SF2457, A NEW ANTIBIOTIC RELATED TO AMICETIN

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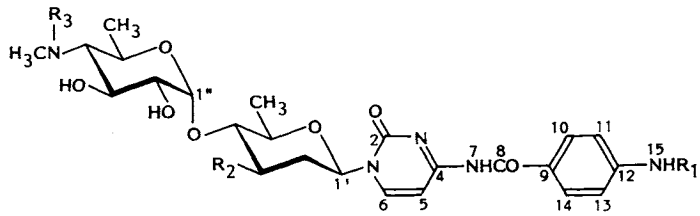
A novel nucleoside antibiotic, SF2457†, was isolated from the fermentation broth of *Nocardia brasiliensis* SF2457. The structure of SF2457 was determined by degradation studies using alkaline hydrolysis and methanolysis. SF2457 is closely related to the amicetin group antibiotics. The antibiotic exhibited inhibitory activity against Gram-positive and Gram-negative bacteria.

In the course of our search for new antibiotics, *Nocardia brasiliensis* SF2457 was found to produce a new antibiotic termed SF2457. Antibiotic SF2457 (**1**) was found to be closely related to the amicetin group of antibiotics which are known to be produced by various species of *Streptomyces*[1~8] and *Arthrobacter*[9~11] (Fig. 1). The antibiotic showed strong activity against Gram-positive bacteria, but weak activity against Gram-negative bacteria. In this paper, we report the taxonomy of the producing strain, as well as the fermentation, isolation, structure elucidation and antimicrobial activity of **1**.

Fig. 1. Structures of antibiotic SF2457 (**1**) and related compounds.



	R_1	R_2	R_3
SF2457 (**1**)	$COCH(CH_3)NH_2$	OH	H
Amicetin A	$COC(CH_3)(CH_2OH)NH_2$	H	CH_3
Plicacetin	H	H	CH_3
Norplicacetin	H	H	H
Oxamicetin	$COC(CH_3)(CH_2OH)NH_2$	OH	CH_3
Bamicetin	$COC(CH_3)(CH_2OH)NH_2$	H	H

† Antibiotic SF2457 has been published in Jpn. Kokai 8393 ('88), Jan. 14, 1988. The producing strain has deposited with the Fermentation Research Institute of Agency of Industrial Science and Technology as accession No. FERM P-8701.

Taxonomy of the Producing Strain

The producing microorganism, strain SF2457[12], was isolated from a soil sample collected at Toba City, Mie Prefecture, Japan. The vegetative mycelium produced on agar medium was rudimentary and branched. The mycelium was also fragmented into coccoid or bacillary elements. Aerial mycelia were formed abundantly on yeast extract-malt extract agar and on tyrosine agar. Aerial hyphae were either unbranched or moderately and irregularly branched, and were straight or irregularly curved. They frequently showed nocardio form zig-zags during sporulation and differentiated into arthrospores. These spores were cylindrical or slightly curved like wiener-sausages, smooth-surfaced and measured 0.4 to 0.5 by 1.0 to 1.5 μm. Mature aerial hyphae had 10 to 50 spores per chain. Spores were not motile, and sclerotia or sporangia were not observed. Cells were Gram-positive and weakly acid-fast. Aerial mass color was in the red color series of TRESNER and BACKUS[13]. The reverse side of colonies was pale brown. Neither water-soluble nor melanoid pigments were produced. The strain did not grow under anaerobic conditions. The temperature range for growth was 15 to 35°C. On ISP medium 9[14], the strain utilized D-glucose, D-fructose, D-mannitol, *myo*-inositol and glycerol, but did not utilize D-xylose, L-arabinose, L-rhamnose, raffinose and sucrose. An analysis of cell wall hydrolysates revealed the presence of *meso*-diaminopimelic acid, arabinose and galactose as major constituents (wall chemotype IV). The whole-cell hydrolysates showed the presence of arabinose and galactose as diagnostic sugars (whole-cell sugar pattern A). Phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides were detected as polar lipids (type PII phospholipid pattern). Mycolic acids with 48 to 68 carbons were present. An MK-8(H_4) was detected as the major menaquinone (more than 90%). Palmitic acid and tuberculostearic acid were detected as the major cellular fatty acids. The guanine-plus-cytosine content of deoxyribonucleic acid was 66 mol%. Strain SF2457 is considered to belong to the genus *Nocardia* Trevisan on the basis of its morphological and chemotaxonomic properties. A comparison of the taxonomic characteristics of strain SF2457 with those of *Nocardia* species described on BERGEY's Manual of Systematic Bacteriology[15] showed that the strain bore a very close resemblance to *N. brasiliensis* (Lindeberg) Pinony[16]. Since the physiological characteristics (Table 1) and the chemotaxonomy of strain SF2457 are in good agreement with those of *N. brasiliensis* JCM 3374 (type strain), we have identified strain SF2457 as a member of *N. brasiliensis* and deposited it in the Institute for Fermentation, Osaka, Japan, with an accession number of IFO 15008.

