STUDIES ON SF-1902 $A_2 \sim A_5$, MINOR COMPONENTS OF SF-1902 (GLOBOMYCIN)

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Four members of globomycin, SF-1902 A_2, A_3, A_{4a} and A_{4b} were newly isolated from the culture of *Streptomyces hygroscopicus* SF-1902. These minor components shared four amino acids in common and the fifth was either valine or *allo*-isoleucine. The fatty acid moiety varied from 3-hydroxy-2-methylheptanoic acid in A_2 to 3-hydroxy-2-methylundecanoic acids in A_{4b}. The length of alkyl chain greatly affected the antibacterial activity, and maximum activity was shown by the homologue (A_5) possessing the longest alkyl chain.

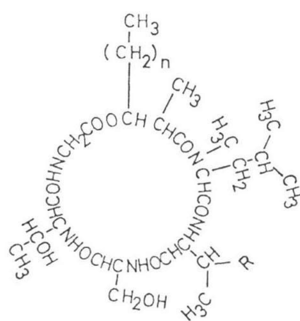
In our screening of new antibiotics, a novel neutral antibiotic complex, SF-1902 A group was isolated from the culture of *Streptomyces hygroscopicus* SF-1902. The complex contained about ten components in which A_1* identical with globomycin[1] was dominant. From the minor components so far five, A_2, A_3, A_{4a}, A_{4b} and A_5 were characterized in this laboratory. Isolation and structure of SF-1902 A_5 were reported previously[2]. In this paper, we wish to describe isolation and structures of SF-1902 A_2, A_3, A_{4a} and A_{4b} as well as structure-activity relationship among them. Characterization of the producing organism and its fermentation have already been reported[3].

Isolation and Purification of Minor Components

The SF-1902 complex was a neutral and solvent soluble material, which could be extracted with ethyl acetate from the fermentation broth and with acetone from the mycelia. SF-1902 A_{4a}, A_{4b} and A_5 (A_{4a}, A_{4b} and A_5) were minute in broth filtrates but substantial amounts ($\sim 8\%$) of these components were found in the organic extract from mycelia.

Isolation of SF-1902 complex was effected by repeated chromatography over silica gel followed by crystallization from aqueous acetonitrile. A crystalline complex thus obtained contained 70% of globomycin (SF-1902 A_1). The minor components were relatively concentrated in the mother liquor, as

Fig. 1. Structures of SF-1902 components.



Component	R	n
A_2	CH_3CH_2	3
A_3	CH_3	5
A_1=globomycin	CH_3CH_2	5
A_{4a}	CH_3CH_2	6
A_{4b}	CH_3	7
A_5	CH_3CH_2	7

* Formerly designated SF-1902.[2]

Chart 1. Isolation and purification of SF-1902 minor components.

