Chem. Pharm. Bull. 27(1) 65—75 (1979)

UDC 547.918.04.09 : 615.281.011.5.015.11

Chemical Modification of Aminoglycoside Antibiotics. Some N-Alkyl Derivatives of Sorbistin A_1 (P-2563 P) and Butirosin A

KIYOSHI NARA, KOUICHI YOSHIOKA, and MAKOTO KIDA

Central Research Division, Takeda Chemical Industries, Ltd.[1]

(Received July 1, 1978)

Some N-alkyl derivatives of sorbistin A_1 (P-2563 P) (**1**) and butirosin A (**10**) were prepared by reductive alkylation with aldehyde and $NaBH_4$ or $NaBH_3CN$. Among them, 4'-N-propylsorbistin A_1 (**8b**) showed antimicrobial activity against gram-positive bacteria comparable to sorbistin A_1 (**1**) and showed broader spectrum of activity against gram-negative bacteria including some of the sorbistin A_1-resistant strains (*K. pneumoniae*, *Ps. aeruginosa*) than sorbistin A_1 (**1**). 2'-N-Propylbutirosin A (**14c**) showed antimicrobial activity similar to butirosin A (**10**) and showed weak antimicrobial activity against some of butirosin A-resistant bacteria including *E. coli* JR 66/W 677 which is reported to produce 3'-phosphotransferase II. It was shown that 2'-N-propylbutirosin A (**14c**) was hardly phosphorylated under the condition where butirosin A (**10**) was almost completely phosphorylated with the purified *E. coli* JR 66/W 677 3'-phosphotransferase II.

Keywords——reductive N-alkylation; N-alkylsorbistin A_1; 2'-N-alkylbutirosin A; sorbistin A_1-resistant bacteria; 3'-phosphotransferase II

Chemical modification of aminoglycoside antibiotics has never received so much attention as those of penicillins and cephalosporins. It is not always easy to modify appropriate hydroxyl or amino groups selectively and preparatively enough. But, the advance in biochemical studies of resistance mechanism[2] has prompted the chemical modification of aminoglycoside antibiotics.[3] The development of useful reagent in sugar and peptide chemistry has apparently contributed to the chemical modification of aminoglycoside antibiotics. Thus, a number of N-acyl or deoxy derivatives of aminoglycoside antibiotics have been prepared. However, N-alkyl derivatives of aminoglycoside antibiotics have rarely been reported hitherto. In recent years, 6'-N-methylkanamycin[4] and 1-N-ethylsisomicin[5] have been shown to have excellent activities against resistant strains; these N-alkyl derivatives showed lower toxicity. This prompted us to prepare N-alkyl derivatives of P-2563 (P)[6] (sorbistin A_1)[7] (**1**), and butirosin A[8] (**10**), expecting to obtain new compounds of improved therapeutic properties.

Preparation of N-Alkyl Derivatives

1,4 and 4'-N-Alkylsorbistin A_1 and 2'-N-alkylbutirosin A were prepared by reductive alkylation with aldehyde and sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaBH_3CN$).

1. 1-N-Alkylsorbistin A_1 (2a, 2b, 3a, 3b)——It was expected the 1-amino group in **1** would be more reactive than the 4-amino group in view of the p*K*a values and steric factors. It was previously demonstrated[6] that 1-N-(*p*-methoxybenzyl) derivative of **1** was exclusively

1) Location: *Jusohonmachi, Yodogawa-ku, Osaka 532, Japan.*

2) H. Umezawa, M. Okanishi, R. Utahara, K. Maeda, and S. Kondo, *J. Antibiot.*, **20**, 136 (1967).
3) S. Umezawa, T. Tsuchiya, R. Muto, Y. Nishimura, H. Umezawa, *J. Antibiot.*, **24**, 274 (1971).
4) H. Umezawa, Y. Nishimura, T. Tsuchiya, and S. Umezawa, *J. Antibiot.*, **25**, 743 (1972).
5) J.J. Wright, *J. Chem. Soc. Chem. Commun.*, **1976**, 206.
6) K. Nara, Y. Sumino, K. Katamoto, S. Akiyama, and M. Asai, *Chem. Pharm. Bull.* (Tokyo), **26**, 1075 (1978).
7) H. Tsukiura, M. Hanada, K. Saito, K. Fujisawa, T. Miyata, H. Koshiyama, and H. Kawaguchi, *J. Antibiot.*, **29**, 1137 (1976).
8) P.W.K. Woo, H.W. Dion, and Q.R. Bartz, *Tetrahedron Lett.*, **1971**, 2617.

