STUDIES ON *ACTINOMYCETALES* PRODUCING ANTIBIOTICS ONLY ON AGAR CULTURE

II. ISOLATION, STRUCTURE AND BIOLOGICAL PROPERTIES OF N-CARBAMOYL-D-GLUCOSAMINE (SUBSTANCE SF-1993)

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N-Carbamoyl-D-glucosamine was isolated from the culture of *Streptomyces halstedii* SF-1993. It showed weak antimicrobial activity against some Gram-negative bacteria and some fungi.

As described in the preceding paper[1], *Streptomyces halstedii* SF-1993 was isolated by screening, in which strains producing antibiotics on agar culture but not in shaken culture were selected. Taxonomy and morphology-productivity relationships of the strain SF-1993 were the subjects of the previous paper. This paper deals with the isolation, characterization and structure of the antibiotic produced by the strain SF-1993.

Production and Isolation

The antibotic SF-1993 could be produced either by agar culture or by submerged culture.

(1) From Agar Culture.

The strain SF-1993 was inoculated on GB agar medium[1] containing 2.0% glycerol, 1.0% Polypeptone, 0.5% meat extract, 0.3% $CaCO_3$ and 2.0% agar in two large Petri-dishes (26.5 cm i.d.) containing 200 ml of the medium and incubated at 28°C for 2 days. In order to separate the culture fluid from agar, the whole cultured agar including mycelia was frozen once at −20°C, then thawed and filtered immediately.

The culture filtrate (280 ml) obtained from the agar culture was treated with charcoal (1.0%, w/v) to decolorize it, followed by filtration and concentration (5 ml). The concentrate was passed through a column of charcoal (6 ml), and developed with water to give substance SF-1993 as a crude powder. The crude powder was purified by a column chromatography over Sephadex LH-20 (300 ml) developed with methanol. From the methanol eluate were obtained fine needles of SF-1993, which were recrystallized from water-ethanol. Yield, 25 mg.

(2) From Submerged Culture.

The submerged fermentation of SF-1993 has been achieved by the use of the diluted medium[1]. The strain SF-1993 was inoculated on medium containing 1.0% glycerol, 0.5% Polypeptone, 0.15% meat extract and 0.15% $CaCO_3$ in thirty 500-ml Erlenmeyer flasks (each flask contained 100 ml of the medium), and incubated at 28°C for 22 hours on a rotary-shaker. After shaking, 2.5 liters of the filtered broth was obtained. The filtered broth was treated with charcoal (1.0%, w/v), followed by filtration and concentration (50 ml). The concentrate was passed through a column of charcoal

(150 ml) and developed with water to give a crude antibiotic powder. The crude antibiotic was purified by a column chromatography over Sephadex LH-20 (300 ml) developed with methanol. Upon standing, the active fractions deposited fine needles of substance SF-1993 which were identical in all respects with substance SF-1993 obtained from agar culture. Yield, 480 mg.