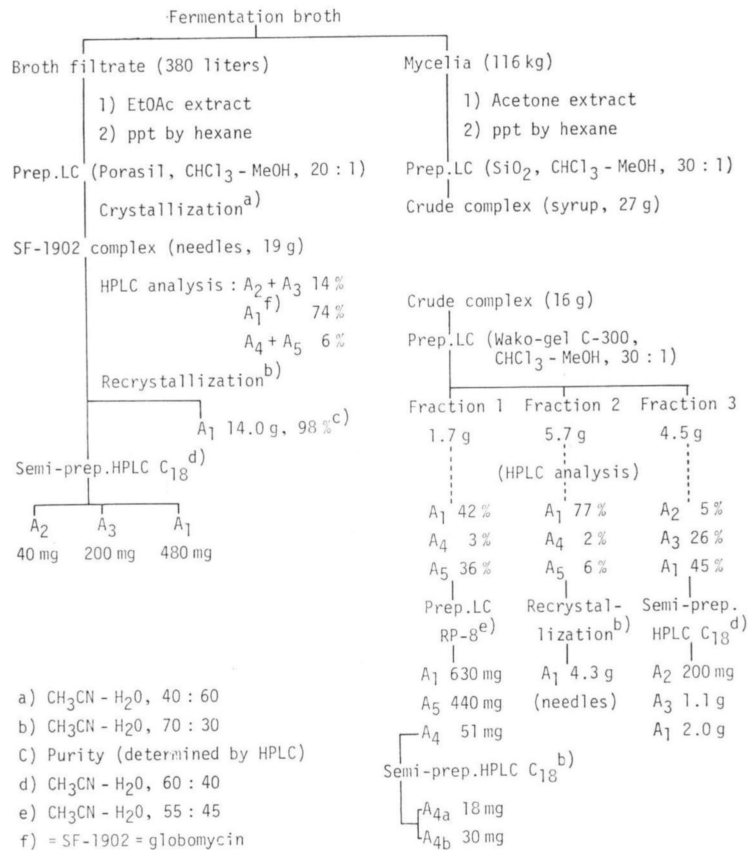


Table 1. Physico-chemical properties of SF-1902 components.

	A_2	A_3	A_1= globomycin	A_{4a}	A_{4b}	A_5
Appearance	Colorless needles					
M.p. (°C)	114~117	115~118	115~117	114~117	115~118	113~117
UV	End absorption					
$[\alpha]_D^{23}$ (*c* 1, $CHCl_3$)[a]	−16°	−16°	−17°	−16°	−16°	−16°
Microanalysis Found: (%)	C, 57.35 H, 8.49 N, 11.01	C, 58.21 H, 8.60 N, 10.78	C, 58.45 H, 8.67 N, 10.51	C, 59.00 H, 8.74 N, 10.26	C, 59.28 H, 8.75 N, 10.35	C, 59.68 H, 8.87 N, 10.12
Molecular formula	$C_{30}H_{53}N_5O_9$	$C_{31}H_{55}N_5O_9$	$C_{32}H_{57}N_5O_9$	$C_{33}H_{59}N_5O_9$	$C_{33}H_{59}N_5O_9$	$C_{34}H_{61}N_5O_9$
Molecular weight	627	641	655	669	669	683
HPLC[b] (minutes)	6.3	7.5	9.0	11.2	11.7	14.8
TLC[c] (Rf value)	0.45					
Color reaction	Positive: Sulfuric acid, LEMIEUX, I_2, GREIG-LEABACK Negative: SAKAGUCHI, ninhydrin					

a) After vacuum drying at 60°C; b) Develosil ODS (4.6 mm×250 mm), CH_3CN-H_2O, 70: 30 (0.7 ml/minute, 15 kg/cm^2); c) Silica gel 60 (E. Merck), $CHCl_3$-MeOH, 5: 1

shown in Chart 1. The crystalline complex seemed to be homogeneous on TLC or normal phase HPLC, but was clearly resolved into multiple peaks in reversed-phase HPLC on ODS-silica gel.

Physico-chemical Properties of Four Minors

Physico-chemical properties of four minor components are listed in Table 1. They showed properties similar to globomycin, but differed from it in the molecular weight by 14 or its multiple. As a representative, SF-1902 A_3 (A_3) was selected, and its IR and PMR spectra are shown in Figs. 2 and 3. CMR chemical shifts of four minors as well as globomycin and A_5 are shown in Table 2. Assignments

Fig. 2. IR Spectrum of SF-1902 A_3 (KBr).