Fermentation

A slant culture of strain SF2457, which contained yeast extract 0.2%, corn starch 1.0% and agar 2.0%, was inoculated into a 100-ml Erlenmeyer flask which contained 20 ml of a seed medium consisting of corn starch 2.0%, glucose 1.0%, wheat germ 0.6%, Polypepton (Daigo Eiyo Kagaku) 0.5%, yeast extract 0.3%, soybean meal 0.2% and $CaCO_3$ 0.1% (pH 7.0 before autoclaving for 30 minutes at 120°C). The inoculated flask was cultured on a rotary shaker (210 rpm) at 28°C for 4 days. Four ml of the first seed culture were inoculated into 80 ml of the same medium contained in a 500-ml Erlenmeyer flask. The inoculated flask was cultured on a rotary shaker (210 rpm) at 28°C for 2 days. Fifty ml of the second seed culture was added to a 5-liter Erlenmeyer flask containing 1 liter of the same medium and fermentation was maintained on a rotary shaker (210 rpm) at 28°C for 2 days. One liter of the third seed culture was transferred to 35 liters of a production medium contained in a 50-liter jar fermenter. The production medium contained glycerol 4.0%, cotton seed meal 1.3%, wheat germ 2.0%, corn steep liquor 1.3%, $CaCO_3$ 0.2% and $CoCl_2 \cdot 6H_2O$ 0.001% (pH 7.0 before autoclaving for 30 minutes at 120°C). The

Table 1. Physiological characteristics of strain SF2457 and *Nocardia brasiliensis* JCM 3374.

Characteristics	SF2457	JCM 3374	Characteristics	SF2457	JCM 3374
Decomposition of			Growth at 45°C	−	−
adenine	−	−	Acid from		
casein	+	+	adonitol	−	−
hypoxanthine	+	+	L-arabinose	−	−
testosterone	(+)	(+)	cellobinose	−	−
tyrosine	+	+	*meso*-erythritol	−	−
xanthine	−	−	D-fructose	+	+
Hydrolysis of			D-galactose	+	+
esculin	+	+	D-glucose	+	+
hippurate	−	−	glycerol	+	+
potato starch	−	−	*myo*-inositol	+	+
Production of			maltose	−	−
catalase	+	+	D-mannitol	+	+
nitrate reductase	(+)	(+)	melezitose	−	−
gelatinase	(+)	−	α-methylglucoside	−	−
urease	+	+	β-methylxyloside	−	−
Resistance to lyzozyme	+	+	D-sorbitol	−	−
Tolerance to 5% NaCl	−	+	trehalose	+	+

Symbols: +, positive; (+), weakly positive; −, negative.

fermentation was carried out at 28°C for 4 days with an aeration rate of 20 liters/minute and an agitation rate of 300 rpm. The activity of the antibiotic was assayed by a paper-disc method using *Staphylococcus aureus* 209P as the test organism.

