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A New Streptothricin Family Antibiotic Producing *Streptomyces* spp. SNUS 8810-111; Characterization of the Producing Organisms, Fermentation, Isolation, and Structure Elucidation of Antibioitics

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A new streptothricin family antibiotic producing *Streptomyces* spp. SNUS 8810-111 was isolated from a soil sample. Study of its morphological and physiological characters indicated that the antibiotic producing organism was a *Streptomyces* spp. Taxonomical studies suggested that the organism might belong to the genus *Streptomyces gougeroti*. The organism produced antibiotics most in calcium carbonate-tryptic soy broth. The active principles were recovered from the broth with a cation exchange resin and eluted from the resin with HCl. Cellulose column chromatography gave two active principles. ^{1}H-^{1}H Homo-COSY study on the first compound revealed four structural components. Total hydrolysis of the antibiotic with HCl allowed isolation of β-lysine. From these data the antibiotic was found to be streptothricin D. The other compound showed one additional signal in the ^{1}H NMR and the ^{13}C NMR spectra. The signal was from a methyl group attached to a nitrogen atom. Comparison of the NMR signals with those of streptothricin D suggested that the compound was *N*-methylstreptothricin D which was a new compound in the family of streptothricin antibiotics.

Key words : *Streptomyces*, Streptothricin D, *N*-methylstreptothricin D, Taxonomy, *Streptomyces gougeroti*, Fermentation, antibiotics

INTRODUCTION

We have been involved in the development of new bioactive compounds from soil microorganisms for last 10 years. We have been interested in the discovery of new useful antifungals and especially, we have tried to find out antibiotics that can be used for treatment of rice blast disease. Thus, we have screened several thousands of microorganisms which produce compounds showing antifungal activities against *Pyricularia oryzae*. During this work we have isolated several antifungal compounds. Structure elucidation of these principles indicated that several organisms produced antibiotics in streptothricin-families (van Tamelen *et al.*, 1961; Bycroft *et al.*, 1972). Detailed study revealed that one organism, a *Streptomyces* spp. SNUS 8810-111 cultured from a soil sample collected at the campus of Seoul National University, produced streptothricin D (**1**) and a new antibiotic, *N*-methylstreptothricin D (**2**). A brief description on the isolation and the NMR data of *N*-methylstreptothricin D was described in a previous report (Kim *et al.*, 1994). In the present paper we would like to describe the morphology and the biological and the physiological characteristics of the producing organism, the production, the isolation and the detailed approach on the structure elucidation of the antibiotics.

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MATERIALS AND METHODS

Isolation of antibiotic producing organisms

Isolation of the organisms which produce antibiotics from a soil sample collected at the campus of Seoul National University was carried out by the method described previously (Goo *et al.*, 1991a; Goo *et al.*, 1991b).

Microbial cultures

The antimicrobial testing organisms, *Bacillus subtilis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Candida albicans*, and *Pyricularia oryzae* were purchased from Korean Type Culture Collection.

Other cultures used for comparison of cell wall analysis were provided by Nothern Regional Research Laboratory in Peoria, Illinois.

Culture maintainance

The antibiotic producing organism SNUS 8810-111 was maintained by transfer of vegetative mycelium on slants of V-8 agar (V-8 juice 200 ml, precipitated $CaCO_3$ 3 g in 1000 ml distilled water, pH not adjusted). Slants were incubated for 7 days at 28-29°C and then stored at 4°C.

Study on growth characteristics

Growth characteristics including colonial morphology were determined with the media recommended by Shirling and Gottlieb (1966) in the International Streptomyces Project (ISP) and by Waksman (1961) or modified Bennett's agar (Difco potato dextrin 1.0%, NZ-Amine Type A 0.2%, BBL beef extract 0.08%, Difco yeast extract 0.1%, KCl 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, $FeSO_4 \cdot 7H_2O$ 4 mg/liter, pH 7.4). Cultrues were observed after incubation at 28°C for 14 days.

Lysozyme resistance of SNUS 8810-111 was tested in YME broth (ISP-2 without agar). Egg yolk reaction (lecithinase) was tested according to Nitsch and Kutzner (1969). Samples of aerial mycelium were observed by scanning electron microscopy (SEM, College of Engineering, Seoul National University) on coverslips coated with a liguid adhesive and touched to the surface of the growth. The coverslips were air dried directly from a 95% ethanol rinse. The samples were coated with gold for viewing by SEM.

