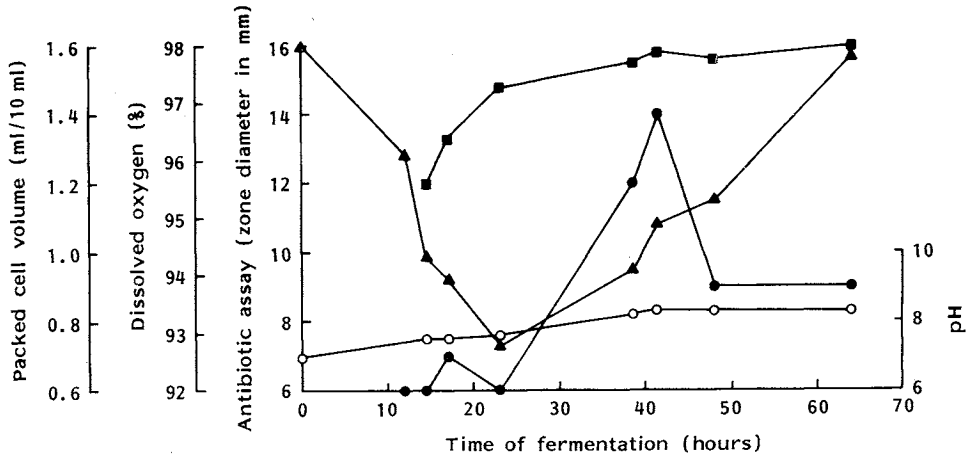
The complete taxonomic analysis will be published elsewhere.

Fermentation

The initial stage inoculum for the fermentation of SCC 1776 was prepared by transferring 2.5 ml of a frozen whole broth (10% glycerol in germination medium stored at −20°C) to 50 ml of germination medium in 250-ml Erlenmeyer flasks. The medium consisted of beef extract, 0.3%; Tryptone, 0.5%; yeast extract, 0.5%; Cerelose, 0.1%; potato starch, 2.4%; calcium carbonate, 0.2%; and Dow-Corning antifoam B, 1 ml per liter of tap water. The pH of the medium was adjusted to 7.0 with 0.1 N NaOH prior to sterilization. The flasks were incubated at 30°C on a rotary shaker at 300 rpm for 48 hours. For the second stage germination, 2-liter flasks containing 350 ml of the same medium were inoculated with 25 ml of the first stage germination. The conditions for incubation were the same as before. Ten-liter fermentations were carried out in 14-liter NBS Laboratory Fermenters in a medium containing PD 650 dextrin, 2.0%; cotton seed flour, 0.75%; crush peas, 0.25%; molasses, 0.5%; maltose, 0.5%; and calcium carbonate, 0.2% in tap water. The pH was adjusted to 7.2 with 0.1 N NaOH prior to the addition of $CaCO_3$. The second state germination (3.5%) was used to initiate the fermentation which was carried out at 32°C with aeration of 3.5 liters per minute and agitation at 350 rpm. Antifungal activity, pH, packed cell volume, and dissolved oxygen were monitored at regular intervals. A typical time course study of a 10-liter fermentation is shown in Fig. 1. The production of the antifungal complex was determined by an agar diffusion assay against *Candida albicans* and by HPLC evaluation of *n*-butanol extract of the broth. Antifungal activity can be detected after 24 hours of incubation. Maximum titers were observed after 48 hours and remained steady over the course of the fermentation.

Fig. 1. Fermentation profile of SCC 1776.

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



Similar fermentation procedures were used for the fermentation of culture SCC 1777. However, the second stage germination contained 500 ml of medium in 2-liter flasks and the production medium consisted of yeast extract, 0.5%; NZ amine, 0.5%; Cerelose, 1.0%; soluble starch, 2.0%; calcium carbonate, 0.4%; cobalt chloride (0.001 M), 0.1%; and antifoam, 0.1% in tap water. The pH of the medium was adjusted to 7.0 with 0.1 N NaOH prior to sterilization. A 5% inoculum from the second stage germination was used to initiate the fermentation. A typical time course study of culture SCC 1777 fermentation is shown in Fig. 2. The optimum antibiotic yields were obtained after 90 hours of incubation. Other studies indicated antibiotic titers did not increase after additional incubation.