Physico-chemical Properties of Substance SF-1993

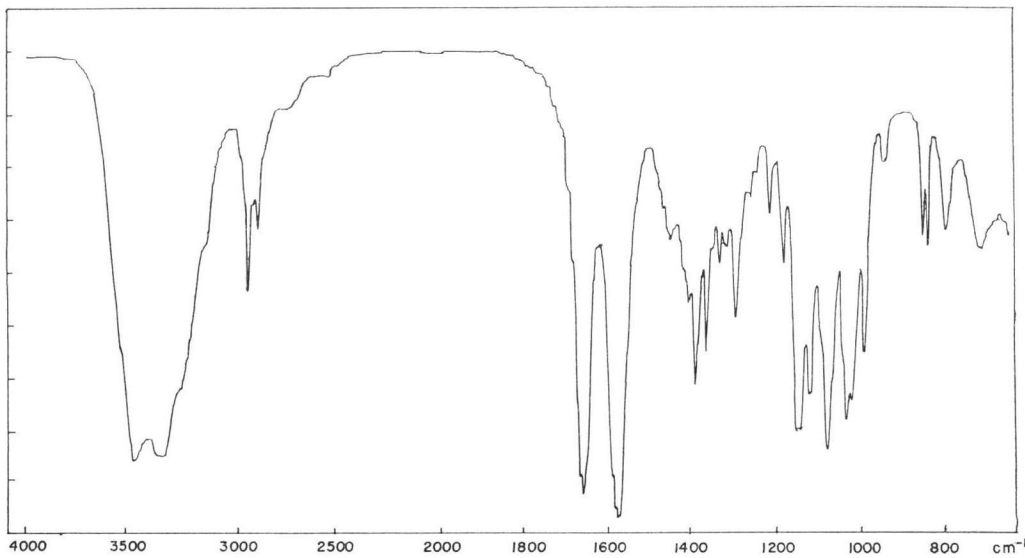
SF-1993 (**1**) is a neutral, water-soluble substance. It is positive to EHRLICH (yellow), naphthoresorcinol-phosphoric acid (pale violet) and RYDON-SMITH reactions, but negative to ninhydrin and 2,3,5-triphenyltetrazolium reactions. SF-1993 melted at 158~159°C and showed mutarotation: $[\alpha]_D^{20}$ +73.6° (10 min.) → +58.1° (overnight) (*c* 0.98, water). Upon standing at room temperature in water for a couple of days, **1** gradually changes to a biologically inactive compound (**2**). The presence of acid or heating (40°C) greatly accelerate the inactivation.

The UV spectrum of SF-1993 in water showed end absorption, and the IR spectrum in a KBr pellet showed characteristic bands at 3450, 3300, 1640, 1560, 1370, 1130, 1060 and 1010 cm^{-1} (Fig. 1). SF-1993 analyzed for $C_7H_{14}N_2O_6$. Found: C, 38.17; H, 6.45; N, 11.84%. Calcd.: C, 37.84; H, 5.93; N, 12.60%.

The FD mass spectrum of SF-1993 showed a base peak at *m/e* 205 (100%, M−17) with a very weak peak at *m/e* 222 (M^+). The EI mass spectrum of its tetraacetate (α-acetate, m.p. 191~194°C, $[\alpha]_D^{20}$ +103° (*c* 1.1, $CHCl_3$), no β-acetate was detected) showed peaks at *m/e* 347 (M−43) and 330 (M−60). The PMR and the CMR spectra of SF-1993 are shown in Figs. 2 and 3. Particularly noteworthy was the similarity of the CMR spectrum to that of D-glucosamine hydrochloride[2], with an exception of additional peaks at 162.3 and 161.9 ppm attributable to amide carbonyls.

These physico-chemical properties of substance SF-1993 suggested that SF-1993 is an N-carbamoyl-D-hexosamine. The PMR spectra of **1** or its tetraacetate in $CDCl_3$ or CD_3COCD_3 (δ 2.02 (s, OAc ×1), 2.04 (s, OAc ×2), 2.19 (s, OAc ×1), 4.0~4.4 (m, 4H), 5.1~5.3 (m, 2H), 5.48 (s, $N\underline{H}_2$),

Fig. 1. IR spectrum of SF-1993 (KBr).



5.93 (d, NH, J=9.2 Hz), 6.15 (d, 1H, J=3.4 Hz)) were of little value in obtaining information about the configuration of SF-1993 because of severe overlapping of signals. However, 2-deoxy-structure was suggested by the fact that irradiation at δ 4.2 collapsed the doublets at δ 5.93 (NH) and 6.15 (H-1). Since acetylation of SF-1993 gave exclusively an α-acetate showing a small $J_{1,2}$ value, the configuration around C-2 could not be obtained.