Study on carbon utilization

The utilization of carbon sources was determined from the growth of the organism after cultivation at 28°C for 7 days in Pridham and Gottlieb's medium (Pridham and Gottlieb, 1948) containing 1% of each carbon source.

Analysis of cell-wall composition

The chemical analysis of the cell-wall composition of the antibiotic producing organism on diaminopimelic acid and sugars was carried out by the method of Staneck and Roberts (1974) using as a control, *Streptomyces fradiae* NRRL B-1195.

Biological activity

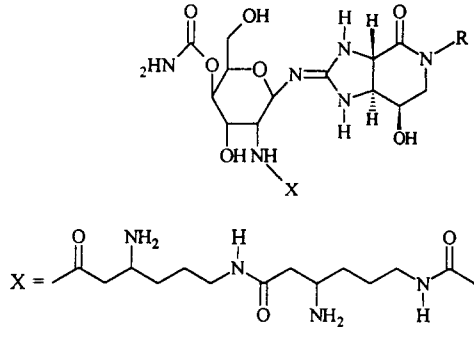
The antimicrobial spectra of the test materials were determined by the agar-diffusion method using 1/4 inch paper discs (Aldrich Chemical Co., Inc.). Bacteria were grown on Mueller-Hinton agar medium (Difco) and fungi (*Pyricularia oryzae* IFO 5994) was grown on Potato-dextrose agar medium. Antimicrobial activities were observed after 24 hours' incubation at 28°C.

Shake flask fermentation

Seed-medium (tryptic soy broth, TSB, Sigma, 50 ml in 300 ml Erlenmeyer flask) was inoculated with a loopful of vegetative mycelium from a V-8 agar slant of SNUS 8810-111. The seed-culture, after incubation at 28°C for 2 days on a reciprocal shaker (180 rpm), is transferred to various antibiotic production media. The media studied were casein-corn steep liquor-soybean flour medium (casein hydrolysate 0.25%, beef extract powder 0.1%, soybean flour 1.0%, distillers solubles 0.2%, corn steep liquor 0.5%, glucose 2.0%, K_2HPO_4 0.2%, $CaCO_3$ 1.0%), oat meal medium (oat meal 4.0%, meat extract 0.3%, NaCl 0.3%, precipitated $CaCO_3$ 0.3%, $Fe_2(SO_4)_3$ 0.04%, $MnCl_2$ 0.04%), tryptic soy broth (TSB: Bacto-tryptone 1.7%, Bacto-soytone 0.3%, glucose 0.5%, NaCl 0.25%, K_2HPO_4 0.25%), calcium carbonate TSB (TSB 3.0%, precipitated $CaCO_3$ 0.3%), V-8 juice broth (V-8 juice 200 ml, precipitated $CaCO_3$ 0.3%), and many other media. The growth of the organism and the production of antibiotics in these media were examined at various culture time.

Production of antibiotic

SNUS 8810-111 was seed-cultured in TSB (TSB 1.5 g in 50 ml distilled water) at 29°C for 48 h on a reciprocal shaker. The seed-culture (50 ml) was transferred to 500 ml of a calcium carbonate-TSB (TSB 3.0%, $CaCO_3$ 0.6%) in an Erlenmeyer flask (2 L). The broth was cultured under the same condition as the seed-culture for 4 days on a reciprocal shaker.



R = H Streptothricin D (**1**)

R = CH_3 *N*-Methylstreptothricin D (**2**)

Fig. 1. The Structures of streptothricin D (**1**) and *N*-methylstreptothricin D (**2**)

Isolation of antibiotics

The fermented broth (3 L) was mixed with Diaion WK-20 (H^+ form, 250 ml per liter of broth) and stirred for 1 h. After confirmation of antibiotic activity-absence in the broth, the resin was poured into a glass column. After washing the column with deionized water (10 L), the column was further washed with HCl solution, the concentration of which was increased gradually from 0.005 N to 0.06 N. The fractions obtained by elution with 0.06 N HCl solution were treated with Diaion WA-30 (OH^- form) and tested for antimicrobial activity against *Pyricularia oryzae*. The active fractions were combined and evaporated under reduced pressure. The residue was dissolved in 95% methanol (100 ml) and the insoluble material was removed by filtration. The filtrate was concentrated and mixed with cellulose powder (10 g). After evaporations of solvent the cellulose powder was applied on the top of a cellulose column. The column was developed with *n*-BuOH-EtOH-AcOH-water (10 : 10 : 2 : 5). The active fractions which showed a single spot on analysis by TLC were combined together and evaporated. The residues were washed with diethyl ether and dried under vacuum. Two compounds (19 mg and 22 mg) were obtained as amorphous solids.

