A NEW AMINOGLYCOSIDE ANTIBIOTIC, SANNAMYCIN C AND ITS 4-N-GLYCYL DERIVATIVE

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Streptomyces sannanensis KC-7038 used for the production of sannamycins A and B has also produced another antibiotic sannamycin C, in the culture broth. Physico-chemical characterization revealed that sannamycin C is a new aminoglycoside antibiotic having 6-N-methylpurpurosamine C and 2-deoxy-3-*epi*-fortamine. Its 4-N-glycyl derivative indicated inhibitory activity against Gram-positive and Gram-negative bacteria containing aminoglycoside resistant strains.

Sannamycins A and B[1,2], new aminoglycoside antibiotics, are produced by a strain belonging to *Streptomyces sannanensis* KC-7038, which simultaneously produce another component, sannamycin C.

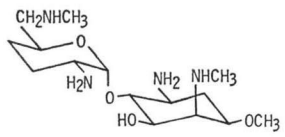
The paper describes the fermentation, isolation, physico-chemical properties and synthesis of the 4-N-glycyl derivative of sannamycin C and its biological properties.

Fermentation

The fermentation of *Streptomyces sannanensis* KC-7038 was performed for the production of sannamycin C in a 200-liter fermentor containing 100 liters of a medium, which contains per liter, 40 g starch, 5 g soybean meal, 40 g corn steep liquor, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4, 3 g NaCl, 1 g $CaCO_3$ and cotton seed oil (pH 6.5 before sterilization). A hundred ml of a good preculture was made by inoculating the broth culture in shake flask into a medium containing the above medium excluding cotton seed oil. The incubated preculture was then inoculated into 100 liters of the fermentation medium as described above. The fermentation was conducted at 27°C under aeration of 50 liters/minute, agitation of 220 rpm at an inner pressure of 0.5 kg/cm^2. The potency of the cultured broth was estimated by a disc-plate method against *Bacillus subtilis* ATCC 6633. After 96 hours incubation a maximum (40 mcg/ml as sannamycin A) was reached.

Isolation

The 96-hour cultured broth (100 liters) containing the sannamycins was filtered at pH 2.0 by using Dicalite (Dicalite Orient Co., Ltd., Japan) as a filter aid, and the filtrate (80 liters) was filtered again at pH 6.4. Sannamycins in the filtrate (70 liters) were adsorbed on a column (15×55 cm) of Amberlite IRC-50 (NH_4^+) resin. The column was washed with deionized water (30 liters) and then eluted with 1 N NH_4OH (6 liters). Active fractions were combined, concentrated and lyophilized to give a pale brown powder of the sannamycin complex (9.7 g). The complex (4.6 g) was dissolved in deionized water (1 liter) and the solution was charged on a column (3×150 cm) of CM-Sephadex C-25 (NH_4^+). After



Sannamycin C

washing with deionized water, the column was eluted with aqueous ammonia with a concentration gradient from water (4.5 liters) to 0.5 N (4.5 liters) at a flow rate of 150 ml/hours. The eluate was cut into 28-ml fractions and each fraction was monitored by bio-activity. After elution of some minor components, sannamycin A (fractions Nos. 207~224) was eluted first, followed by sannamycin B (fractions Nos. 236~244) and then sannamycin C (fractions Nos. 245~253). Fractions containing sannamycin C were concentrated and lyophilized to give a colorless solid (340 mg) of pure sannamycin C.

Physico-Chemical Properties

Physico-chemical properties data of sannamycin C are listed in Table 1. Sannamycin C is a colorless solid and shows $[\alpha]_D^{25}+59°$ (c 1, H_2O). Sannamycin C is readily soluble in water. Sannamycin C gives positive ninhydrin and RYDON-SMITH reactions. Sannamycin C is stable at pH 2.0, 7.0 and 10.0 at 60°C for 4 hours. Molecular ion peak of the mass spectrum and analytical data for sannamycin C agreed with the molecular formula of $C_{15}H_{32}N_4O_4$ (332). The IR and NMR spectra of sannamycin C are shown in Figs. 1 and 2, respectively. The 100 MHz ^1H NMR spectrum of sannamycin C indicated one anomeric proton (5.57 ppm) and three methyl groups assigned to N-CH_3 (2.87 and 2.93 ppm) and O-CH_3 (3.87 ppm).

Sannamycin C was clearly differentiated from known aminoglycoside antibiotics by the physico-chemical properties as shown in Tables 1 and 2. From these results mentioned above one

Table 1. Physico-chemical properties of sannamycin C.