Isolation

The procedure for the isolation and purification of Sch 38518 (**1**) and Sch 39185 (**2**) from culture

Fig. 2. Fermentation profile of SCC 1777.

● Packed cell volume, □ glucose utilization, ▲ dissolved oxygen, △ antibiotic assay, ○ pH.

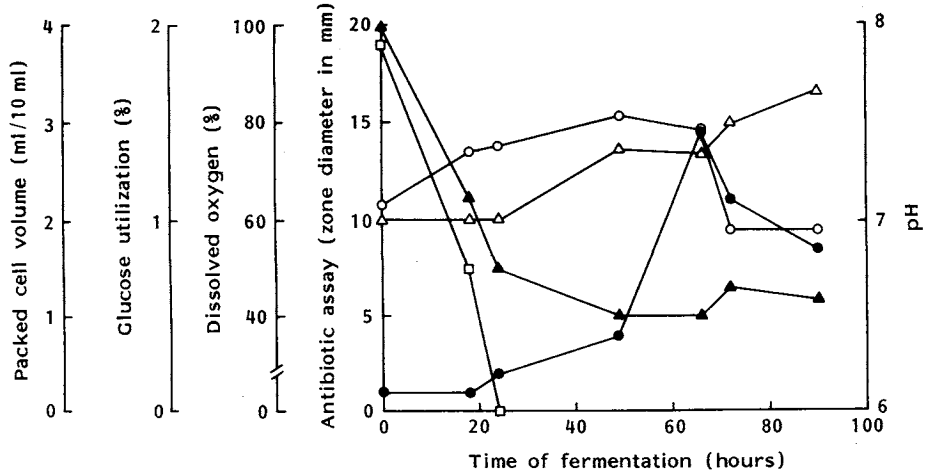
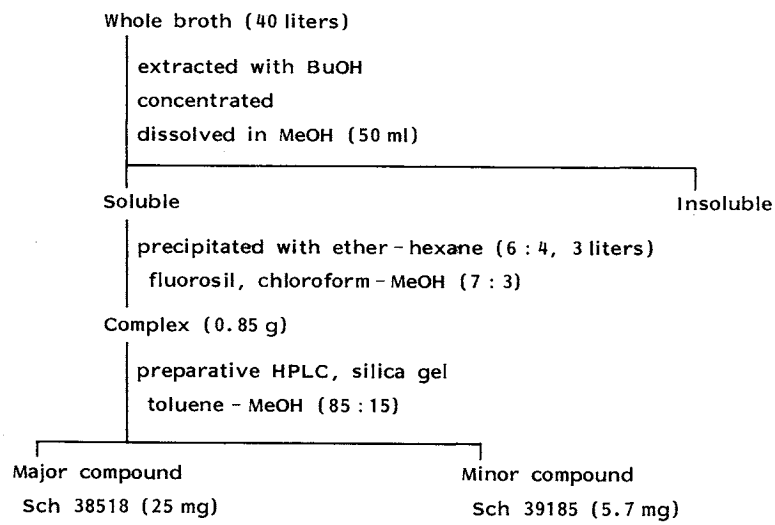


Fig. 3. Isolation and purification procedures for Sch 38518 and Sch 39185.



broth of SCC 1776 is described in Fig. 3. A 40-liter portion of the fermentation broth was extracted with 60 liters of *n*-butanol to remove the antifungal mixture from the aqueous broth. The organic layer was concentrated to 2 liters, washed with water and dried. The residue was dissolved in methanol and the antibiotic complex was precipitated by addition to a mixture of diethyl ether and hexane (1:4). The resulting precipitate was filtered and then dissolved in a chloroform and methanol (7:3) mixture and applied to a fluorosil column (5.0 × 10.2 cm) and eluted with the same solvent mixture. Both antifungals eluted as a mixture and were detected by TLC (Analtech silica gel plates, $CHCl_3$-MeOH (8:2), Rf's for **1** and **2** are 0.25 and 0.3, respectively) and agar diffusion assay against *C. albicans*. Final purification was achieved by preparative HPLC on a silica gel cartridge (Waters Div., Millipore Corp.), eluting with a mixture of toluene and methanol (85:15). The minor component Sch 39185 elutes first followed by Sch 38518.