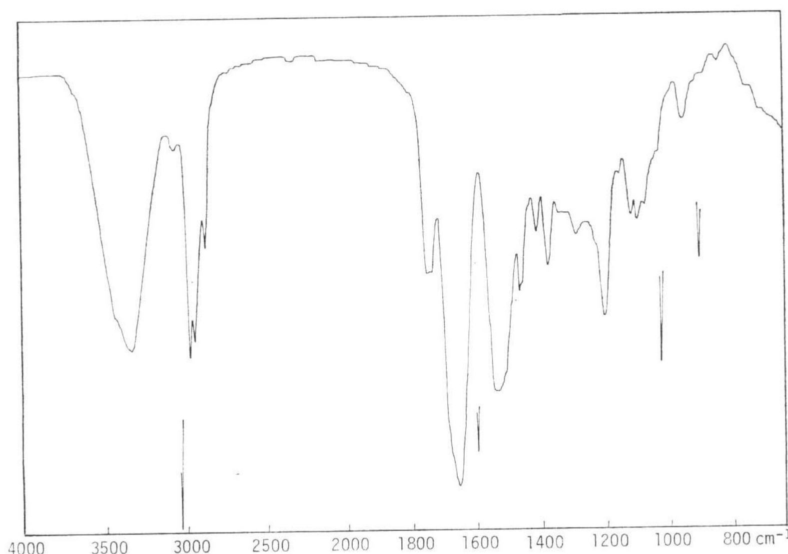


Fig. 3. PMR Spectrum of SF-1902 A_3 (200 MHz, $CDCl_3$).

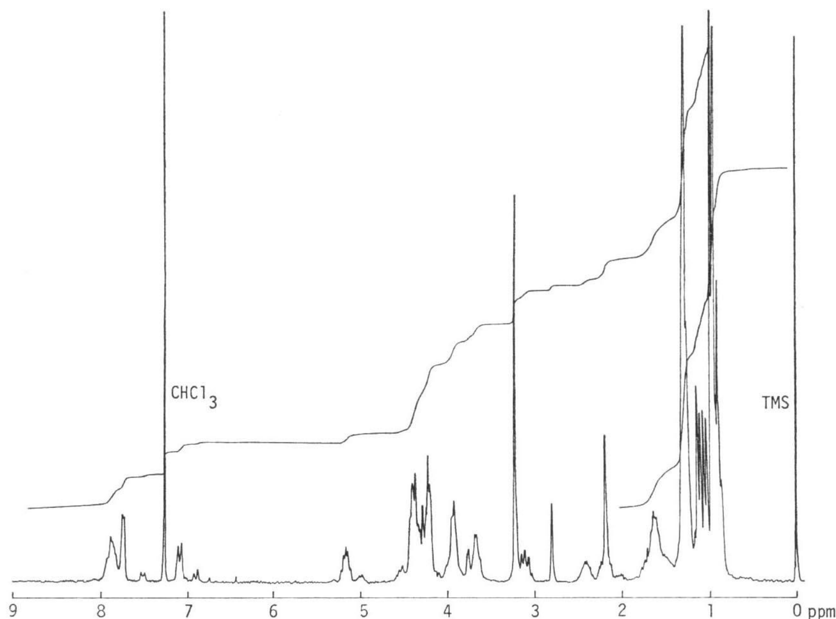


Table 2. ^{13}C Chemical shifts of SF-1902 minor components (ppm).