NII-Electronic Library Service

obtained by reductive alkylation of **1** with *p*-methoxybenzaldehyde by $NaBH_4$. Further, when the proton magnetic resonance (PMR) spectrum of a monoacetate[6] obtained by acetylation of **1** with Ac_2O in water was compared with that of **1**, the C-1 methylene multiplet of the monoacetate was shifted to 3.2 ppm and 3.6 ppm, while the C-1 methylene of **1** was observed at 3.4 ppm, indicating that the acetylation took place at the 1-amino group. On

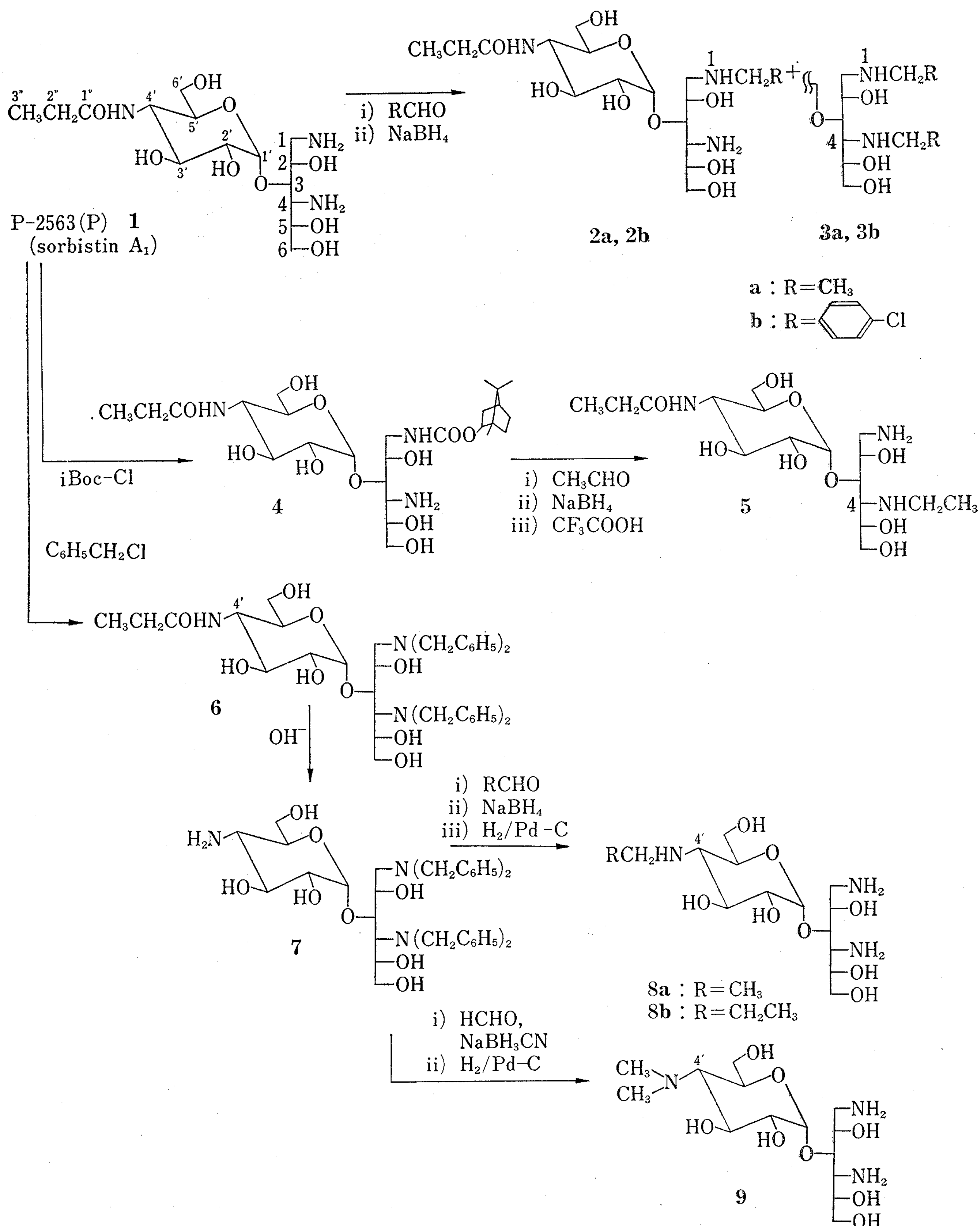


Chart 1

NII-Electronic Library Service

the basis of this observation, 1-N-alkyl derivatives (**2a**, **2b**) were prepared as shown in Chart 1. **1** was condensed with acetaldehyde or *p*-chlorobenzaldehyde and then, the reaction product was reduced with $NaBH_4$ to afford the 1-N-alkyl derivatives (**2a**, **2b**). The 1,4-di-N-alkyl derivatives (**3a**, **3b**) were also obtained as by-products.

2. **4-N-Ethylsorbistin A$_1$ (5)**——4-N-Ethylsorbistin A$_1$ (**5**) was prepared as shown in Chart 1. Acylation of **1** with isobornyloxycarbonyl chloride (iBoc-Cl) gave 1-N-iBoc derivative of **1** (**4**), which was alkylated with CH_3CHO and $NaBH_4$, followed by removal of the iBoc with trifluoroacetic acid (CF_3COOH) to afford **5**.

3. **4′-N-Alkylsorbistin A$_1$ (8a, 8b, 9)**——4′-N-Alkyl derivatives (**8a**, **8b**, **9**) of **1** were prepared according to the route shown in Chart 1. Protection of the two primary amino groups of **1** with benzyl chloride gave the tetrabenzyl derivative (**6**),[9] which was hydrolyzed in 0.5 N NaOH to afford the key, 1,4-di-N-protected intermediate (**7**). **7** was condensed with an excess of CH_3CHO or propionaldehyde and the reaction product was reduced with $NaBH_4$ to give 4′-N-alkyl derivative, which was hydrogenated over Pd–C to remove the benzyl groups to afford the 4′-N-ethyl derivative (**8a**) and the 4′-N-propyl derivative (**8b**).[*,10]

