Sch 37137, A NOVEL ANTIFUNGAL COMPOUND PRODUCED BY A *MICROMONOSPORA* SP.

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE AND BIOLOGICAL PROPERTIES

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A new antifungal compound, Sch 37137, was isolated from the cultured broth of a *Micromonospora* sp., SCC 1792. Sch 37137 is structurally related to A 19009, a dipeptide previously discovered from a *Streptomyces* sp. The compound was weakly active against species of *Candida* and dermatophytes (mean MICs ≥ 128 μg/ml) in Sabouraud dextrose, yeast-nitrogen and modified Eagles minimum essential media; however activity against *Candida* sp. (mean MICs ≥ 12 μg/ml) and dermatophytes (mean MICs ≥ 0.8 μg/ml) significantly improved in MA medium.

In the course of screening for new antifungal agents from culture broths, we have discovered a novel antifungal compound, Sch 37137, produced by a *Micromonospora* sp. The structure and biological properties of Sch 37137 resemble A 19009[1], a dipeptide isolated from a *Streptomyces* sp.

This paper details the producing organism, production, isolation, structure and biological properties of Sch 37137.

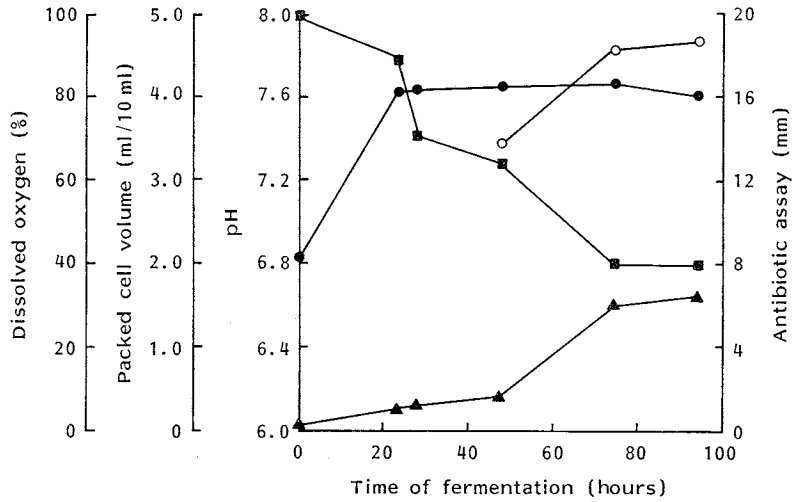
The Producing Culture

The producing culture, SCC 1792, was isolated from a South African soil by plating soil dilutions on yeast-starch agar (yeast extract 0.01%, potato starch 0.01%, agar 1.5%; pH 7.0) containing 5 μg/ml novobiocin. The isolated organism grew well on most media described by SHIRLING and GOTTLIEB[2] forming raised and tightly folded orange colonies and a fluorescent yellow diffusible pigment. Aerial mycelia were not formed. Single spores (0.8~1.2 μm i.d.) either sessile or on short sporophores occurred along the length of fine, branching vegetative mycelia. Whole cell analysis by the method of LECHEVALIER[3] indicated the presence of hydroxy- and *meso*-diaminopimelic acid along with arabinose and xylose as characteristic sugars. Based on morphological and chemical characteristics the producing culture was identified as a *Micromonospora* sp.

Fermentation

The culture SCC 1792 was stored as a stock suspension at -20°C in a growth medium containing 12% sucrose solution. Three ml of a thawed suspension was used to inoculate 70 ml of growth medium consisting of beef extract 0.3%, Tryptone 0.5%, yeast extract 0.5%, Cerelose 0.1%, potato starch 2.4%, $CaCO_3$ 0.2% and Dow-Corning antifoam-B 0.1%, in a 250-ml Erlenmeyer flask. After 48 hours incubation at 30°C on a rotary shaker operating at 300 rpm, 25 ml of the resulting cell suspen-