Isolation

The fermentation broth from four fermenters was filtered using Hyflo Super-Cel (Johns-Manville). The mycelial cake was extracted with 50% aqueous acetone (40 liters). The extract was concentrated to 20 liters and the combined solution (120 liters) of the broth filtrate and the concentrate was applied to a column of Diaion HP-20 (6 liters). The column was eluted with 50% aqueous acetone (18 liters) and the eluate was concentrated to 5 liters under reduced pressure. The concentrate was applied to a column of Amberlite CG-50 (H^+, 500 ml). The column was washed with water and then eluted fractionally with 0.1 N HCl. The active fractions were combined and adjusted to pH 7.0 with 1 N NaOH. The solution was applied to a column of Diaion HP-20 (300 ml). After washing with water, the column was eluted with 50% aqueous acetone (1.5 liters). The active eluate was concentrated to dryness. The residue was dissolved in a small amount of water and adsorbed onto a column of CM-Sephadex C-25 (Na^+, 500 ml). After washing with water, the column was eluted fractionally with 0.5 M NaCl. The active fractions were combined and then adsorbed onto a column of Diaion HP-20 (150 ml). After washing with water, the column was eluted with 50% aqueous acetone. The active eluate was concentrated and lyophilized to afford 700 mg of **1**.

Physico-chemical Properties

The free base of **1** was obtained as a water soluble colorless amorphous powder. It showed positive color reactions with H_2SO_4, $KMnO_4$ and ninhydrin reagent. The physico-chemical properties of **1** are listed in Table 2. The UV spectrum of **1** shows a strong absorption maximum at 303 nm similar to those of the amicetin group antibiotics. The IR spectrum showed absorptions due to hydroxyl groups (3350 cm^{-1}), an amide carbonyl (1650 cm^{-1}) and conjugated C=C bonds (1600 cm^{-1}). **1** gave positive Dragendorff and

Table 2. Physico-chemical properties of SF2457 (**1**).

Appearance	Colorless powder
Molecular formula	$C_{27}H_{38}N_6O_9$
MS (*m/z*)	590 (M^+)
Anal Calcd:	C 54.91, H 6.48, N 14.23
Found:	C 54.59, H 6.20, N 14.02
$[\alpha]_D^{22}$ (*c* 1.0, MeOH)	+99.2°
MP (dec)	172~174°C
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	303 (573)
$\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm ($E_{1cm}^{1\%}$)	260 (sh, 260), 317 (493), 330 (sh, 407)
$\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm ($E_{1cm}^{1\%}$)	272 (360), 325 (500)
IR ν_{max}^{KBr} cm^{-1}	3350, 1650, 1600, 1560, 1480, 1405, 1380, 1330, 1300, 1250, 1190, 1080, 790
Rf value*	
Solvent 1	0.67
Solvent 2	0.82

* Kieselgel 60 F_{254} (E. Merck). Solvent 1: Isopropanol-MeOH-10% CH_3COONH_4 (4:2:3), Solvent 2: *n*-BuOH-MeOH-H_2O (4:1:2).

Table 3. ^1H NMR data of SF2457 (**1**) and the degradation products (**2** and **4**).

Proton	**1**	**2**	**4**
	m	m	m
5-H	7.31 d (7.6)	6.04 d (7.6)	6.06 d (7.7)
6-H	8.23 d (7.6)	7.75 d (7.6)	7.76 d (7.7)
10,14-H	7.85 d (9.0)		
11,13-H	7.63 d (9.0)		
17-H	4.29 q (7.2)		
18-H_3	1.67 d (7.2)		
1′-H	5.76 dd (2.1, 11.0)	5.79 dd (2.0, 11.3)	5.81 dd (2.1, 11.3)
2′-Hax	1.82 ddd (11.0, 11.3, 12.8)	1.90 ddd (11.3, 11.3, 12.6)	1.90 ddd (11.3, 11.3, 12.3)
2′-Heq	2.37 ddd (2.1, 5.0, 12.8)	2.24 ddd (2.0, 4.9, 12.6)	2.27 ddd (2.1, 4.9, 12.3)
3′-H	4.10 ddd (5.0, 8.8, 11.3)	4.05 ddd (4.9, 8.7, 11.3)	3.84 ddd (4.9, 9.2, 11.3)
4′-H	3.40 dd (8.8, 9.2)	3.32 dd (8.7, 9.0)	3.19 dd (9.2, 9.2)
5′-H	3.76 dq (6.2, 9.2)	3.70 dq (5.9, 9.0)	3.61 dq (6.2, 9.2)
6′-H_3	1.40 d (6.2)	1.35 d (5.9)	1.33 d (6.2)
1″-H	5.41 d (4.0)	5.34 d (3.8)	
2″-H	3.74 dd (4.0, 9.5)	3.62 dd (3.8, 9.7)	
3″-H	4.04 dd (9.5, 10.3)	3.72 dd (9.7, 10.0)	
4″-H	3.13 dd (10.3, 10.3)	2.28 dd (10.0, 10.0)	
4″-NCH_3	2.82 s	2.39 s	
5″-H	4.27 dq (6.2, 10.3)	3.94 dq (6.2, 10.0)	
6″-H_3	1.43 d (6.2)	1.30 d (6.2)	