Nature	Basic colorless solid
$[\alpha]_D^{25}$ (c 1, H_2O)	+59°
UV	End absorption
Elementary analysis for	$C_{15}H_{32}N_4O_4 \cdot \frac{1}{2}H_2O$
(%)	Found Calcd.
C	53.29 52.76
H	9.19 9.74
N	16.22 16.41
MW (Mass)	332
IR ν_{max}^{KBr} cm^{-1}	3400, 1570
^1H NMR (D_2O) δ	
N-CH_3	2.87, 2.93
O-CH_3	3.87
Anomeric H	5.57

Fig. 1. IR spectrum of sannamycin C.

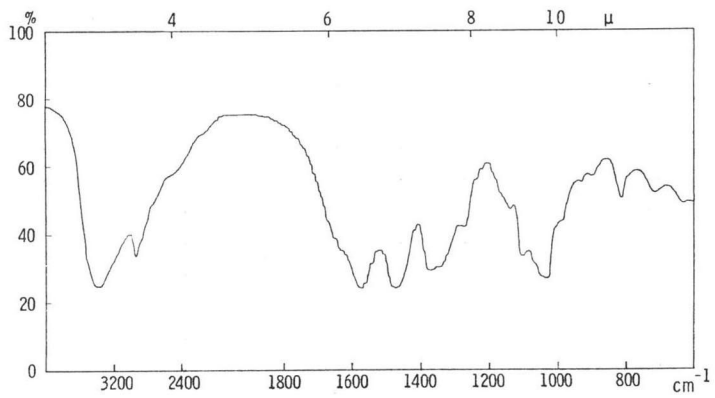
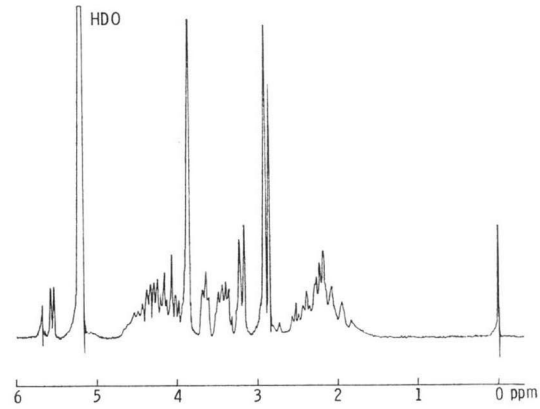


Table 2. Comparison between sannamycins B and C on thin-layer chromatography.

	Sannamycin B	Sannamycin C
Rf	0.51	0.43

Solvent system: $CHCl_3$-CH_3OH-17% NH_4OH, 2 : 1 : 1 lower layer
Silica gel TLC: TLC aluminium sheets silica gel 60 F_{254} pre-coated

Fig. 2. ^1H NMR spectrum of sannamycin C free base in D_2O.



can conclude that sannamycin C is a new aminoglycoside antibiotic with a structure very closely related to sannamycin B.

Biological Properties

Antimicrobial activity of sannamycin C was determined in a nutrient agar. It has a weak activity against all of the microorganisms as shown in Table 5.

The Structure of Sannamycin C (1)

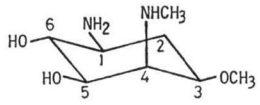
Methanolysis of tetra-N-acetylsannamycin C (**2**) with hydrogen chloride in anhydrous methanol followed by re-N-acetylation afforded methyl N-acetyl-6-N-methyl-α- and β-purpurosaminides C (**3** and **4**) and a N-acetylaminocyclitol (**5**). ^1H NMR spectra of **3** and **4** were superimposable with that of the authentic sample obtained from sannamycin B. Hydrolysis of **5** with 4 N sodium hydroxide afforded

Table 3. Chemical shifts and coupling constants of ^1H NMR spectra of sannamycin C and aminocyclitol (**6**).

Protons	Sannamycin C		Aminocyclitol (**6**)	
	ppm	J (Hz)	ppm	J (Hz)
1′	5.57	3.5		
2′	3.4 ~	3.5		
3′	2.0 ≀			
4′	2.5			
5′	4.5 ~	6.0		
6′	3.09	6.0		
1	3.33	12.5, 4.5, 8.5	3.07	12.0, 4.5, 9.0
2 ax.	2.05	12.5, 12.5, 10.5	2.00	12.0, 12.0, 12.0
2 eq.	2.49	4.5, 12.5, 4.5	2.44	4.5, 12.0, 4.5, 1.5
3	4.13	4.5, 3.5, 10.5	4.04	3.3, 12.0, 4.5
4	3.66	3.7, 3.5	3.75	3.3, 3.3, 1.5
5	4.32	3.7, 8.5	4.03	3.3, 9.5
6	4.07	8.5, 8.5	3.93	9.0, 9.5
N-CH_3	2.87			
N-CH_3	2.93		2.96	
O-CH_3	3.87		3.88	