4′-N,N-Dimethyl derivative (**9**) was prepared by the reductive methylation of **7** with 37% aqueous formaldehyde and $NaBH_3CN$,[11] followed by removal of the protecting groups. $NaBH_3CN$ has been recommended for reductive methylation of amines by virtue of the mild conditions, and high yield of pure product.

4. **2′-N-Alkylbutirosin A (14a, 14b, 14c, 14d, 14e, 15)**——Haskell[12] has prepared the 1-N-alkyl and 4‴-N-methylbutirosin A and recognized that the alkylation of the 1-amino group of the deoxystreptamine moiety lowered the antimicrobial activity and 4‴-N-methylbutirosin A destroyed the anti-pseudomonas activity. In the light of these results, we tried the alkylation of the 2′-amino group of the aminosugar moiety of butirosin A (**10**). Attempt to prepare 3,6′,4‴-tri-N-Cbz-butirosin A (**13**), only the 2′-amino group of which is free, by hydrolysis of the 3′-phosphate of 3,6′,4‴-tri-N-Cbz-butirosin A 3′-phosphate (**12**)[13] in hot aqueous acetic acid was unsatisfactory. It was found that a deal of undesired 1-N-(γ-amino-α-hydroxybutyryl)-neamine was produced causing a serious yield drop. Another attempt to prepare **13** was successful; it was found that **13** is obtained directly from butirosin A (**10**). Thus butirosin A (**10**) in a mixture of tetrahydrofuran (THF)–water was allowed to react with 3 molar equivalents of Cbz ester of N-hydroxy-5-norbornen-2,3-dicarboximide (HONB–Cbz)[14] in THF under ice-cooling while keeping the pH at 7 with saturated sodium carbonate to afford **13** as a major product; the product (**13**) was purified by recrystallization from MeOH. The structure of **13** was confirmed by infrared (IR), PMR spectrum and mixed melting point.