δ: ppm from TSP (0 ppm) in D_2O as the internal reference.
m: Multiplicity.
Coupling constants (J=Hz) are in the parentheses.

anthrone reactions but was negative to FEHLING's and TOLLEN's reagents.

Structure Elucidation

The ^1H NMR spectrum of **1** showed three C–CH_3 (three doublets at δ 1.40 ppm, δ 1.43 ppm and δ 1.67 ppm) and one N–CH_3 (one singlet at δ 2.82 ppm) (Table 3). The characteristic vinyl protons appeared as doublets at δ 7.31 ppm and δ 8.23 ppm (J=7.6 Hz), and four aromatic protons as an AB quartet at δ 7.63 ppm and δ 7.85 ppm (J=9.0 Hz). In addition, two anomeric protons were observed at δ 5.41 ppm (d,

Fig. 2. Procedure for the isolation of degradation compounds.

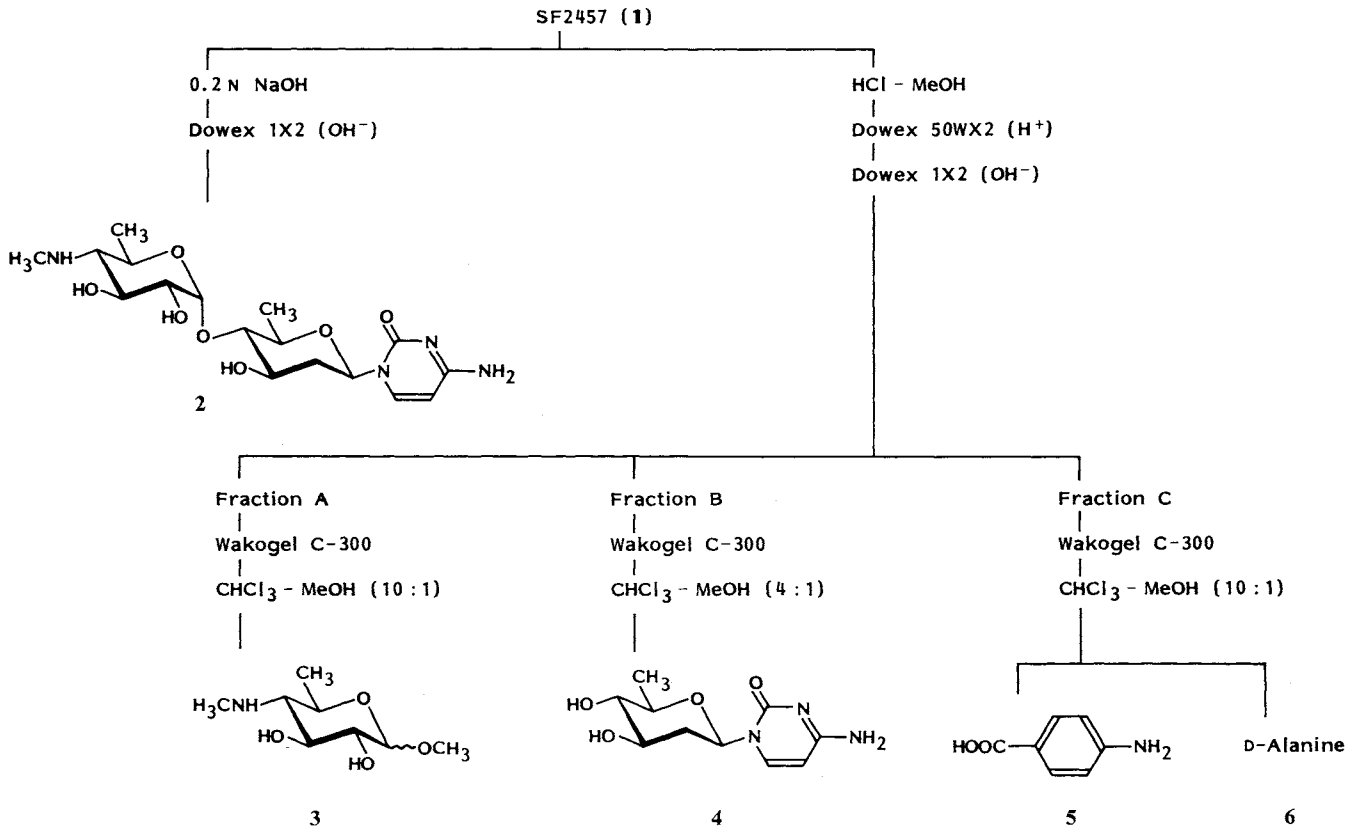