Fig. 1. Fermentation profile of Sch 37137.
■ Dissolved oxygen, ▲ packed cell volume, ● pH, ○ antibiotic assay.



sion was used to inoculate 500 ml of the growth medium in a 2-liter Erlenmeyer flask. The culture was incubated as described above. After 48 hours the entire contents of the flask were used to inoculate 10 liters of fermentation medium consisting of NZ-amine A 0.5%, yeast extract 0.5%, Cerelose 1.0%, soluble starch 2.0%, $CaCO_3$ 0.4%, $CoCl_2$ 0.0004% and Dow-Corning antifoam-B 0.1% in a 14-liter fermentor (New Brunswick Scientific, Edison, N.J.). The fermentation was carried out at 30°C with aeration of 3.5 liters/minute and agitation of 350 rpm. The pH and dissolved oxygen levels were continuously monitored without adjustment during the entire fermentation by means of probes submerged in the vessel. Microbial growth was determined by packed cell volume. A typical time course for the fermentation is shown in Fig. 1. The antibiotic production started 48 hours after inoculation, then gradually increased reaching a maximum at 96 hours. The amount of antibiotic produced was determined by a paper-disk agar diffusion method using *Candida albicans* strain 406 as the test organism.

Scheme 1. Procedure for the isolation and purification of Sch 37137.

Fermentation broth (110 liters, pH 7)
↓ filter
Filtrate
↓ adsorb onto BioRad AG 50X8 (H^+), 2 liters, elute with 0.5 N NH_4OH, concentrate
BioRad AG50 eluate (78.8 g)
↓ adsorb onto BioRad AG 1X8 (HCO_3^-), 2 liters, elute with CO_2 - saturated water, lyophilize
BioRad AG1X8 eluate (3.4 g)
↓ chromatograph on charcoal, 1 liter, elute gradient of 0 to 20% aq MeOH
Sch 37137 (1.3 g)

Isolation

Sch 37137 was recovered from the broth filtrate by adsorbing on the cation exchanger BioRad AG 50X8 (H^+). Further purification steps leading to the isolation of Sch 37137 are presented in Scheme 1. Sch 37137 was isolated as a white amorphous solid upon lyophilization.

Physico-chemical Properties and Structure

Sch 37137 is an amphoteric water soluble compound: MP 198°C (dec); $[\alpha]_D^{26}$ −30.6°. The compound is stable in the pH 2 to 9 range at room temperature. Sch 37137 has an Rf of 0.3 on TLC

(silica gel) developing with butanol - acetic acid - water - ethyl acetate (1 : 1 : 1 : 1), and gives a positive response to both ninhydrin and Rydon spray reagents.

The structure of Sch 37137 was deduced on the basis of spectroscopic and chemical data. The molecular weight of the compound was established as 288 from fast atom bombardment (FAB) mass spectral data that showed a sodiated ion $M+Na^+$, *m/z* 311, and a protonated molecular ion, M+H, *m/z* 289, (found 289.1163, calcd 289.1148), corresponding to a molecular formula $C_{10}H_{17}N_4O_6$. The UV spectrum (H_2O) showed no absorbance maxima greater than 215 nm. The IR spectrum (KBr) showed bands at 3300 cm^{-1} (br, NH and OH) and 1650 cm^{-1} (amide).