2′-N-Alkyl butirosin A (**14a**—**14e**) were prepared by reductive alkylation of **13** with aldehyde by $NaBH_4$ followed by removal of the N-protecting groups. 2′-N,N-Dimethylbutirosin A (**15**) was prepared in a good yield from **13** by reductive methylation with 37% formaldehyde and $NaBH_3CN$ followed by removal of the Cbz (Chart 2). The ^{13}C-nuclear magnetic resonance (NMR) spectrum was compared with that of butirosin A (**10**).[15] Table I shows the chemical shifts of 2′,6′-diaminoglucose moiety in **15** and those of **10**. From Table

9) K. Nara, Y. Sumino, K. Katamoto, S. Akiyama, and M. Asai, *Chem. Pharm. Bull.* (Tokyo), **26**, 1075 (1978).

*) During the preparation of this manuscript, 4′-N-propyl derivative (**8b**) appeared in an article.[11] **8b** has been prepared by reducing the persilylated **1** with lithium aluminum hydride in refluxing dioxane.

10) M.M. Ponpipom, R.L. Bugianesi, and T.Y. Shem, *J. Med. Chem.*, **21**, 221 (1978).
11) R.F. Borch and A.I. Hassid, *J. Org. Chem.*, **37**, 1673 (1972).
12) T.H. Haskell, R. Rodebaugh, N. Plessas, D. Watson, and R.R. Westland, *Carbohydrate Research*, **28**, 263 (1973).
13) K. Yoshioka, S. Yamamoto, H. Mabuchi, and K. Hiraga, *Chem. Pharm. Bull.* (Tokyo), in preparation.
14) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1857 (1974).
15) P.W.K. Woo and R.D. Westland, *Carbohyd. Res.*, **31**, 27 (1973).

NII-Electronic Library Service

I, it is clear that the chemical shift of 2′-C of **15** shows about 10 ppm downfield shift and those of 1′- and 3′-C show upfield shift of about 4 ppm, indicating the N-alkylation took place at the 2′-amino group.

butirosin A **10**

HONB-Cbz (3mol)
pH 7.0
ice-cooling
THF-water

13

i) R′CHO
ii) $NaBH_4$
iii) H_2/Pd-C

14a : $R=CH_3$
14b : $R=CH_2CH_3$
14c : $R=CH_2CH_2CH_3$
14d : $R=CH_2$-C_6H_5
14e : $R=CH_2$-C_6H_4-Cl

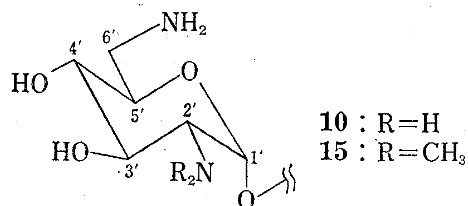
i) 37%HCHO, $NaBH_3CN$, pH 7.0
ii) H_2/Pd-C

15

Chart 2

TABLE I. ^{13}C-NMR Chemical Shifts of 2′, 6′-Diaminoglucose Moiety of **10** and **15**

C-NO	**10** (ppm)	**15** (ppm)
1′	99.7	95.6
2′	56.4	66.3
3′	74.0	70.0
4′	72.2	72.0
5′	73.7 or 75.3	73.4
6′	42.5	42.4