TMS as the external reference

Fig. 3. Aminocyclitol (**6**)



the aminocyclitol (**6**). The structure of **6** was shown to be 2-deoxy-3-*epi*-fortamine or its mirror image by the ^{1}H NMR and ^{13}C NMR spectra (Tables 3 and 4). The $\Delta[M]_{436}$ CuAm value of **5** was +1800°[8]. Thus the absolute structure of **6** was 2-deoxy-3-*epi*-fortamine (Fig. 3). The position of glycosidic linkage was determined by the ^{13}C NMR spectra of **1** and **6** (Table 4). The significant difference in the chemical shifts in assignment for C-6 in **1** (85.8 ppm) and **6** (75.7 ppm) indicated the aminocyclitol was glycosylated at C-6 in **6**. The coupling constant ($J_{1',2'}$=3.5Hz) of the anomeric proton in the ^{1}H NMR of **1** was due to the α-configuration of the glycoside. In this way the absolute structure of sannamycin C was determined to be 3-*epi*-sannamycin B.

Table 4. Chemical shifts of ^{13}C NMR spectra of sannamycin C and aminocyclitol (**6**).

Carbons	ppm	
	Sannamycin C	Aminocyclitol
1′	102.3	
2′	50.5	
3′	27.1	
4′	28.9	
5′	69.2	
6′	55.8	
1	50.0	50.9
2	32.2	32.7
3	78.3	78.1
4	61.1	61.5
5	73.4	73.6
6	85.8	75.7
N-CH_3	35.9	
N-CH_3	37.3	37.8
O-CH_3	57.0	56.5

Table 5. Antimicrobial spectra of sannamycin C and 4-N-glycylsannamycin C (**7**).

Test organisms	MIC in mcg/ml		
	Sannamycin C	**7**	Sannamycin A
Staphylococcus aureus FDA 209P	25	0.39	0.39
Bacillus anthracis	6	0.20	0.20
〃 *cereus*	100	0.78	1.56
〃 *subtilis* ATCC 6633	12	0.20	0.20
Streptococcus faecalis	>100	50	50
Escherichia coli NIHJ	100	3.13	3.13
〃 〃 K-12 ML 1410	>100	3.13	3.13
〃 〃 〃 R- 81[i]	>100	6.25	6.25
〃 〃 〃 R- 82[ii]	>100	6.25	6.25
〃 〃 〃 R-101[iii]	100	3.13	6.25
Proteus vulgaris OX-19	50	3.13	3.13
〃 *inconstans*[iv]	>100	12.5	6.25
Klebsiella pneumoniae PCI 602	>100	0.78	1.56
Pseudomonas aeruginosa Shibata	>100	6.25	6.25
〃 〃 99[v]	>100	>100	>100
〃 〃 A_3	>100	12.5	12.5
〃 〃 GN 315[vi]	>100	50	25
Serratia marcescens	50	3.13	1.56
Mycobacterium smegmatis ATCC 607	>100	3.13	1.56

Medium: nutrient agar (Eiken Chemical Co., Ltd., Japan)
i) APH (3′)-I ii) APH (3′)-II iii) AAD (2″) iv) AAC (2′) v) AAC (3)-I vi) AAC (6′)-IV

Synthesis of 4-N-Glycylsannamycin C

Since sannamycin C was shown to exhibit weak antibiotic properties as shown in Table 5, it appeared possible that 4-N-glycylation of sannamycin C might lead to a substance with improved antibiotic properties because the useful antibiotics in this class have a N-glycyl group at 4-position. For this reason the synthesis of 4-N-glycyl sannamycin C (**7**) was attempted.

Selective 1,2′,6′-tris-N-benzyloxycarbonylation of sannamycin C using benzyloxycarbonyloxysuccinimide with aid of nickel (II) acetate[4], followed by N-benzyloxycarbonylglycylation afforded 1,2′,6′-tris-N-benzyloxycarbonyl-4-N-(N-benzyloxycarbonylglycyl)sannamycin C. Finally, the N-benzyloxycarbonyl groups were removed by catalytic hydrogenolysis to give 4-N-glycylsannamycin C in an overall yield of 53%. In the ^{1}H NMR spectrum of 4-N-glycylsannamycin C sulfate, the large coupling derived from *trans*-diaxial protons was not exhibited by the cyclitol ring protons. The resonance of H-3 was observed as a quartet (J=3 Hz) at 4.54 ppm. This requires that the aminocyclitol ring has inverted from the ^{4}C$_1$ conformation as shown for sannamycin C (**1**) to the ^{1}C$_4$ conformer which incorporates four axial substituents at 1, 3, 5 and 6 positions in 4-N-glycylsannamycin C sulfate (**7**).