On the other hand, treatment of **1** with 5% methanolic hydrogen chloride followed by acetylation gave a 7: 5 mixture of α- and β-methyl glycoside tetraacetates. The mixture was purified over silica gel column chromatography followed by crystallization from ethanol to give each pure anomer: α-anomer; syrup, $[\alpha]_D^{20}$ +83.0° (*c* 1.1, $CHCl_3$), MS, *m/e* 404 (M^+), PMR (100 MHz, d_6-benzene+d_6-acetone 3:1) δ 1.78, 1.79, 1.80 and 1.83 (s, OAc×4), 3.09 (s, OMe), 3.86 (dq, an X part of an ABMX

Fig. 2. WEFT-PMR spectrum of SF-1993 at 100 MHz in D_2O (an equilibrium solution)
*; the signal of **2** formed during accumulation.

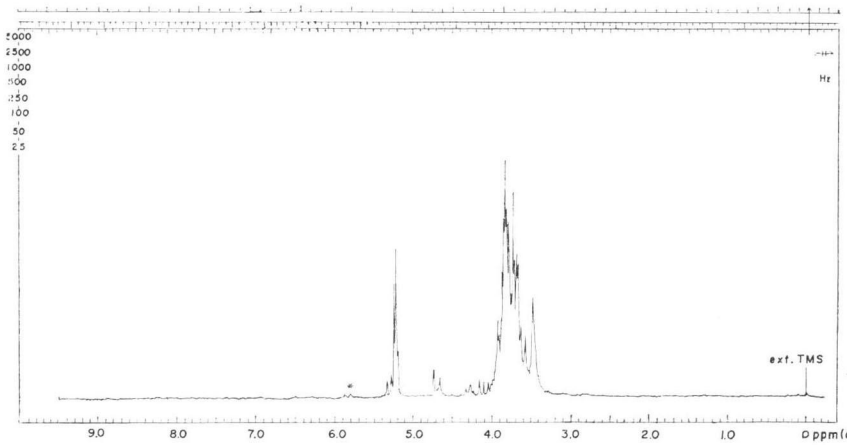
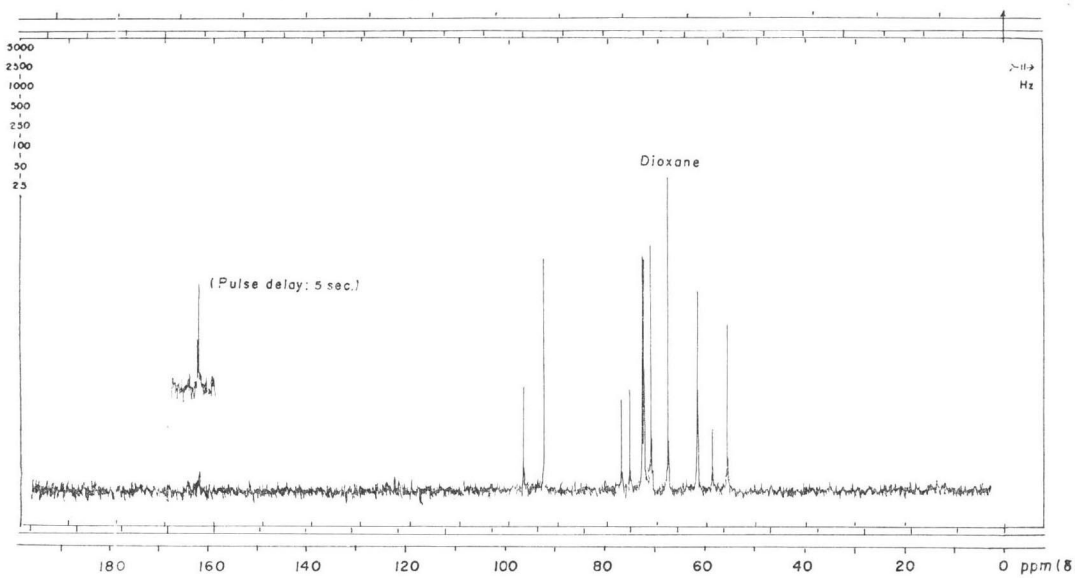
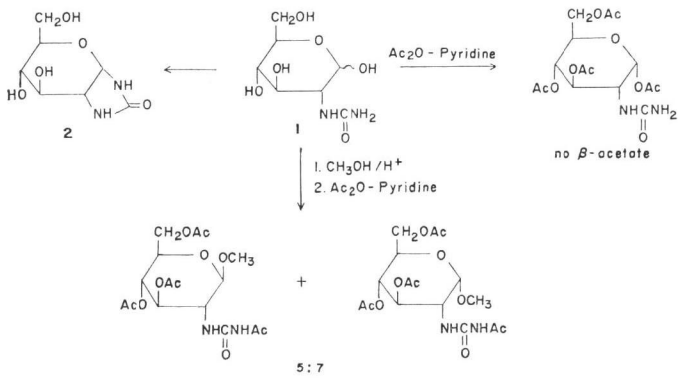