The steps leading to the isolation and purification of **3** from culture broth of SCC 1777 are outlined in Fig. 4. The active principle from 130 liters fermentation broth was extracted twice with 50-liters *n*-butanol. The organic layer was evaporated to 2 liters, washed with water and then dried. The residue was then dissolved in methanol and the antibiotic complex was precipitated from a mixture

Fig. 4. Isolation procedure for Sch 38516.

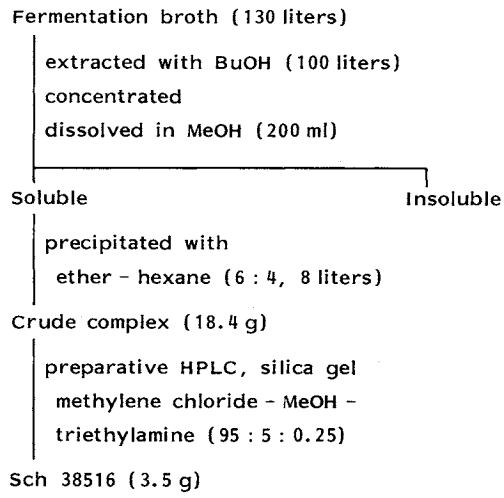
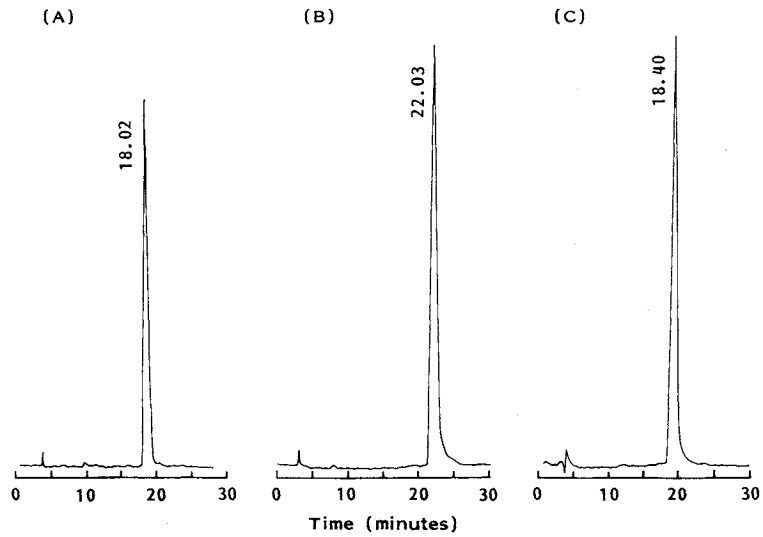


Fig. 5. HPLC profiles of the macrolactams Sch 38518 (A), Sch 39185 (B) and Sch 38516 (C).



Column: Shandon MOS Hypersil (5 μm) 25 cm × 4.6 mm; mobile phase: methanol: 0.01 M sodium acetate (pH 4.0 with glacial acetic acid) 57:25; flow rate: 1 ml/minute.

of diethyl ether and hexane (1 : 4). Final purification of Sch 38516 was achieved by preparative HPLC on a silica gel column using a mixture of dichloromethane, methanol and triethylamine (95 : 5 : 0.25) as eluting solvent. Presence of triethylamine assists in sharper elution of Sch 38516.

To indicate the purity of the compounds isolated an HPLC system was developed.[14] Fig. 5 shows HPLC traces of purified components, detected by fluorescence after post-column derivatization with *o*-phthalaldehyde. A detailed report discussing the detection of these compounds using post-column derivatization will be published in a separate paper.[14]

Experimental

The melting points are uncorrected. IR spectra were determined on a Nicolet FTIR model 10-MX instrument. UV 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 Fennigan 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 ^1H and ^{13}C NMR, respectively. ^1H and ^{13}C NMR spectra recorded relative to TMS as an internal standard.