		A_2	A_3	A_1= globomycin	A_{4a}	A_{4b}	A_5
N-Me-Leu	C_α	68.1	68.2	68.8	68.4	68.1	68.5
	C_β	38.2	38.4	38.4	38.3	38.2	38.3
	C_γ	24.1	24.1	24.1	24.2	24.2	24.2
	$C_{\delta 1}$	23.1	23.1	23.0	23.0	23.1	23.0
	$C_{\delta 2}$	21.9	21.7	21.9	21.9	21.8	21.9
	N-Me	40.0	40.0	40.0	40.3	39.8	40.0
allo-Ileu	C_α	56.6		56.5	56.4		56.6
	C_β	36.7		37.2	37.0		37.0
	$C_{\gamma 1}$	27.3		27.3	27.2		27.3
	$C_{\gamma 2}$	14.6		14.6	14.6		14.6
	C_δ	11.7		11.7	11.7		11.7
Val	C_α		58.1			58.1	
	C_β		30.6			30.5	
	$C_{\gamma 1}$		20.2			20.2	
	$C_{\gamma 2}$		17.1			17.2	
Ser	C_α	58.0	58.4	58.8	58.3	58.5	58.5
	C_β	61.4	61.0	61.2	61.2	61.1	61.3
allo-Thr	C_α	59.1	59.1	59.4	59.1	59.0	59.2
	C_β	66.9	66.5	66.8	66.7	66.8	66.8
	C_γ	18.7	18.1	18.5	18.5	18.5	18.5
Gly	C_α	40.6	40.5	40.4	40.4	40.4	40.5
Fatty acid	C-2	41.4	41.7	41.7	41.5	41.5	41.6
	C-3	76.9	76.5	76.6	76.5	76.5	76.7
	C-4	31.2	31.6	31.6	31.4	31.5	31.5
	C-5	25.3	25.2	25.3	25.2	25.2	25.3
	C-6	22.6	29.2	29.2	29.0	29.5	29.6
	C-7	13.9	31.6	31.6	29.5	29.3	29.4
	C-8		22.6	22.6	31.8	29.2	29.3
	C-9		14.0	14.0	22.6	31.8	31.8
	C-10				14.1	22.6	22.7
	C-11					14.1	14.1
	C-2-Me	15.0	15.0	15.0	15.0	15.0	15.0
C=O		169.0	168.6	168.4	168.4	168.5	168.1
		170.3	169.8	169.8	169.7	169.8	169.4
		171.1	171.6	171.1	170.2	170.9	170.9
		173.3	173.4	173.4	173.2	173.0	173.2
		174.9	174.6	175.0	174.8	174.1	174.8
		177.1	177.0	177.1	176.9	176.1	176.9

were made by conventional methods and PRFT experiments. α-Carbon peaks of amino acids were broader (W1/2$\sim$60 Hz), than those of the other peaks and were relatively temperature independent. However, in methanol-d_4 or in case of diacetate, these carbons showed sharp signals (W1/2$\sim$15Hz), suggesting the conformational isomerism involving hydrogen bonding.

The Structures of the Four Minor Components

Acid hydrolysis of each component liberated five amino acids and one fatty acid. Those are summarized in Table 3. These degradation products were identified by comparison with authentic samples by amino acid analysis, GLC and GC-MS. Configurations of amino acids were determined by optical rotation or by GLC after conversion into *N*-trifluoroacetyl-L-prolyl-peptide methyl ester of *N*-trifluoroacetyl-L-prolyl-*O*-TMS-peptide methyl ester[4,5]. Conventional hydrolysis with HCl gave only racemized *N*-methyl-leucine, but hydrolysis with methanesulfonic acid[6] gave optically active one, though partially racemized. Its L-configuration was determined by optical rotation.

Structures of fatty acids were deduced by NMR and MS analysis and confirmed by chemical synthesis *via* the REFORMATSKY reaction according to BELLARSOUED[7]. Relative configuration of hydroxy fatty acids were considered to be *threo*, because $J_{2,3}$ in R–$\overset{3}{C}$H(OH)–$\overset{2}{C}$H(CH_3)–COOH was 7.3 Hz. CANCEILL *et al.* studied in detail the stereochemical outcome of the REFORMATSKY reaction, and reported that $J_{2,3}^{threo}$ of 3-hydroxy-2-methyl-phenyl-carboxylic acid was 8.6 Hz, while that of *erythro* isomer, 4.7 Hz. Indeed the synthetic mixture showed two $J_{2,3}$ values, 5 Hz and 7 Hz which could be assigned to *erythro* and *threo* respectively. The hydroxy fatty acid from $A_2 \sim A_4$ as well as A_1 and A_5 all showed $J_{2,3}$ of 7.3 Hz corresponding to a *threo* isomer. The amino acid sequence was determined by mass spectrometry of the permethylated open chain derivatives and the corresponding perdeuteromethylated analogues, as shown in Table 4. The site of lactone ring formation was considered to be identical with that in A_1 from the similarity of CMR.

Table 3. Amino acid and fatty acid constituents of SF-1902 minor components.