10 : R=H
15 : $R=CH_3$

NII-Electronic Library Service

Results and Discussion

Minimum inhibitory concentrations (MICs) of sorbistin A_1 derivatives prepared in this study were determined against gram-positive and gram-negative bacteria. The 1-N-alkyl and 4-N-alkyl derivatives (**2a, 2b, 3a, 3b, 5**) resulted in a complete loss of activity. The 4′-N-ethyl and dimethyl derivatives (**8a, 9**) showed a marked decrease in the activity. While the 4′-N-propyl derivative (**8b**) showed antimicrobial activity against gram-positive and gram-negative bacteria comparable to the sorbistin A_1 (**1**) (Table II). In order to compare

Table II. Antimicrobial Activities of N-Alkyl Derivatives of Sorbistin A_1 (**1**)

Organism		MIC (μg/ml)			
		Sorbistin A_1(**1**)	**8b**	**8a**	**9**
Gram-positive bacteria					
Staphylococcus aureus	IFO 209P	50	25	100	>100
Staphylococcus aureus	1840	100	50	>100	>100
Staphylococcus aureus	1-F-12-C	50	50		
Staphylococcus aureus	D-30-1	50	50		
Staphylococcus aureus	308A-1	50	50	>100	>100
Staphylococcus epidermidis	IFO 3762	25	12.5		
Staphylococcus epidermidis	IFO 12993	50	25		
Staphylococcus saprophyticus	1-F-15-D	50	6.25		
Bacillus subtilis	PCI 219	25	25	50	>100
Gram-negative bacteria					
Escherichia coli	NIHJ JC-2	100	100	>100	>100
Escherichia coli	TN 659	100	100		
Escherichia coli	O-26	100	100		
Escherichia coli	O-139	100	>100		
Escherichia coli	103	100	>100		
Proteus vulgaris	B-174	100	100		
Proteus mirabilis	IFO 3849	100	100	>100	>100
Proteus mirabilis	B-221	100	50		
Proteus morganii	IFO 3168	100	50	>100	>100
Salmonella typhimurium	6466	50	100		
Salmonella typhimurium	10	50	100		
Salmonella typhimurium	1	50	>100		
Salmonella enteritidis	414	50	100		
Salmonella pullorum	1064	50	100		
Klebsiella pneumoniae	DT	12.5	25	50	100
Klebsiella pneumoniae	B 175	50	100		
Klebsiella pneumoniae	B 207	>100	50		
Serratia marcescens	IFO 12648	>100	100	>100	>100
Serratia marcescens	B 205	>100	>100		
Serratia liquefaciens	B-187	>100	>100		
Enterobacter cloacae	B-176	>100	100		
Enterobacter cloacae	B 214	>100	100		
Pseudomonas aeruginosa	Kanagawa	100	50		
Acinetobacter anitratus	TN 1140	100	50	>100	>100
Citrobacter freundii	TN 518	>100	100		

the antimicrobial activity of 4′-N-propylsorbistin A_1 (**8b**) with sorbistin A_1 (**1**) against gram-negative bacteria of clinical isolates, susceptibility of 100 strains of *Klebsiella pneumoniae* and 98 strains of *Pseudomonas aeruginosa* to sorbistin A_1 (**1**) and 4′-N-propylsorbistin A_1 (**8b**) were tested. The susceptibility patterns of these strains are shown in Fig. 1. The 4′-N-propylsorbistin A_1 (**8b**) was almost the same activity against *K. pneumoniae* as sorbistin A_1 (**1**),

NII-Electronic Library Service

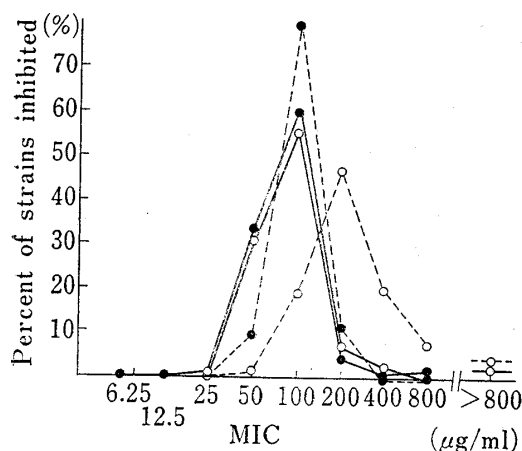


Fig. 1. Sensitivity Distribution of Clinical Isolates

Sorbistin A_1 (1)
—●—: *Klebsiella pneumoniae* (100 strains).
—○—: *Pseudomonas aeruginosa* (98 strains).
4'-N-Propylsorbistin A_1 (8b)
---●---: *Klebsiella pneumoniae* (100 strains)
---●---: *Pseudomonas aeruginosa* (98 strains).