RESULTS AND DISCUSSION

Morphology of SNUS 8810-111

The vegetative mycelium of SNUS 8810-111 grew abundantly on many complex and synthetic media. Cultural characteristics of SNUS 8810-111 grown on various media are summarized in Table I. The organism produced substrate and aerial mycelia, and spores on solid culture (Fig. 2). Colony grown on the ISP No. 2, 4, 5, and 7 media were tough and leathery. A pale yellowish brown diffusible pigment was formed in ISP No. 2, 3, and 4 media, and in nutrient medium. Melanin pigment was not produced on tyrosine agar (ISP No. 7 medium). White aerial spore mass was developed on the surface of colonies on ISP No. 4 medium. The spore chain was rectiflexible and the spore surface ornamentation was smooth (Fig. 3).

Chemical composition, physiological properties and identification of the organism

The analysis of whole-cell diaminopimelic acids and sugars showed SNUS 8810-111 to contain LL-DAP and only a trace of ribose (no characteristic sugar pattern). This indicated that the strain had a cell-wall chemotype I and a sugar pattern C.

The ability of SNUS 8810-111 to use a number of sole carbon sources was tested and the results are summerized in Table II. Other physiological properties of the organism are listed in Table III. Based

Fig. 2. Light micrograph of mycelium of SNUS 8810-111 (× 1,000)

Table I. Cultural properties of SNUS 8810-111

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract-Malt extract agar (ISP 2)	Abunt thick	Pale yellowish white	Dull yellow	Pale yellowish brown
Oatmeal agar (ISP 3)	Good thin	None	Pale yellow	Pale yellowish brown
Inorganic salts-Starch agar (ISP 4)	Abunt thick	Pale yellowish white	Light reddish yellow	Pale yellowish brown
Glycerol-Asparagine agar (ISP 5)	Abunt thick	Pale yellowish white	Pale yellow	Nonw
Peptone-yeast extraction agar (ISP 6)	Good	None	Dull yellow	None
Tyrosine agar (ISP 7)	Abunt thick	Pale yellowish white	Pale yellow	Pale yellow
Nutrient agar	Poor thin	None	Pale yellowish brown	Pale yellowish brown

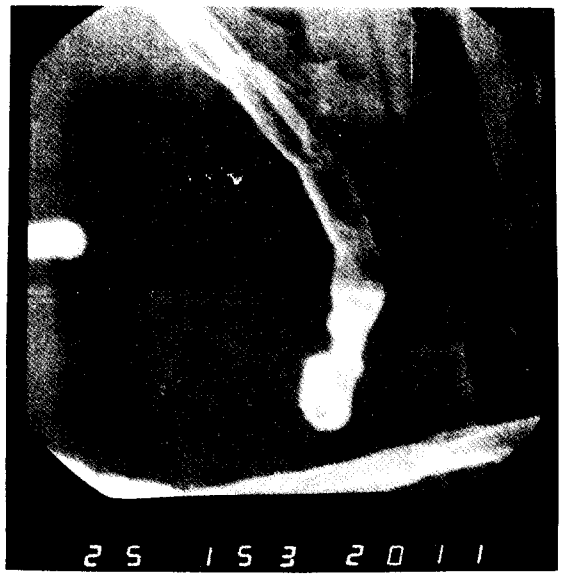


Fig. 3. Scanning electron micrograph of spores of SNUS 8810-111 (x 18,000)