Components	Fatty acid* n	Amino acid					
		L-MeLeu	L-*allo*-Ileu	L-Val	L-Ser	L-*allo*-Thr	Gly
A_2	3	+	+	−	+	+	+
A_3	5	+	−	+	+	+	+
A_1=globomycin	5	+	+	−	+	+	+
A_{4a}	6	+	+	−	+	+	+
A_{4b}	7	+	−	+	+	+	+
A_5	7	+	+	−	+	+	+

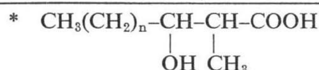

* $CH_3(CH_2)_n$–CH(OH)–CH(CH_3)–COOH

Table 4. Mass spectra of permethylated (perdeuteromethylated) seco-acids of SF-1902 components (*m/z*).

	Acyl-MeLeu	Me-*allo*-Ile (Val)	Me-Ser (OMe)	Me-*allo*-Thr (OMe)	Me-Gly (OMe)
A_2	284 (287)	411 (417)	526 (538)	655 (673)	757 (781)
A_3	312	425	540	669	771
A_1	312 (315)	439 (445)	554 (566)	683 (701)	785 (809)
A_{4a}	326 (329)	453 (459)	568 (580)	697 (715)	799 (823)
A_{4b}	340	453	568	697	799
A_5	340 (343)	467 (473)	582 (594)	711 (729)	813 (837)

These results lead to the structures shown in Fig. 1 for the minor components of SF-1902. Thus, A_2 shared five amino acids in common with A_1, but the fatty acid alkyl chain was shorter than that of A_1 by two methylenes. A_3 shared four amino acids in common with A_1 and *allo*-isoluecine was replaced by valine. The fatty acid in A_3 was identical with that in A_1. A_{4a} shared five amino acids in common with A_1, but the fatty acid contained one more carbon than that of A_1. A_{4b} shared five amino acids in common with A_3, but the fatty acid contained two more carbons than that of A_1.

Biological Properties

Antibacterial spectra of $A_2 \sim A_{4b}$ were compared with those of A_1 and A_5. They were active against Gram-negative bacteria, but not against Gram-positive bacteria. Of particular interest was that the antibacterial activity is quite sensitive to the length of alkyl side chain, either in a fatty acid or in an amino acid, as shown in Table 5. Replacement of valine with *allo*-isoleucine, resulting in single carbon increment, exerted 2 to 4 fold decrease in MIC value. As seen between globomycin (A_1) and A_{4a} or between A_{4a} and A_5, single carbon increase in a fatty acid side chain resulted in also 2 to 4 fold activity enhancement. SF-1902 A_5 which had the longest side chain showed the highest antibacterial activity. A_2, with the shortest side chain showed the weakest activity, though it still exhibited considerable activity against *Salmonella interitidis* and *Shigella flexneri*. Globomycin (SF-1902 A_1) was intermediate in both

Table 5. Antimicrobial spectrum of SF-1902 minor components.