but was approximately two-folded less susceptible to *Ps. aeruginosa* than sorbistin A_1 (**1**). However, the antimicrobial activity of 4'-N-propylsorbistin A_1 (**8b**) against sorbistin A_1-resistant strains of *K. neumoniae* which were not inhibited at concentrations higher than 400 μg/ml of sorbistin A_1 (**1**), was found more potent than that of sorbistin A_1 (**1**) (Table III).

Sorbistin A_1-inactivation by ATP with cell free extracts prepared from these resistant strains was examined. The remaining sorbistin A_1 was determined by microbioassay and thin-layer chromatography (TLC) method. Sorbistin A_1-inactivation was not observed with any of the 4 resistant strains (*K. pneumoniae* GN 7555, GN 7558; *Ps. aeruginosa* GN 8551, GN 8786). The result indicated aminoglycoside-modifying enzymes such as phosphorylating, adenylating and acetylating enzymes were not involved here.

Table III. Antimicrobial Activities of 4'-N-Propylsorbistin A_1 (**8b**) against Resistant Organisms

Organism		MIC (μg/ml) Sorbistin A_1 (**1**)	4'-N-Propylsorbistin A_1 (**8b**)
Klebsiella pneumoniae	GN 7555	800	100
Klebsiella pneumoniae	GN 7558	400	100
Pseudomonas aeruginosa	GN 8551	>800	400
Pseudomonas aeruginosa	GN 8786	>800	>800

MICs of 2'-N-alkylbutirosin A (**14a—14e, 15**) were determined against 26 gram-positive and gram-negative bacteria. The 2'-N-benzyl (**14d**), *p*-chlorobenzyl (**14e**) and dimethylbutirosin A (**15**) lost most of the activity. The 2'-N-methyl (**14a**), ethyl (**14b**), and propylbutirosin A (**14c**) showed activity comparable to butirosin A (**10**) (Table IV). Table V shows the activity of **14a, 14b, 14c** against butirosin A-resistant bacteria. It was found that the 2'-N-propylbutirosin A (**14c**) was also weakly active against *E. coli* JR 66/W 677 which is reported to produce 3'-phosphotransferase II,[16] but was inactive against *Ps. aeruginosa* GN 315, 6'-N-acetyltransferase producer,[17] and *B. brevis* IFO 12334, 4'-nucleotidyltransferase producer.[18] The 2'-N-propyl group of **14c** might have affected the phosphorylation of the 3'-hydroxyl group by the 3'-phosphotransferase II. In order to confirm this, the rate of phosphorylation was examined using the purified enzyme prepared from *E. coli* JR 66/W 677 cells. As is shown in Fig. 2, 2'-N-propylbutirosin A (**14c**) was hardly phosphorylated under the condition where butirosin A (**10**) was almost completely phosphorylated in about 2 hr. This decrease in the rate of the phosphorylation is ascribable to the presence of

16) M. Yagisawa, H. Yamamoto, H. Naganawa, S. Kondo, T. Takeuchi, and Y.A. Chabbert, *J. Antibiot.*, **26**, 407 (1973).
17) M. Yagisawa, S. Kondo, T. Takeuchi, and H. Umezawa, *J. Antibiot.*, **28**, 486 (1975).
18) H. Shirafuji, M. Kida, and M. Yoneda, The Annual Meeting of the Agricultural Chemical Society of Japan, Kyoto, April, 1976, p. 261.