Physico-chemical Properties and Structures

Sch 38518, Sch 39185 and Sch 38516 are lipophilic compounds, basic in nature and show positive color response to ninhydrin. They are soluble in methanol, DMSO, partially soluble in chloroform, ethyl acetate and insoluble in ether, hexane and H_2O. The physico-chemical properties of these antifungals are shown in Table 1.

These compounds showed end absorption in the UV spectrum, and –OH and –NH (3600, 3300 cm^{-1}) stretching and an amide (1640, 1550 cm^{-1}) functionality in the IR spectrum. The preliminary characterization of **1** and **2** indicated that the two compounds are isomers having molecular formulae $C_{25}H_{48}N_2O_5$. However, the optical rotations were different (+9.7° and −5.8°, respectively). A FAB mass spectrum of **3** showed a molecular peak at 442, 14 mass units less than **1** and **2**. High resolution mass measurements established the molecular formula as $C_{24}H_{46}N_2O_5$: (calcd for $C_{24}H_{47}N_2O_5$: 443.3485 and found: 443.3517).

The ^1H NMR spectrum of Sch 39185 (**2**) was similar to that of Sch 38518 (**1**), both spectra showed the presence of three primary methyl groups centered at δ 0.9, one secondary methyl at δ 1.25 and one anomeric proton at δ 4.8. Differences were observed for proton signals on oxygenated carbons. The ^{13}C

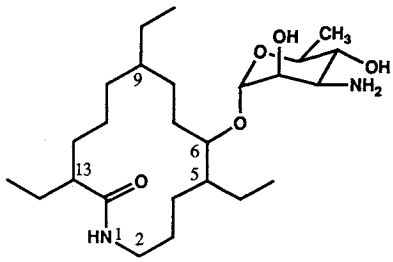
Table 1. Physico-chemical properties of Sch 38518, Sch 39185 and Sch 38516.

	Sch 38518	Sch 39185	Sch 38516
MP	220°C (dec)	216~220°C (dec)	156~160°C (dec)
$[\alpha]_D^{26}$	+9.7° (*c* 0.5, MeOH)	−5.8° (*c* 0.5, MeOH)	−6.7° (*c* 0.5, DMSO)
UV λ_{max} nm	End absorption	End absorption	End absorption
IR (KBr) ν cm^{-1}	3420, 3300, 2930, 1640, 1550, 1455, 1045	3420, 3300, 2920, 1640, 1555, 1385, 1050	3430, 3340, 2940, 1645, 1550, 1460, 1055
FAB-MS	457 $(M+H)^+$, 312, 294, 163, 146	457 $(M+H)^+$, 312, 294, 164, 146	443 $(M+H)^+$, 298, 280, 164, 146
HR-MS	$C_{25}H_{48}N_2O_5$	$C_{25}H_{48}N_2O_5$	$C_{24}H_{46}N_2O_5$
^1H NMR	3 Primary methyls (δ 0.9)	3 Primary methyls (δ 0.9)	2 Primary methyls
($CDCl_3+CD_3OD$) δ	1 Secondary methyl (δ 1.25)	1 Secondary methyl (δ 1.25)	2 Secondary methyls
	Several $-CH_2-$ and $-CH<$	Several $-CH_2-$ and $-CH<$	Several $-CH_2-$ and $-CH<$
	7–N–CH– and –O–CH–	7–N–CH– and –O–CH–	7–N–CH– and –O–CH–
	1 Proton at δ 4.8—Anomeric	1 Proton at δ 4.8—Anomeric	1 Proton at δ 4.8—Anomeric

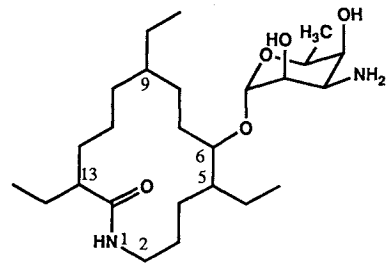
Table 2. ^{13}C NMR chemical shifts of Sch 38518, Sch 39185 and Sch 38516.