Table 4. ^{13}C NMR data of SF2457 (**1**) and the degradation products (**2** and **4**).

Carbon	1	2	4	Carbon	1	2	4
	m	m	m		m	m	m
C-2	155.2 s	157.8 s	157.9 s	C-2′	38.1 t	37.3 t	37.6 t
C-4	163.0 s	166.8 s	166.9 s	C-3′	71.6 d	71.9 d	71.3 d
C-5	99.1 d	97.2 d	97.3 d	C-4′	85.3 d	84.8 d	76.6 d
C-6	147.0 d	142.3 d	142.4 d	C-5′	74.5 d	74.2 d	75.8 d
C-8	168.4 s			C-6′	18.7 q	18.2 q	17.7 q
C-9	121.0 s			C-1″	100.7 d	100.8 d	
C-10,14	130.2 d			C-2″	73.2 d	73.5 d	
C-11,13	128.8 d			C-3″	67.6 d	70.5 d	
C-12	142.2 s			C-4″	63.7 d	65.8 d	
C-16	169.8 s			4″-NCH_3	30.8 q	33.4 q	
C-17	50.8 d			C-5″	64.7 d	68.7 d	
C-18	17.7 q			C-6″	17.9 q	18.2 q	
C-1′	82.1 d	81.0 d	81.2 d				

δ: ppm from TMS in D_2O using dioxane (67.4 ppm) as the internal reference.
m: Multiplicity.