NII-Electronic Library Service

Table IV. Antimicrobial Activities of 2′-N-Alkylbutirosin A

Organism		MIC (μg/ml)			
		Butirosin A	**14a**	**14b**	**14c**
Staphylococcus aureus	FDA 209P	1.56	6.25	6.25	12.5
Escherichia coli	NIHJ JC 2	6.25	12.5	12.5	12.5
Escherichia coli	O-111	3.13	12.5	6.25	6.25
Escherichia coli	ML 1410 R5	25	25	100	100
Klebsiella pneumoniae	DT	0.78	1.56	0.78	1.56
Klebsiella pneumoniae	GN 3853	3.13	6.25	6.25	6.25
Klebsiella pneumoniae	TN 802	25	100	100	>100
Pseudomonas aeruginosa	IFO 3080	3.13	12.5	25	25
Pseudomonas aeruginosa	TI-13	6.25	25	12.5	50
Pseudomonas aeruginosa	GN 3347	25	50	50	>100
Proteus vulgaris	GN 4413	50	50	12.5	25
Proteus morganii	IFO 3168	12.5	50	50	100
Proteus morganii	GN 4381	100	100	50	>100
Proteus rettgeri	GN 4425	12.5	12.5	12.5	25
Proteus rettgeri	GN 4424	50	25	12.5	50
Proteus rettgeri	GN 4427	100	100	50	100
Proteus inconstans	TN 800	100	12.5	3.13	25
Serratia marcescens	IFO 12648	50	50	25	50
Serratia marcescens	TN 24	50	50	25	50
Citrobacter freundii	GN 99	25	100	25	100
Citrobacter freundii	GN 726	100	50	12.5	25
Enterobacter cloacae	TN 581	3.13	6.25	6.25	6.25
Enterobacter cloacae	TN 594	12.5	25	12.5	25
Salmonella typhosa	58	3.13	6.25	3.13	6.25
Shigella flexneri	EW 10	6.25	12.5	12.5	12.5
Shigella sonnei	EW 33	6.25	25	12.5	25

Table V. Antimicrobial Activities of 2′-N-Alkylbutirosin A against Resistant Organisms

Organism		MIC (μg/ml)			
		Butirosin A	**14a**	**14b**	**14c**
Escherichia coli	JR 66/W 677	>100	>100	>100	100
Escherichia coli	GN 3464	>100	>100	100	50
Pseudomonas aeruginosa	GN 315	>100	>100	>100	>100
Bacillus brevis	IFO 12334	>100	>100	>100	>100
Proteus morganii	GN 4392	>100	100	50	100
Proteus rettgeri	TN 798	>100	>100	100	>100

2′-N-propyl group which should prevent the phosphorylation of the 3′-hydroxyl group by the 3′-phosphotransferase II.

In summary, the authors have prepared some N-alkyl derivatives of sorbistin A_1 (p-2563 P) (**1**) and those of butirosin A (**10**) by reductive alkylation with aldehyde and $NaBH_4$ or $NaBH_3CN$. The N-alkylation of the amino group, particularly 4′-amino group in sorbistin A_1 (**1**) and 2′-amino group in butirosin A (**10**) led to some improved antimicrobial activity. In case of the N-alkyl derivatives of sorbistin A_1, it was found that 4′-N-propylsorbistin A_1 (**8b**) showed antimicrobial activity against gram-positive and gram-negative bacteria comparable to sorbistin A_1 (**1**), and also showed antimicrobial activity against some of the sorbistin A_1-resistant bacteria found in the clinical isolates. In case of the 2′-N-alkyl derivatives of