Acid hydrolysis of Sch 37137 (6 N HCl, 105°C, 17 hours) gave alanine and 2,3-diaminopropanoic acid (as the only ninhydrin detectable products). The presence of these compounds was confirmed using the following chromatographic systems: TLC, electrophoresis and HPLC of the *o*-phthalaldehyde amino acid derivatives. In all these experiments the amino acids were identified by comparison to authentic standards. These amino acids were separated by ion-exchange chromatography on Dowex 50X8 (pyridine form) eluting with a pyridine - acetic acid gradient. Alanine eluted with 0.2 M pyridine and 2,3-diaminopropanoic acid eluted with 0.5 M pyridine. Optical rotations of the HCl salts of alanine ($[\alpha]_D^{25}$ observed +6.9°, literature[4] +8.5°) and 2,3-diaminopropanoic acid ($[\alpha]_D^{25}$, observed +23°, literature[4] +25°) indicated both amino acids are in the L-form. Mild acid hydrolysis of Sch 37137 (2 N HCl, 5 hours) gave alanine as the only ninhydrin detectable product. This result indicated that alanine is terminally linked in the Sch 37137 molecule. The configuration of alanine as the 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC) derivative[5] was confirmed to be in the L-form by HPLC using peak enhancement with an authentic sample.

Mass spectral data provided further evidence for the presence of terminal alanine. The FAB spectrum of Sch 37137 showed a strong ion peak at *m/z* 218 amu, ($M+H^+-71$), that can arise from the loss of a terminal alanyl residue. Alanine was shown to be linked through the *C*-carboxy terminus in the following way; dansylation of Sch 37137, followed by acid hydrolysis (6 N HCl, 105°C, 17 hours) gave only one hydrolysis product identified as dansylated alanine†. This compound was identified, using a standard reference of dansylated alanine. This experiment indicated that the amine of the terminal alanine group of Sch 37137 was free. This conclusion was further supported by ^1H NMR studies at different pH values, discussed below.

The ^{13}C NMR spectrum of Sch 37137 showed the presence of 10 carbon atoms, four of which were carbonyls, (see Table 1). Two carbon signals at 54.2 and 54.6 ppm were assigned to the epoxide carbons in the molecule.

The ^1H NMR spectrum in D_2O was consistent with the presence of an epoxide. The two epoxide protons were located at δ 3.69 (1H, d, J=1.5 Hz) and δ 3.72 (1H, d, J=1.5 Hz), respectively. These protons were assigned *trans* to each other on the basis of the small coupling constant[6]. A doublet at δ 1.55 (3H, d, J=6.5 Hz) coupled to a proton at δ 4.11 (1H, q, J=6.5 Hz) was assigned to the alanine methyl group. The presence of the CH_2CH group was indicated by an ABX spin system at δ 3.56 (1H, dd, J_{AB}=14.0 and J_{AX}=6.0 Hz), δ 3.78 (1H, dd, J_{AB}=14.0 and J_{BX}=4.0 Hz) and δ 4.38 (1H, dd, J=6.0 and 4.0 Hz). In summary, the above data were consistent with an alanyl, a 2,3-diaminopropanoyl and an epoxide moiety in the molecule.

The unique assembly of the Sch 37137 parts was verified by a pH-dependence ^1H NMR chemical

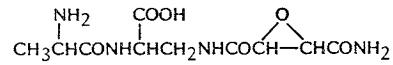
† Identification on polyamide TLC plates irrigating with 1.5% aq formic acid.

shift study on the compound and structure **1** proposed as shown in Fig. 2. ^1H NMR spectra of the compound were recorded in D_2O over the pH range 1.9 to 10.5. A graph showing the dependence of the chemical shifts of the protons versus the pH of the solution is shown in Fig. 3.

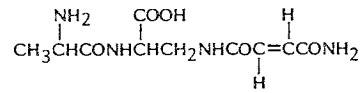
Analysis of Fig. 3 showed that the CH_2CH methine proton was adjacent to a free COOH (titration in the range pH 2 to 4). The *CH* alanyl proton was adjacent to a free NH_2 (titration in the pH range 7 to 10), and the methylene and epoxide protons were unaffected by the change in pH. These results were in accord with the proposed structure **1** shown in Fig. 2. Furthermore, the upfield shift values shown in Fig. 3 were comparable to data[7] reported for the related compound **2**.