J=4.0 Hz) and δ 5.76 ppm (dd, J=2.1 and 11.0 Hz). The ^1H NMR data were also suggestive of the close structural similarity of **1** to oxamicetin[9~11]. **1** was hydrolyzed in 0.2 N NaOH solution under conditions similar to those used for oxamicetin[10]. The isolation procedure for the hydrolysis product is shown in Fig. 2. The basic fragment in the hydrolysis product was extracted with *n*-butanol and purified by Dowex 1X2 (OH^-). The degradation product (**2**) was analyzed for $C_{17}H_{28}N_4O_7$. It showed UV absorption maxima at 278 nm in 0.1 N HCl and at 270 nm in 0.1 N NaOH. The ^1H NMR and ^{13}C NMR data for **2** are shown in Tables 3 and 4. The UV and NMR spectra are very similar to those of the degradation product of oxamicetin[10]. The coupling constant of the anomeric proton (d, J=3.8 Hz) indicated an α-linkage for glucosamine. **1** was hydrolyzed in HCl-MeOH under the same conditions used for oxamicetin. The methanolysis product was filtered and the filtrate was concentrated under reduced pressure. The concentrate was adsorbed on a column of Dowex 50WX2 (H^+). The isolation procedure for the methanolysis product is shown in Fig. 2. The fractions containing the sugar fragment were detected by anthrone reagent. The degradation product (**3**) was obtained as an anomeric mixture of methyl glucosaminides. It analyzed for $C_8H_{17}NO_4$. The degradation product (**4**) was a colorless crystalline powder and analyzed for $C_{10}H_{15}N_3O_4$. It melted at 135~137°C. It showed UV absorption maxima at 278 nm in 0.1 N HCl and at 270 nm in 0.1 N NaOH. It gave positive reactions to ninhydrin and anthrone reagents, but was negative to FEHLING's solution. These properties suggested a nucleoside structure for **4**, but the negative FEHLING's solution along with the acid stability of **4** excluded the possibility of an *O*-glycosidic linkage in the compound. The data of UV, ^1H NMR and ^{13}C NMR suggested the structure of **4** is 1-(β-3-hydroxyamicetosyl)cytosine. This structure of **4** is identical to that of the degradation product of oxamicetin. The degradation product (**5**) was obtained as colorless crystals which melted at 185~187°C and analyzed for $C_7H_7NO_2$. **5** showed a UV absorption maximum at 266 nm (ε 1,470) in water. **5** was identified with 5-aminobenzoic acid using UV, ^1H NMR and ^{13}C NMR methods. A mixture of **5** with an authentic sample showed no melting point depression. The degradation product (**6**) was obtained as a colorless powder and analyzed for $C_3H_7NO_2$. The optical rotations of **6** were $[\alpha]_D^{25}$ −1.8° (*c* 1.0, H_2O) and $[\alpha]_D^{25}$ −12.3° (*c* 1.0, 6 N HCl). **6** was shown to be identical to D-alanine using ^1H NMR and optical

Table 5. Antibacterial activities of SF2457 (1).

Test organisms	MIC (μg/ml)	Test organisms	MIC (μg/ml)
Staphylococcus aureus 209P JC-1	1.56	*Salmonella typhimurium* LT-2	50
S. aureus Smith S-424	1.56	*Salmonella* sp. D-0001	50
S. aureus No. 26	1.56	*Shigella sonnei* EW33 Type I	25
S. epidermidis ATCC 14990	1.56	*Klebsiella pneumoniae* PCI602	100
S. epidermidis 109	1.56	*K. pneumoniae* 22#3038	100
Enterococcus faecium ATCC 8043	12.5	*Proteus vulgaris* OX19	50
Bacillus anthracis No. 119	1.56	*P. mirabilis* GN310	>100
Escherichia coli JC-2	100	*Providencia rettgeri* J-0026	>100
E. coli No. 29	100	*Morganella morganii* Kono	100
E. coli W3630 RGN823	100	*Serratia marcescens* MB-3848	100
E. coli JR66/W677	50	*Pseudomonas aeruginosa* MB-3829	>100
Citrobacter freundii GN346	>100	*P. cepacia* M-0527	>100
Salmonella typhi O-901-W	1.56	*Xanthomonas maltophilia* M-0627	>100
S. enteritidis No. 11	1.56		

MIC values were determined by an agar dilution method using Sensitivity Disk Agar-N (Nissui). Inoculum size was 10^6 cfu/ml.

rotation methods. In order to determine which of the amino groups were free, **1** was acetylated by acetic anhydride in MeOH at room temperature. The ^{1}H NMR spectrum of the *N*-acetyl derivative showed that the acetylation resulted in a down-field shift of the methyl proton of C-18. This fact indicated that the amino group of D-alanine was free. From these results, the structure of **1** was assigned to SF2457 (Fig. 1).

Antibacterial Activity

The antibacterial activity of **1** is shown in Table 5. The antibiotic showed strong activity against Gram-positive bacteria but weak activity against Gram-negative bacteria. It is interesting that the antibiotic was inhibitory against *Salmonella typhi* and *Salmonella enteritidis* at a concentration of 1.56 μg/ml, but was active against *Salmonella typhimurium* at a concentration of 50 μg/ml.