Carbons	Sch 38518	Sch 39185	Sch 38516	Carbons	Sch 38518	Sch 39185	Sch 38516
2	39.2	39.2	38.2	11	25.5	25.5	24.3
3	28.1	28.1	27.0	12	33.9	33.9	32.8
4	25.5	25.6	24.5	13	50.7	50.9	49.8
5	41.2	41.2	40.3	13′	26.9	26.9	26.0
5′	21.5	21.6	19.8	13″	12.3	12.3	11.5
5″	8.9	9.0	8.3	14	178.2	178.2	176.8
6	76.9	77.6	76.0	1′	97.5	97.8	97.1
7	21.7	21.8	20.7	2′	72.9	70.3	72.6
8	22.7	22.7	24.5	3′	53.8	48.7	47.6
9	39.0	39.2	30.5	4′	71.0	69.3	71.4
9′	27.6	27.7	—	5′	69.8	67.5	67.2
9″	12.6	12.6	19.8	6′	17.7	16.7	16.0
10	32.5	32.6	33.5				

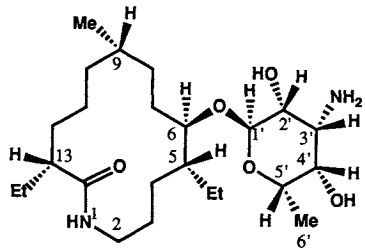
Fig. 6. Structures of Sch 38518 (**1**), Sch 39185 (**2**) and Sch 38516 (**3**).



1



2



3

NMR chemical shifts of all the three compounds are listed in Table 2. Both **1** and **2** showed 25 and Sch 38516 (**3**) showed 24 carbon signals, respectively, consistent with their molecular formulae. The APT ^{13}C experiment for **1** and **2**, indicated that both have 4 methyls, 11 methylenes, 9 methines and a quaternary carbon. Most of the ^{13}C NMR signals of Sch 39185 (**2**) were similar to those of Sch 38518 (**1**) except for one methyl group and methines connected to heteroatoms. Analysis of the spectral data and comparison of the acetates of aglycones and sugars obtained by hydrolysis with 6 N HCl, indicated that **1** and **2** differ in having isomeric sugars, mycosamine and 3,6-dideoxy-3-amino-L-talopyranose, respectively. The structures of these antifungals are shown in Fig. 6. Details of the structure determination have been reported elsewhere.[6,7]

For Sch 38516 (**3**), the lack of a methylene group compared to Sch 39185 (**2**) could result from a ring contraction to a 13 membered ring or a loss from one of the three ring substituted ethyl groups. ^{1}H NMR revealed that Sch 38516 (**3**) contains two primary methyl groups (δ 0.75 and 0.80) and two secondary methyl groups (δ 0.81 and 1.18). These data indicated that Sch 38516 has one methyl instead of an ethyl side chain.

The APT ^{13}C NMR of **3** established 4 methyls, 10 methylenes, and 9 methines and a quaternary carbon signal (Table 2). From ^{13}C chemical shifts (C-8 through C-10) it is apparent that the methylene attached to C-9 of Sch 38518 (**1**) is lost and there is a methyl instead of ethyl group. Also comparison of

Fig. 7. 2D (^{13}C-^{13}C) chemical shift correlation of aglycone monoacetate of Sch 38516.