Fig. 3. CMR spectrum of SF-1993 at 25.16 MHz in D_2O.





system; H-5, $J_{5,6}=4.9$ Hz, $J_{5,6'}=2.7$ Hz, $J_{5,4}=9.5$ Hz), 4.09 & 4.26 (an AB part of an ABX system: H-6 & 6′, $J_{6,6'}=12.0$ Hz), 4.47 (td, H-2, $J_{2,3}=10.0$ Hz, $J_{1,2}=3.1$ Hz, $J_{2,NH}=8.8$ Hz), 4.69 (d, H-1), 5.26 (t, H-4, $J_{4,3}=9.5$ Hz), 5.56 (t, H-3), 9.00 (d, NH), 9.48 (s, NH): β-anomer; fine needles, m.p. 202~204°C, $[\alpha]_D^{20}$ +19.6° (*c* 0.7, $CHCl_3$), MS, *m/e* 404 (M^+), PMR (d_6-benzene + d_6-acetone 1:2) δ 1.89, 1.91, 1.93 and 1.98 (s, OAc×4), 3.40 (s, OMe), 3.76 (dq, an X part of an ABMX system; H-5, $J_{5,6}=4.1$ Hz, $J_{5,6'}=2.9$ Hz, $J_{5,4}=9.2$ Hz), 4.03 (bq, H-2, $J_{2,1}=8.2$ Hz, $J_{2,3}=10.0$ Hz, $J_{2,NH}=9.0$ Hz), 4.15 & 4.32 (an AB part of an ABX system: H-6,6′, $J_{6,6'}=12.0$ Hz), 4.70 (d, H-1), 5.12 (t, H-4, $J_{4,3}=9.2$ Hz), 5.47 (dd, H-3), 8.80 (d, NH), 9.28 (s, NH).

These PMR data, especially the coupling constants of each adjacent protons of the β-anomer, suggested the *gluco*-configuration for SF-1993. The structure of SF-1993 was finally confirmed to be N-carbamoyl-D-glucosamine by synthesis from D-glucosamine hydrochloride. An authentic sample of N-carbamoyl-D-glucosamine was obtained by the treatment of D-glucosamine hydrochloride with 1.1 equivalent of potassium cyanate in water for 20 minutes, followed by chromatography over Sephadex G-10 and concentration. Addition of ethanol to the concentrate gave fine needles of N-carbamoyl-D-glucosamine in 81% yield. The synthetic sample was identical in all respects including biological activity.

The N-carbamoyl derivatives of D-galactosamine, syrup, and D-mannosamine, syrup, were newly synthesized as reference compounds, and they were quite different from SF-1993. These isomers were much less stable than **1**, especially D-galacto-isomer, and changed to inactive compounds like **2** within a day at room temperature. N-Carbamoyl-D-glucosamine has been synthesized by MICHEEL *et al.*[3] in 1956, but this is the first report of it as an antibiotic.