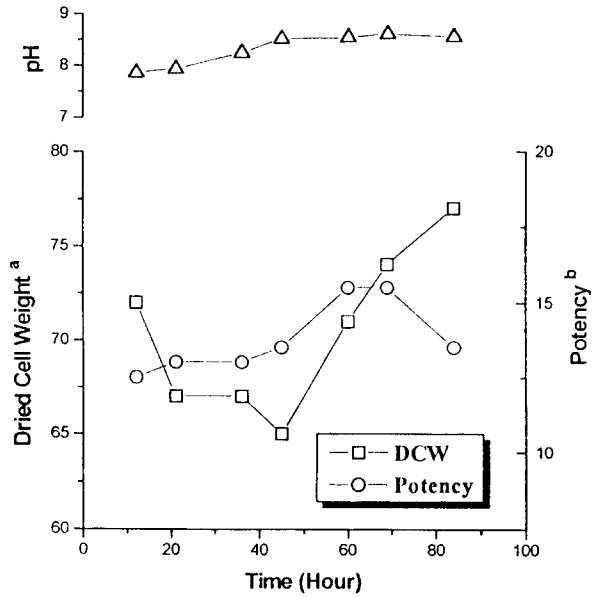


Fig. 4. Growth of SNUS 8810-111 (dry cell weight in mg per 5 ml of broth: DCW) in TSB, pH change of the broth, and the inhibition zone diameter (Potency; 6.25 mm paper disk wetted with 30 µl of btoth).

Table II. Utilization of carbon sources by SNU 8810-111

Carbon source	Utilization
D-Glucose	+
D-Fructose	+
L-Rhamnose	+
D-Mannitol	+
Sucrose	+
D-Xylose	+
Raffinose	-
Inositol	-
L-Rhamnose	-
L-Arabinose	-
Cellulose	-

Table III. Physiological properties of SNU 8810-111

Optimum growth temperature	25-30°C
Liquefaction of gelatine	Positive
Starch hydrolysis	Positive
Degradation of tyrosine	Positive
Degradation of casein	Positive
Degradation of xanthine	Positive
Growth on Czapek's solution agar	Moderate
pH Tolerance	Neutral
Melanine formation	Negative

on these morphological and chemotaxonomical characteristics strain SNUS 8810-111 was considered to belong to the genus *Streptomyces gougeroti.*

Selection of antibiotic producing organisms and fermentation of antibiotics

About 40% of 2,000 microbes screened from soils collected at the Kwanak campus of Seoul National University showed antibacterial compounds production on the culture of agar discs. The antibiotic producing microorganisms were cultured in TSB for 4 days and the cutured broth was tested for antimicrobial activity against *B. subtilis, E. coli, Pr. vulgaris, Ps. aeruginosa, C. albicans,* and *Py. orizae.* We selected several species of microorganisms including SNUS 8810-111 which showed strong antimicrobial and antifungal activities.

The selected organisms were seed-cultured in TSB and the seed-culture was used to inoculate 10 different media, most of the cultures showed good production of antibiotics. Different activities against different test organisms were observed in different culture media. Compared to other media, No. 7 medium showed good growth and the cultured broth showed good antibacterial and antifungal activities.

A typical time course for the fermentation is shown in Fig. 4. The antibiotic production started after 12 hours' incubation and reached a maximum after 60 hours'. The amount of antibiotic produced was determined by a paper disc agar diffusion method using *Pyricularia oryzae* as the test organism.

Physico-chemical properties and structure elucidation of the antibiotic

Examination of the chemical properties of antibiotics indicated that the antibiotics were water soluble and partially extractable with *n*-butanol. Thin layer chromatographic and bioautographic analysis of the residue obtained from *n*-butanol extract on a silica gel TLC plate with *n*-BuOH-EtOH-AcOH-H_2O (10 :

10 : 2 : 5) indicated that the antibiotics were composed of 2-3 components around R_f value 0.1-0.2. The antibiotics were adsorbed on a cation exchange resin (WK-20, H^+ form) and were eluted with ammonia water or hydrochloric acid. The antibiotics showed stability under basic and acidic condition. Thus, we eluted the antibiotics from the resin with HCl as increasing its concentration gradually. The antibiotic was eluted with 0.06 N HCl and the eluate was treated with WA-30 (OH^- form). Evaporation of the solution gave a residue which was contaminated with a lot of salts. Thus, the residue was treated with methanol and the insoluble salt material was removed by filtration. The methanol soluble components were chromatographed over a column packed with cellulose powder (Sigma). The column was developed with *n*-BuOH-EtOH-AcOH-H_2O. The active fraction showed two components, which were isolated by column chromatography on cellulose with *n*-BuOH-EtOH-0.1 N HCl (1 : 1 : 1). Both compounds showed antibacterial activities against *P. oryzae* and showed inhibition zones of 18.0 and 19.0 mm diameter around 6 mm diameter paper discs wetted with 30 μl of the solution of the compounds at the concentration 1 mg/ml.