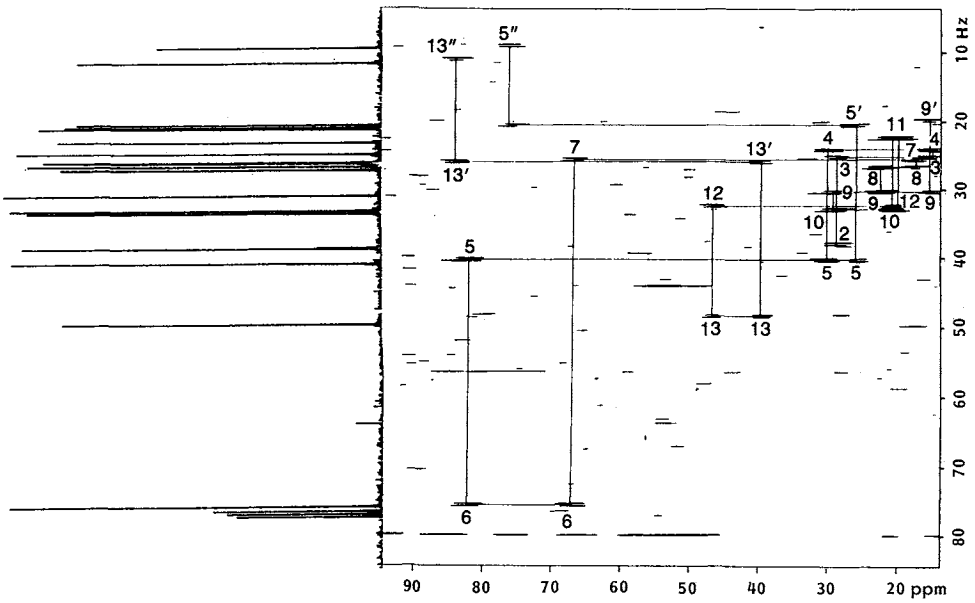


Table 3. Antifungal activities of Sch 38518, Sch 39185 and Sch 38516.

Strains	No. of strains	Geometric mean MIC (μg/ml)					
		SDB[a]			EMEM[b]		
		Sch 38518	Sch 39185	Sch 38516	Sch 38518	Sch 39185	Sch 38516
Candida	7	1.8	2.0	0.91	5.9	6.6	3.62
Dermatophytes	6	≥40.3	≥101.6	≥80.6	—	—	—

[a] SDB: SABOURAUD-dextrose broth, pH 5.7, 28°C.
[b] EMEM: EAGLE's minimum essential medium, pH 7.0, 37°C, CO_2.

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		

the ^{13}C chemical shifts of this antifungal with Sch 38518 (**1**) and Sch 39185 (**2**) revealed that it closely resembles Sch 39185 (**2**), suggesting the presence of an identical sugar. The carbon skeleton of the aglycone was established by 2D (^{13}C-^{13}C) chemical shift correlation studies of the aglycone monoacetate as shown in Fig. 7.

The structure of this antifungal along with absolute stereochemistry is shown in Fig. 6. Details of structure elucidation and determination of absolute stereochemistry by X-ray crystallographic analysis have been published elsewhere.[7]

Biological Activity

The minimum inhibitory concentration (MICs) of these antifungals against various strains of *Candida* sp. and dermatophytes are shown in Table 3. They exhibit good antifungal activity and are more active against *Candida* than dermatophytes. Sch 38518 showed moderate activity at 2% concentration against a topical guinea pig *Trichophyton mentagrophytes* infection, and an intravaginal hamster *Candida* infection. Intravenous toxicity studies in mice showed an iv LD_{50} of 25 mg/kg.

Discussion

Sch 38518 (**1**), Sch 39185 (**2**) and Sch 38516 (**3**) are novel compounds discovered in our search for antifungals from microbial sources. Sch 38518 (**1**) and Sch 39185 (**2**) have identical aglycones but have isomeric sugars. Sch 38518 (**1**) was found to contain 1-mycosamine[6] and the sugar from Sch 39185 (**2**) was identified as 3,6-dideoxy-3-amino-L-talopyranoside, a novel sugar first isolated from these naturally occurring compounds.

The aglycone is a 14 membered macrocyclic lactam having three ethyl groups attached to carbon atoms 5, 9, and 13 and the sugar is attached at carbon atom 6. These two antifungals represent the members of two homologous series formed by homologous aglycones attached to isomeric sugars.[8] Sch 38516 (**3**) is a lower homolog of the antifungal Sch 39185 (**2**), containing a 14 membered macrocyclic lactam ring system with two ethyl side chains at C-5 and C-13 and a methyl group at C-9. The sugar is identical to that of Sch 39185 (**2**), namely 3,6-dideoxy-3-amino-L-talopyranoside.

These antifungals are novel compounds belonging to a new family of natural products containing a lactam ring. Although there are several compounds with mixed lactone-lactam rings and pseudo-lactam rings known[15~18] the compounds we have isolated represent a new class of compounds. Recently compounds **1** and **2** have been reported as antivirals.[19,20]

Acknowledgment

The authors wish to acknowledge Mr. MURAWSKI for initial isolation studies and Mr. MIERZWA for the development of an HPLC system for detection.

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