Structure **2** is a revision of structure assignment of antibiotic A 19009[1]. Structure **2** for A 19009 was confirmed on the basis of spectro-

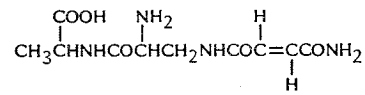
Fig. 2. Structures of Sch 37137, A 19009 and its isomer.



Sch 37137 (**1**)



A 19009 (**2**)



Isomer of A 19009 (**3**)

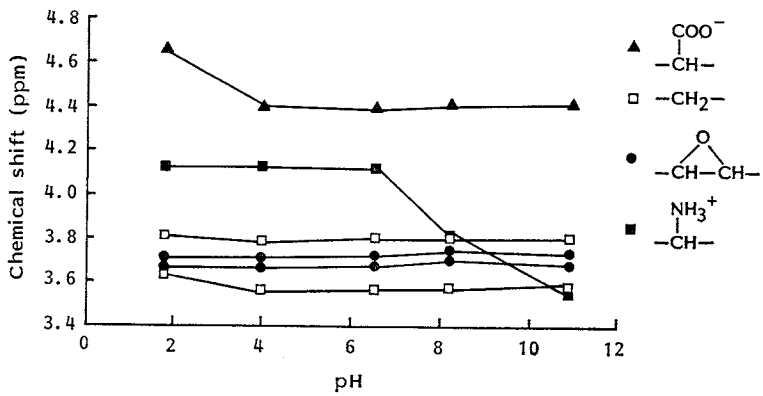
Table 1. ^{13}C NMR assignments for Sch 37137 and A 19009[7].

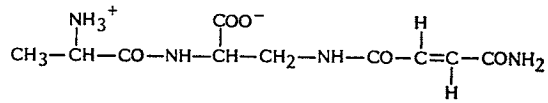
Carbon type	Sch 37137 (**1**)	A 19009 (**2**)
Ala-CH_3	17.3	16.6
DAP-CH_2	41.6	41.2
Ala-CH	50.0	49.5
DAP-CH	56.0	49.5
CH=CH	—	132.5, 133.4
	54.2, 54.6	—
4×CO	169.7, 171.4 (2), 171.9	167.3, 169.2, 170.6, 175.5

Spectra of **1** were obtained in D_2O solution on a Varian XL-400 instrument.

DAP: Diaminopropanoic acid.

Fig. 3. pH-dependence ^1H NMR study of Sch 37137.





+0.31 +0.62 (+0.27)(+0.59)

+0.29 +0.13, +0.12 (+0.24) (+0.09, 0.0)

Literature[7] values (Δ in ppm); corresponding Δ values for **1** in parenthesis.

scopy and a total synthesis[7,8] of both compounds **2** and **3**. We compared the spectroscopic data obtained for **1** with **2** and **3**, (see Table 2). Although there is a close correlation of data between **1**, **2** and **3**, the structure of **1** was proposed on the basis of all the above data and lends support to the reassignment[7] of structure **2** to A 19009[1].

It should be noted, however, that in revising A 19009 to **2**, reference to other possible structural isomeric forms were omitted. Although the data for **1** is consistent with the proposed structure, we considered the possibility of structure **4**, whereby the coupling of 2,3-diaminopropanoic acid to alanine in **1** is reversed. Structure **4** was eliminated and **1** favored in the following manner.

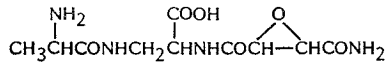


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Table 2. ^{1}H NMR assignments for Sch 37137 (**1**), A 19009 (**2**) and its isomer **3**.