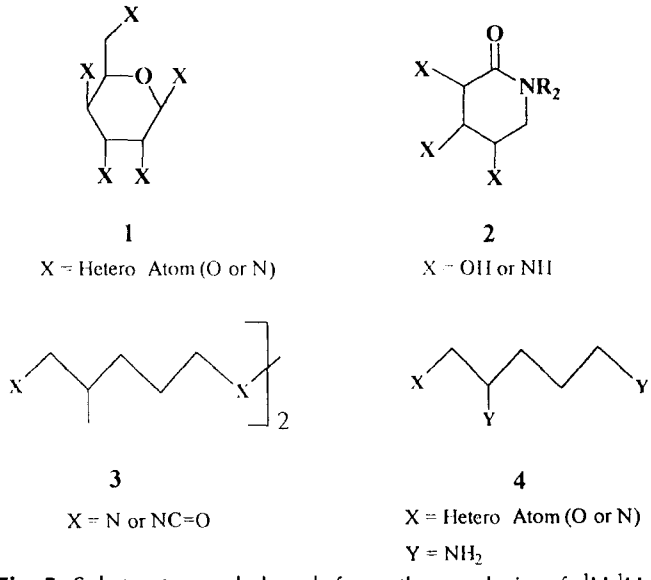


Fig. 5. Substructures deduced from the analysis of ^1H-^1H Homo-COSY spectrum.

Characterization of antibiotics

Both compounds are white amorphous powders melted above 200°C with decomposition and showed ions at m/z 758 and m/z 773 in their FAB mass spectra. They were soluble in water and methyl alcohol, and insoluble in other organic solvents. It gave strong purple color by ninhydrin and a positive response to the Dragendorff reagent but gave no color by anisaldehyde-sulfuric acid or by iodine. They exhibited end absorption in UV spectra.

The compound which showed a molecular ion at m/z 758 in the FAB mass spectrum gave a doublet (J= 10 Hz) which was correlated with a double doublet at 4.22 ppm. The latter signals showed correlation with the signals at 4.73 ppm, which correlated further to a triplet at 4.29 ppm that was correlated to a doublet at 3.68 ppm. These correlation data implied that the compound contained a hexose unit in which, since the coupling constants are 10.0, 3.3 and 6.0 Hz in sequence, the protons are at axial, equatorial, axial, and axial positions, respectively. The signals at 5.05 ppm were from the anomeric proton in the sugar. Since the signals were actually observed at a lower field than those of other anomeric protons, we assumed that an electron withdrawing group was attached to the oxygen atom which was attached to the anomeric proton-attached carbon atom. The existence of the sugar unit in the molecule was further confirmed by its ^{13}C NMR spectrum which showed many oxygen-attached carbon signals around 50-90 ppm. The ^1H-^1H correlation spectrum data revealed substructure 1 in Fig. 5 as the sugar component.

The doublet at 4.59 ppm (J=14 Hz) showed correlation with a doublet at 4.07 ppm (J=14 Hz), which was correlated with signals at 4.69 ppm. The signals at 4.69 ppm were hidden under the signal of water, but it showed further correlation with those at 3.80 ppm (dd, J=6.0, 15.0 Hz) and at 3.40 ppm (d, J=15.0 Hz), which implied the presence of a methylene group. From these correlations we could propose substructure 2 of Fig. 5.

Further correlations were observed between signals at 3.60 and 1.59 ppm, signals at 1.59 and signals at 3.20 ppm, and signals at 3.60 and 2.54-2.77 ppm. Analysis of the signals observed around 2.54-2.77 ppm implied that they were from two protons in a methylene group which were coupled geminally. Actually the signals were part of an ABX system. The chemical shifts of these protons suggested that the methylene group was attached to a carbonyl group, the protons at 3.60 and 3.20 ppm were those attached to the carbon atoms which have an oxygen or a nitrogen atom. The integrated values of protons for the signals at 1.66 and 1.59 ppm implied two methylene groups attached to the carbon atoms. These analysis suggested substructure 3 of Fig. 5. From another correlations observed between the signals at 3.60 ppm and 3.01 ppm, 3.60 ppm and 2.54-2.77 ppm and the signals which are from two methylene groups at 1.76 ppm implied substructure 4 of Fig. 5.