4-N-Glycylsannamycin C has strong activity against Gram-positive and Gram-negative bacteria especially aminoglycoside resistant strains excluding an AAC (3)-I producing organism (*Pseudomonas aeruginosa* 99) as shown in Table 5.

Experimental

The spectrometric data were obtained by the following instruments. Infrared spectra; Japan Spectroscopic Co., Ltd. Model DS-403 G spectrometer. Mass spectra; Japan Electron Optics Lab. Model JMS-D-100 mass spectrometer and Hitachi Model RMU-6MG mass spectrometer. *^{13}C NMR* spectra; Japan Electron Optics Lab. Model JNM-FX-100 spectrometer. ^{1}H NMR spectra; Japan Electron Optics Lab. Model JNM-MH-100 spectrometer (TMS as the external reference in D_2O). Optical rotations were measured on a Digital polarimeter DIP-4 of Japan Spectroscopic Co., Ltd. Chromatographies were performed with the following reagents. Silica gel; Wako gel C-200 (column chromatography), E. Merck DC-Alufolien 60 F_{254} (thin-layer chromatography).

Tetra-N-acetylsannamycin C (**2**)

To a solution of sannamycin C (**1**, 280 mg) in methanol (50 ml), acetic anhydride (5 ml) was added and the solution was kept at room temperature for 5 hours. The solution was concentrated and the residue was chromatographed on a column of silica gel with chloroform - methanol (20: 1) to give **2** (313 mg) as a colorless solid; $[\alpha]_D^{23}$ +125° (*c* 1, H_2O); MS: *m/z* 500 (M^+), 257, 227, 207; ^{1}H NMR ($CDCl_3$) δ 1.94, 2.00, 2.11, 2.14, 2,24, (s, N-COCH$_3$ rotamer); 2.91, 3.13, 3.34, 3.37, 3.40 (s, N-CH$_3$, O-CH$_3$ rotamer); 4.75 (1H, d, J=3.5 Hz, H-1′).

Anal. Calcd. for $C_{23}H_{40}N_4O_8\cdot\frac{1}{2}H_2O$: C, 54.21; H, 8.11; N, 10.99.
Found: C, 54.35; H, 7.96; N, 11.01.

Methanolysis of tetra-N-acetylsannamycin C

A solution of **2** (300 mg) with 13 ml of 6 N HCl in dry methanol was heated at 80°C for 8 hours in a sealed tube. The methanolysate was concentrated *in vacuo*. The residue was dissolved in 100 ml of deionized water and passed through a column (2×7 cm) of Dowex 1-X2 (OH^-). The effluent was evaporated to dryness under reduced pressure and co-evaporated with toluene to remove residual water. To a solution of the residue in 10 ml of methanol, 1 ml of acetic anhydride was added and the mixture was allowed to stand overnight at room temperature. The reaction mixture was evaporated under reduced pressure and chromatographed on a column (1.5×30 cm) of silica gel with chloroform - acetone (1: 2) to give three products. From the initial fractions methyl 2,6-di-N-acetyl-6-N-methyl-α-purpurosaminide C (**3**, 55 mg) was obtained as a colorless syrup; $[\alpha]_D^{23}$ +172° (*c* 1, MeOH); ^{1}H NMR ($CDCl_3$)

δ 1.97 (3H, s, N-$COCH_3$); 2.08, 2.13 (s, N-$COCH_3$ rotamers); 2.98, 3.12 (s, N-CH_3 rotamers); 3.33 (3H, s, O-CH_3); 4.61 (1H, d, J=3.5 Hz, H-1′).

From the second fractions methyl 2,6-di-N-acetyl-6-N-methyl-β-purpurosaminide C (**4**, 26 mg) was obtained as a colorless syrup; $[\alpha]_D^{23}+2°$ (c 1, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.98 (3H, s, N-$COCH_3$); 2.10, 2.14 (s, N-$COCH_3$, rotamers); 3.00, 3.14 (s, N-CH_3, rotamers); 3.45, 3.46 (s, O-CH_3, rotamers); 4.22, 4.27 (d, J=8.5 Hz, H-1′, rotamers).