Proton	Sch 37137 (**1**)	A 19009 (**2**)[7]	Isomer of A 19009 (**3**)[7]
CH_3	1.55 (3H, d, J=6.5)	1.32 (3H, d, J=7)	1.17 (3H, d, J=7.3)
CH_3CH	4.11 (1H, q, J=6.5)	3.84 (1H, q, J=7)	3.97 (1H, q, J=7.3)
CH_2CH	3.56 (1H, dd, J_{AB}=14), 3.78 (1H, dd)	3.38 (1H, dd, J_{AB}=14), 3.59 (1H, dd)	3.55 (1H, dd, J_{AB}=14.5), 3.65 (1H, dd)
CH_2CH	4.38 (1H, dd, J_{AX}=4, J_{BX}=6)	4.16 (1H, dd, J_{AX}=4.2, J_{BX}=7.8)	3.99 (1H, dd, J_{AX}=4.5, J_{BX}=7.5)
$\overset{O}{\diagup\diagdown}$ CHCH	3.69, 3.72 (2H, d, J=1.5)	—	—
CH=CH	—	6.66 (2H, s)	6.66 (2H, br s)

Spectra of **1** obtained in D_2O solution on a Varian XL-400 instrument, δ ppm, J (Hz).

Table 3. *In vitro* activity of Sch 37137 (**1**), A 19009 (**2**) and its isomer **3** against various fungi and bacteria.

Organism (No. of strains)	Medium	Geometric mean MICs (μg/ml) in various media				
		Sch 37137 (**1**)		A 19009 (**2**)		Isomer of A 19009 (**3**)
		Exp 1	Exp 2	Exp 1	Exp 2	Exp 2
Candida (8)[e]						
	SDB[a]	≥128	≥128	≥128	≥128	≥128
	EMEM[b]	≥128	≥128	≥128	≥128	≥128
	MA[c]	≥12	≥14	≥27	≥18	≥45
Dermatophytes (7)[f]						
	SDB	≥86	≥128	≥128	≥128	≥128
	MA	≥0.8	—	≥18	—	—
Gram-negative bacteria (77)[g]	MHB[d]		≥128		≥128	—
Gram-positive bacteria (31)[g]	MHB[d]		≥128		≥128	—

[a] Sabouraud dextrose broth, pH 5.7.
[b] Eagles minimum essential medium, pH 7.0.
[c] MA medium[9], pH 5.7.
[d] Mueller-Hinton broth, pH 7.4.
[e] 48 hours.
[f] 72 hours.
[g] 24 hours.

An Edman degradation was performed on **1** yielding the terminal alanine derivative, and the remaining peptide was isolated by purification on SP-Sephadex (pyridine$^+$) eluting with pyridine-acetic acid. ^{1}H NMR chemical shift data from this product obtained in neutral and acidic solution indicated that the free amine group is attached to a methine group as expected when cleavage occurs from **1**. If cleavage had occurred from **4** an H_2NCH_2-group would have been generated. In the ^{1}H NMR spectrum, the methine proton shifts from δ 3.8 (1H, m) in neutral to δ 4.2 (1H, m) in acid solution; the other protons underwent virtually no shift.

Biological Properties

The antibacterial and antifungal activity of Sch 37137 compared with A 19009 (**2**) and its isomer **3** is shown in Table 3. Sch 37137 was inactive ($\geq$128 μg/ml) in Sabouraud dextrose, yeast-nitrogen and modified Eagles minimum essential media. Activity significantly improved against *Candida* and dermatophytes in MA medium[9] (a dextrose-salt medium containing glutamate and biotin). Similar results were seen with both isomers. In MA medium, **1** is more active than either **2** or **3**. Compound **2** is slightly more active than its isomer **3**, as previously shown by BOROWSKI *et al.*[9].

The intravenous LD_{50} of Sch 37137 in mice was 600 mg/kg.

Conclusion

Antifungal compounds from a *Micromonospora* sp. are rare. Recently the novel antifungal compounds, rustmicins (galbonolides)[10~13], were reported from a *Micromonospora* sp. These macrolides were also produced by a *Streptomyces*. We now report on a new antifungal dipeptide, Sch 37137, from a *Micromonospora* sp. This compound is closely related to A 19009 previously isolated from a *Streptomyces* sp., where an epoxide replaces a double bond in the molecule. This change of functionality may explain the increased level of biological activity.

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