Organisms	MIC (mcg/ml)					
	A_2	A_3	A_1=Globomycin	A_{4a}	A_{4b}	A_5
Staphylococcus aureus 209P JC-1	>100	>100	>100	>100	>100	>100
Staphylococcus aureus Smith	>100	>100	>100	100	100	100
Bacillus anthracis No. 119	>100	>100	>100	100	100	100
Escherichia coli NIHJ JC-2	50	25	6.25	3.13	3.13	1.56
Escherichia coli No. 29	50	25	12.5	3.13	6.25	1.56
Escherichia coli W3630 RGN-823	25	6.25	3.13	1.56	3.13	0.78
Escherichia coli K-12 IAM 1264	50	12.5	6.25	3.13	6.25	1.56
Escherichia coli A-0014	12.5	6.25	3.13	1.56	3.13	0.78
Citrobacter freundii GN 346	100	50	25	12.5	25	6.25
Salmonella typhimurium O-901-W	25	50	12.5	3.13	6.25	3.13
Salmonella paratyphi A	100	50	25	6.25	6.25	3.13
Salmonella enteritidis No. 11	1.56	0.78	0.78	0.39	0.10	0.10
Shigella flexneri 2a	3.13	1.56	0.78	0.39	0.39	0.10
Shigella sonnei EW33 Type 1	12.5	6.25	3.13	1.56	1.56	0.78
Klebsiella pneumoniae PCI-602	>100	50	25	6.25	12.5	1.56
Klebsiella pneumoniae F-0004	6.25	3.13	1.56	0.39	0.78	0.10
Proteus vulgaris OX-19	>100	>100	>100	100	100	100
Proteus rettgeri J-0026	>100	>100	>100	25	25	6.25
Serratia marcescens No. 1	>100	>100	>100	50	50	25
Serratia marcescens MB-3848	>100	>100	>100	100	100	50
Pseudomonas aeruginosa MB-3829	>100	100	50	50	25	12.5
Vibrio cholerae var. *eltor* Inaba #930	25	6.25	3.13	0.78	1.56	0.78
Vibrio cholerae var. *eltor* Ogawa NIH-41	25	6.25	6.25	0.78	1.56	0.39
Yersinia enterocolitica O: 3 (2H20)	>100	25	25	25	25	6.25
Yersinia enterocolitica O: 5 (334)	>100	100	100	100	50	25

the side chain length and activity.

Since acetylation of hydroxyl groups in serine and *allo*-threonine diminished activity, the two hydroxyl groups seem to be essential for antibacterial activity. However, the diphosphate of globomycin in which hydroxyl groups of serine and *allo*-threonine were phosphorylated retained antibacterial activity to some selected bacteria (MIC; 12.5 mcg/ml against *Escherichia coli* W3630 RGN 823, and 25 mcg/ml against *Shigella sonnei* EW 33 Type I). On the other hand mono-phosphate (Ser) showed no antibacterial activity. Open chain compounds (seco-acids) also completely lost bioactivity. These results indicate that the presence of long side chain of lipophilic nature and a cyclic structure with inner hydrophilic hole are essential for the antibacterial activity of globomycin analogues.

INUKAI *et al.* reported that globomycin inhibited the prolipoprotein processing enzyme of Gram-negative bacteria[8]. Since the new analogues of globomycin probably possess the same mechanism of action, these new antibiotics, especially A_5 will be suitable tools in the study of bacterial lipoprotein synthesis in outer membrane.

Intraperitoneal administration of each component at a dose of 180 mg/kg caused no death of mice.

Experimentals

HPLC Analysis

HPLC analysis of SF-1902 minor components was carried out on Nucleosil $5C_{18}$ (4.6 mm × 150 mm), μBondapak C_{18} (4.6 mm × 250 mm) or Develosil ODS-7 (Nomura Kagaku, Osaka) (4.6 mm × 250 mm) developed with 60 ~ 70 % aqueous acetonitrile. Components were detected with a UV detector at 220 nm.

Separation of Four Products

a) Preliminary separation: Crude extract (16 g) was applied to silica gel column (Wako-gel C-300, 40 mm × 250 mm) and developed with a mixture of chloroform - methanol (40: 1 ~ 20: 1) (flow rate: 6 ~ 10 ml/minute, 10 ~ 25 kg/cm²). Biologically active effluents were divided roughly into three fractions after HPLC analysis. Most of A_4 was distributed in the first fraction (1.7 g; $A_{4a}+A_{4b}$: 3%, A_5: 36%, globomycin: 42%). A_2 and A_3 were concentrated in the third fraction (4.5 g; A_2: 5%, A_3: 26%, globomycin: 45%). The second fraction gave crystalline globomycin containing less than 10% of minors. After concentration, followed by addition of acetonitrile, all three fractions afforded crystalline mixture of SF-1902 components. Separation of minor components by fractional crystallization failed.