The last product was the 2,5-di-N-acetylaminocyclitol (**5**, 48 mg); $[\alpha]_D^{24}+3°$ (c 1, MeOH); MS: m/z 275 (M^++1); ^{1}H NMR ($CDCl_3$) δ 1.95, 2.13 (s, N-$COCH_3$), 3.30, 3.35 (s, N-CH_3, rotamers), 3.35 (s, O-CH_3).

Anal. Calcd. for $C_{12}H_{22}N_2O_5\cdot\frac{1}{2}H_2O$: C, 50.87; H, 8.18; N, 9.89.
Found: C, 50.63; H, 8.07; N, 9.65.

Aminocyclitol (**6**)

A solution of **5** (230 mg) in 7 ml of 4 N NaOH was heated at 110°C for 5 hours. The reaction mixture, neutralized with diluted hydrochloric acid, was charged on a column (2×15 cm) of CM-Sephadex C-25 (NH_4^+) and after washing the column with deionized water, **6** was eluted by gradient elution between 0.025 N NH_4OH and 0.5 N NH_4OH. The eluate containing **6** was concentrated to give a solid of **6** (113 mg); $[\alpha]_D^{25}+11°$ (c 1, H_2O); MS: m/z 190 (M^+); ^{1}H NMR (D_2O) Table 3.

Anal. Calcd. for $C_8H_{18}N_2O_3\cdot\frac{1}{2}H_2O$: C, 48.22; H, 9.61; N, 14.46.
Found: C, 48.47: H, 9.33; N, 14.29.

Tris-N-benzyloxycarbonyl-4-N-benzyloxycarbonylglycylsannamycin C (**8**)

To a solution of sannamycin C (160 mg) in methanol (5 ml), nickel (II) acetate dihydrate (248 mg) was added and the mixture was stirred for 30 minutes. After the mixture became clear, N-benzyloxycarbonyloxysuccinimide (415 mg) was added and the mixture was stirred for 2 hours at room temperature. After addition of concentrated aqueous ammonia (2.5 ml), the solution was stirred for 1 hour. The reaction mixture was concentrated *in vacuo* and the concentrate was extracted with chloroform (20 ml). The organic layer was washed successively with 3 N aqueous ammonia and water, dried with Na_2SO_4, and concentrated to give a syrupy residue. To a solution of the residue in dioxane (9 ml), N-hydroxysuccinimide ester of N-benzyloxycarbonylglycin (300 mg) and triethylamine (0.3 ml) were added and the mixture was heated overnight at 60°C. Evaporation, extraction of the residue with chloroform, washing of the solution with water and drying with Na_2SO_4, followed by evaporation of the solvent gave a syrup, which was chromatographed on a column of silica gel with chloroform - methanol (50: 1) to give a colorless solid of **8** (280 mg); $[\alpha]_D^{25}+46°$ (c 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.98 (3H, s, 6′-N-CH_3), 3.14 (3H, s, 4-N-CH_3), 3.29 (3H, s, O-CH_3).

Anal. Calcd. for $C_{49}H_{59}N_5O_{12}$: C, 63.55; H, 6.42; N, 7.56.
Found: C, 63.82; H, 6.20; N, 7.27.

4-N-Glycylsannamycin C (**7**)

A solution of **8** (270 mg) in acetic acid (4 ml) was hydrogenated with palladium black under an atmospheric pressure of hydrogen. After filtration, the filtrate was diluted with water (400 ml) and neutralized with aqueous ammonia. The solution was charged on a column of CM-Sephadex C-25 (NH_4^+) and, after washing the column with water, the column was eluted with aqueous ammonia with a concentration gradient from 0.05 N to 0.35 N. The eluate containing **7** was lyophilized to give a colorless solid, which was neutralized with diluted sulfuric acid and lyophilized to afford a colorless solid (148 mg) of 4-N-glycylsannamycin C sulfate; $[\alpha]_D^{25}+71°$ (c 1, H_2O); ^{1}H NMR (D_2O) δ 3.33 (3H, s, 6′-N-CH_3), 3.87 (3H, s, 4-N-CH_3), 3.99 (3H, s, O-CH_3), 4.39 (1H, m, H-1), 4.54 (1H, q, J=3Hz, H-3), 4.66 (2H, s, gly-CH_2), 4.94 (2H, H-5, 6), 5.30 (1H, t, J=3Hz, H-4), 5.99 (1H, d, J=3.5 Hz, H-1′).

Anal. Calcd. for $C_{17}H_{35}N_5O_5\cdot 2H_2SO_4\cdot H_2O$: C, 33.82; H, 6.85; N, 11.60; S, 10.63.
Found: C, 33.50; H, 6.58; N, 11.35; S, 10.38.

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