The inactive compound **2** melted at 150~153°C and did not show mutarotation: $[\alpha]_D^{20}$ −49.2° (*c* 1, water). Lack of mutarotation, together with the elemental analysis (Calcd. for $C_7H_{12}N_2O_5$: C, 41.17; H, 5.92; N, 13.72%, Found: C, 40.87; H, 5.94; N, 13.13%) and EI mass spectrum of its triacetate (m.p. 173~175°C, $[\alpha]_D^{20}$ +17.9° (*c* 0.6, $CHCl_3$) showing a molecular ion at *m/e* 330 suggested the ureylene structure **2** for the inactive compound.

Biological Properties of Substance SF-1993

The antimicrobial spectrum of the antibiotic SF-1993 was tested by the paper-disc agar diffusion method and the results are listed in Table 1. The antibiotic SF-1993 is weakly active against some Gram-negative bacteria and some fungi. In contrast, N-carbamoyl-D-galactosamine and -D-mannosamine, isomers of **1**, showed no activity so far tested.

The antibacterial activity of the antibiotic SF-1993 was reversed by several sugars. As shown in Table 2, the activity of SF-1993 was antagonized by hexoses, especially by D-glucose, but not by

Table 1. Antimicrobial spectrum of the antibiotic SF-1993.

Test organism	Inhibitory diameter (mm) 8 mg/ml	2 mg/ml	Medium
Escherichia coli IFO 13168	25.2	18.3	a
Escherichia coli K12	20.3	14.5	〃
Escherichia coli K12R*	25.2	21.0	〃
Shigella sonnei	22.1	13.8	〃
Salmonella typhi	26.0	18.6	〃
Proteus vulgaris	26.6	14.5	〃
Klebsiella pneumoniae	0	0	〃
Pseudomonas aeruginosa	0	0	〃
Bacillus subtilis	0	0	〃
Staphylococcus aureus	0	0	〃
Sarcina lutea	0	0	〃
Mycobacterium smegmatis	0	0	b
Candida albicans	(15.0)	0	c
Cryptococcus neoformans	(11.7)	0	〃
Aspergillus niger	(30.0)	(17.5)	〃
Mucor spinescens	(33.3)	(24.2)	〃
Rhizopus niveus	(18.2)	(10.0)	〃
Trichoderma koningi	(20.3)	(11.7)	〃

Method: paper-disc agar diffusion method.
Medium: a; bouillon agar, b; glycerol bouillon agar, c; malt extract-yeast extract agar.
*resistant strain against kanamycin, tetracycline and chloramphenicol.
( ): hazy zone.

Table 2. Effect of various sugars on the antibacterial activity of SF-1993.

Sugar	Concentration (M)	Percent of amount reversed
None		0%
D-Glucose	0.04	100
	0.2	100
D-Glucosamine	0.04	50
	0.2	100
N-Acetyl-D-glucosamine	0.04	10
	0.2	100
D-Galactose	0.04	50
	0.2	100
D-Fructose	0.04	0
	0.2	100
D-Mannose	0.04	0
	0.2	70
L-Arabinose	0.2	0
D-Xylose	0.2	0
D-Ribose	0.2	0

The reversal by the sugar was examined by the agar diffusion assay method using *Escherichia coli* IFO 13168 as a test organism. Equal volume of the antibiotic solution (0.04 M) and the sugar solution (0.08 or 0.4 M) were mixed and assayed.

pentoses. It was also antagonized by D-glucosamine and N-acetyl-D-glucosamine. Acute toxicity of SF-1993 was examined using mice. No mice died on administering the antibiotic at a dose of 1,000 mg/kg intravenously.

Discussion

As nitrogen-containing monosaccharide antibiotics, 3-amino-3-deoxy-D-glucose[4], nojirimycin[5] and streptozotocin[6] are known. Substance SF-1993 is a new type of amino-sugar antibiotic containing an N-carbamoyl group. In this connection, it is interesting that LL-BM-123β[7] and its analogues contain N-carbamoylamino-sugar derivatives, and show strong activity against both Gram-positive and negative bacteria. SF-1993 substance is a new glucose analogue having antibacterial activity, but it showed no inhibition against glucosidase, differing from nojirimycin[8].

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