Experimental

General Procedure

UV and IR spectra were recorded on Shimadzu UV-260 and Hitachi 260-10 IR spectrophotometers. Mass spectra were recorded with a Hitachi M-80B mass spectrometer. Optical rotations were measured with a Perkin Elmer model 241 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded using a Jeol JNM-GSX400 spectrometer. TLC was done on silica gel 60 F_{254} plates (E. Merck, Art. 5715).

Alkaline Hydrolysis of SF2457 (1)

A solution of **1** (500 mg) in 10 ml of 0.2 N NaOH was stirred for 15 hours at room temperature. The reaction mixture was extracted with *n*-butanol. The extract (20 ml) was washed with a small amount of water and concentrated under reduced pressure to afford 200 mg of crude solid. The product was loaded on a column of Dowex 1X2 (OH^-, 2.0×23 cm) and developed with water. The anthrone positive fractions were concentrated under reduced pressure and lyophilized to give 107.5 mg of colorless crystals of **2**: MP 175~176°C; UV $\lambda_{max}^{0.1\,N\,HCl}$ 278 nm (ε 12,800), UV $\lambda_{max}^{0.1\,N\,NaOH}$ 270 nm (ε 8,300); MS *m/z* 464 (M^+); *Anal* calcd for $C_{17}H_{28}N_4O_7$: C 43.97, H 6.08, N 12.06, found: C 43.70, H 6.02, N 12.25.

Methanolysis of SF2457 (1)

A solution of **1** (1.014 g in 20 ml of MeOH) was cooled in a dry ice-acetone bath and saturated with dry HCl and then kept at room temperature for 48 hours. The mixture was concentrated to 5 ml and applied to a column of Dowex 50WX2 (H^+, 1.5×20 cm) which had been washed with MeOH. The column

was eluted with 1 N NaOH - MeOH (1 : 1). The active fraction was concentrated under reduced pressure and applied to a column of Dowex 1X2 (OH^-, 1.8 × 17 cm). The anthrone positive fractions (A and B) and negative fraction (C) were eluted with water and 0.1 N HCl, respectively. The first eluate (fraction A) was applied to a column of silica gel (Wakogel C-300, 30 g) and the column was developed with $CHCl_3$ - MeOH (10 : 1). The fractions which gave a positive anthrone reaction were collected and concentrated to give 38.8 mg of anomeric mixture of methyl glucosaminides (**3**). The second eluate (fraction B) was applied to a column of silica gel (Wakogel C-300, 50 g) and the column was developed with $CHCl_3$ - MeOH (4 : 1). The fractions which gave a positive anthrone reaction were concentrated and lyophilized to give 41.1 mg of colorless crystals of **4**: MP 135~137°C; UV $\lambda_{max}^{0.1\,N\,HCl}$ 278 nm (ε 11,500), UV $\lambda_{max}^{0.1\,N\,NaOH}$ 270 nm (ε 8,100); MS *m/z* 241 (M^+); *Anal* calcd for $C_{10}H_{15}N_3O_4$: C 49.79, H 6.27, N 17.42, found: C 49.40, H 6.08, N 17.25. The third eluate (fraction C) was applied to a column of silica gel (Wakogel C-300, 30 g) and the column was developed with $CHCl_3$ - MeOH (10 : 1) to give two fractions with Rf values of 0.85 and 0.19 (*n*-BuOH - MeOH - H_2O, 4 : 1 : 2) on a silica TLC plate. The first fraction was concentrated to give 92.8 mg of colorless crystals of **5**: MP 185~187°C; UV $\lambda_{max}^{H_2O}$ 266 nm (ε 1,470); MS *m/z* 137 (M^+); *Anal* calcd for $C_7H_7NO_2$: C 61.31, H 5.15, N 10.21, found: C 61.05, H 5.30, N 10.08. The second fraction was concentrated to give 40.2 mg of a colorless powder of **6**: $[\alpha]_D^{25}$ −1.8° (*c* 1.0, H_2O), $[\alpha]_D^{25}$ −12.3° (*c* 1.0, 6 N HCl); MS *m/z* 87 (M^+); *Anal* calcd for $C_3H_7NO_2$: C 40.44, H 7.92, N 15.72, found: C 40.15, H 7.70, N 15.58.

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