Total hydrolysis of the compounds in 6 N HCl at 100°C overnight gave several spots on TLC analysis; one of the spots was much bigger and intenser than

the others when ninhydrin was sprayed. Analysis of the total hydrolysate with an automatic amino acid analyzer gave a signal which was much bigger than the rest. The retention time of the signal was almost same as that of L-lysine. To confirm the structure of major component, the residue obtained from total hydrolysis of the antibiotic (43 mg) was chromatographed on a cellulose column to give KBT-111-272B (5 mg). KBT-111-272B showed signals at 1.74 (m, 4H, $-CH_2-CH_2-$), 2.35 (dd, 1H, J=16.6, 7.7 Hz, $-CH_2CO_2-$), 2.52 (dd, 1H, J=16.6, 4.9 Hz, $-CH_2CO_2-$), 2.98 (m, 2H), 3.42 (m) in its ^{1}H NMR spectrum and 23.3, 29.4, 38.5, 39.6, 49.1, and 178.2 ppm in its ^{13}C NMR spectrum. These signals were also observed in the spectra of the original antibiotic. The signals at 2. 35 and 2.52 ppm in its ^{1}H NMR spectrum were characteristic for the AB pattern of an ABX system and the signals implied existance of a methylene group adjacent to a chiral center. Analysis of these data indicated that the isolated compound might be β-lysine. Comparison of the ^{13}C NMR data with those (39.3, 49.6, 29.8, 23.7, 38.8 ppm, the carbonyl signal which was unobserved) reported by other people confirmed its structure.

Several β-lysine-containing antibiotics are found in the literature. Tuberactinomycin (Yoshioka *et al.*, 1971), capreomycin (Nomoto *et al.*, 1978), lysinomycin (Kurath *et al.*, 1984) and streptothricin (Waksman *et al.*, 1942) are the most typical ones. Among these antibiotics the NMR and other spectral data of the isolated compounds fitted best to the structure of streptothricin. Comparison of the spectral data with those reported for streptothricins led to the conclusion that the antibiotic isolated from SNUS 8810-111 was streptothricin D (**1**). The other compound which showed an ion at m/z 773 was confirmed to be *N*-methylstreptothricin D (**2**) which was a new compound isolated first from SNUS 8810-111. In addition to the signals observed in NMR spectra of streptothricin D *N*-methylstreptothricin D showed additional signals in ^{1}H NMR and ^{13}C NMR spectrum. Comparison of the NMR data of two compounds revealed that the compound showing an ion at m/z 773 in FAB mass spectrum was *N*-methylstreptothricin D (Kim *et al.*, 1994)

Since streptothricin F was isolated first from culture media of *Streptomyces lavenduelae* (Waksman *et al.*, 1942), many streptothricin-group antibiotics have been reported. Three components 3,6-diaminohexanoic acid (L-β-lycine), 2-amino-2-deoxy-D-gulose and streptolidine were isolated from the hydrolysis products of streptothricin F and Its structure was proposed by van Tamelen *et al.* (1961) for the first time. Afterwards, Khokhlov *et al.* (1972) suggested that all the streptothricin analogues, streptothricin A-F, contain the same common moiety, *N-guan*-streptolidyl gulosaminide, and they differ in the number of β-lysine residues in the unbranched peptide chain, and all the β-lysine residues are linked by amide bond between the carbonyl groups and the ε-amino groups leaving the β-amino groups free. Kusumoto *et al.* (1981) synthesized *N-guan*-streptolidyl gulosaminide and from that the exocyclic guanidine nitrogen atom of streptolidine is linked to gulosamine by β-glycosidic bond in streptothricin F. The location of the carbamoyl group was confirmed later to be at the 4-hydroxyl group of gulosamine moiety from comparison of ^{1}H NMR spectra of streptothricin F with two model compounds obtained by synthesis (Kusumoto *et al.*, 1982). In the present study we confirmed that several soil microorgamisms produced streptothricins and a new streptothricin antibiotic, *N*-methylstreptothricin D, were isolated.

Biological activity

Streptothricin D and *N*-methylstreptothricin D showed strong antimicrobial activities against fungi as well as Gram-positive and Gram-negative bacteria as reported in the previous paper (Kim *et al.*, 1994).

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