b) Complete separation: 1) A sample of the first fraction (1.7 g) was chromatographed over LiChroprep RP-8 (25 ~ 40 μ, E. Merck) column (40 mm × 250 mm) and developed with 55% aqueous acetonitrile (4.5 ml/minute; 20 kg/cm²). Pure components except A_{4a} and A_{4b} were obtained by this methods. Separation of A_{4a} from A_{4b} was still incomplete under this condition. A_4 mixture sample (51 mg) was chromatographed over Nucleosil $5C_{18}$ (10 mm × 250 mm) and eluted with 70% aqueous acetonitrile (2.0 ml/minute; 120 kg/cm²) to carry out the final separation. Complete separation of A_{4a} from A_{4b} needed repeated chromatography over the same column.

2) A sample of the third fraction (4.5 g) was chromatographed over Nucleosil $5C_{18}$ (10 mm × 250 mm) and developed with 60% aqueous acetonitrile (1.3 ~ 1.7 ml/minute; 55 ~ 78 kg/cm²), in order to obtain pure samples of A_2 and A_3. Each component was finally purified by recrystallization from 60% aqueous acetonitrile.

Isolation of Optically Active *N*-Methylleucine

A sample of a minor component (300 mg) was hydrolyzed in 4 M methanesulfonic acid (30 ml) containing 0.2% tryptamine at 110°C for 20 hours. After neutralization by aqueous NaOH, the hydrolysate was passed through a small column of Dowex 50W-×2 (H^+), which was washed with water followed by elution with 0.5 N NH_4OH. After concentration, the eluate was subjected to chromatography

on cellulose (70% isopropanol) and then Dowex 50W-×2 (pyridine - formic acid, pH 3.1; 0.1 M) to give a pure sample of *N*-methylleucine (25 mg): $[\alpha]_D^{20}$ +10° (*c* 1.1, 5 N HCl). (Ref. $[\alpha]_D^{15}$ +31.3° (*c* 0.9, 5 N HCl)[9]).

Permethylation of Seco-acid

To a solution of seco-acid of a minor component (3~5 mg) in freshly distilled dry DMSO (2 ml) was added methylsulfinyl carbanion in DMSO (0.5 ml; prepared from NaH; 50%, 200 mg and DMSO; 2 ml) under nitrogen. After 15 minutes at room temperature, 200 µl of CH_3I was carefully added and stirred for 2 hours. After usual work-up, the product was subjected to MS-analysis.

Preparation of Mono- and Di-phosphates

Globomycin (SF-1902 A_1) (2.0 g) was reacted with diphenylphosphorochloridate (2.43 g) in dry pyridine (45 ml) at −20°C for 2 hours. The reaction product, after ethyl acetate extraction and concentration, was immediately subjected to catalytic hydrogenolysis for 2.5 hours. PtO_2 (500 mg) was used in 70% aqueous ethanol (200 ml) containing acetic acid (0.5 ml). After usual work-up, the product was subjected to silica gel column chromatography ($CHCl_3$ - MeOH=10: 1~5: 1) to give diphosphate (530 mg): glass, m.p. 147~159°C, $[\alpha]_D^{22}$ +12° (*c* 1, $CHCl_3$) and monophosphate (220 mg): glass, m.p. 139~142°C, $[\alpha]_D^{20}$ +26° (*c* 1, $CHCl_3$).

In the CMR spectrum of monophosphate, β-C of serine underwent low field shift of 3.4 ppm (J_{COP}= 3.4 Hz) (in CD_3OD - $CDCl_3$). Neutralization with 1 N NaOH gave the respective sodium salts; diphosphate sodium salt: m.p. 140~143°C, $[\alpha]_D^{20}$ +7.3° (*c* 1, H_2O), ^{31}P NMR (in D_2O) −3.71 and −4.65 ppm from external Pi, monophosphate sodium salt: m.p. 125~127°C, $[\alpha]_D^{22}$ +14° (*c* 1, H_2O), ^{31}P NMR (in D_2O) −4.18 ppm from external Pi.

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