NII-Electronic Library Service

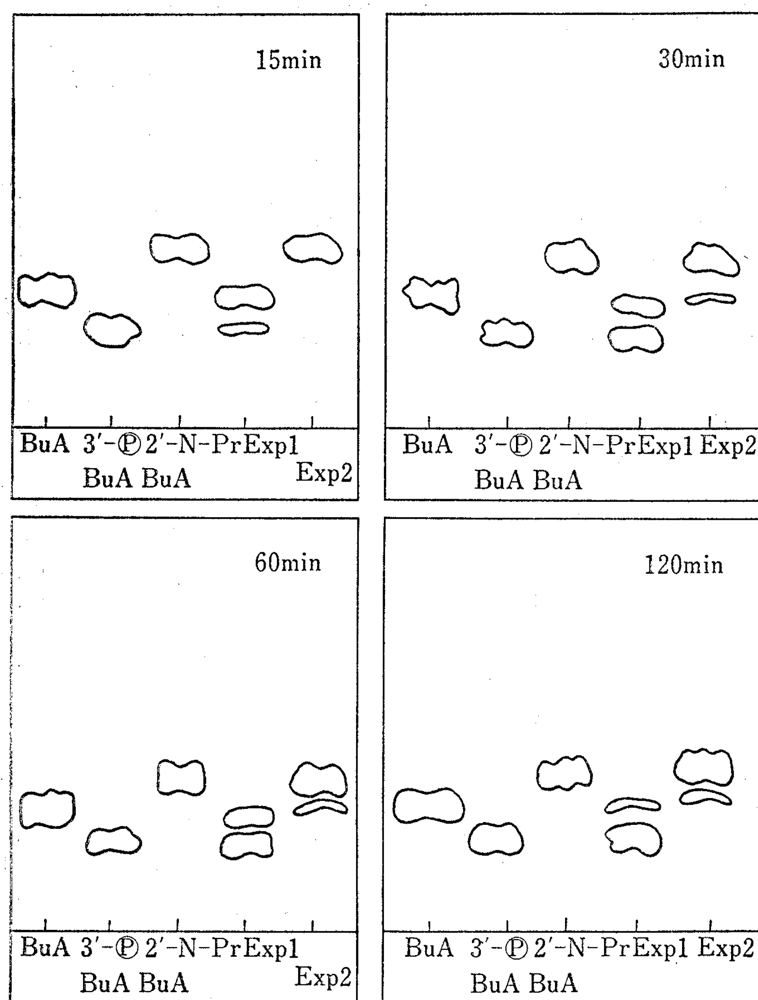


Fig. 2. TLC Patterns showing the Rate of Phosphorylation of Butirosin A (10) and 2'-N-Propylbutirosin A (14c) by 3'-Phosphotransferase II Prepared from *E. coli* JR 66/W 677

Exp. 1. substrate; butirosin A (10).
Exp. 2. substrate; 2'-N-propylbutirosin A (14c).
TLC; silica gel, NH_4Cl (2 mol)–MeOH–NH_4OH=2: 3: 1, detection; ninhydrin.
Abbreviation; BuA: butirosin A, 3'-Ⓟ BuA: butirosin A 3'-phosphate, 2'-N-Pr BuA: 2'-N-propylbutirosin A.

butirosin A (**10**), 2'-N-propylbutirosin A (**14c**) showed antimicrobial activity similar to butirosin A and showed weak antimicrobial activity against some of butirosin A-resistant bacteria including *E. coli* JR 66/W 677 which is reported to produce 3'-phosphotransferase II. We have shown that 2'-N-propylbutirosin A (**14c**) was hardly phosphorylated with the purified *E. coli* JR 66/W 677 3'-phosphotransferase II. This trial involving the N-alkylation of the amino group close to the hydroxyl groups or amino groups susceptible to the aminoglycoside-modifying enzymes presents a new approach to chemical modifications of aminoglycoside antibiotics.

Experimental

Melting points were determined by Yanagimoto's microscope hot stage and uncorrected. ^{13}C-NMR spectra were determined by Varian XL-100 at 25.2 MHz. The samples were examined as 10% solution containing about 2% (v/v) of 1,4-dioxane as an internal reference. The ^{13}C-shifts obtained were converted to the tetramethylsilane (TMS) scale. MIC was determined by the agar dilution method. One loopful of bacterial suspension containing about 10^7 cells per ml was inoculated on a modified Mueller–Hinton agar

NII-Electronic Library Service

(Table II) or Trypticase soy agar (BBL) (Tables III, IV, and V) containing twofold serial dilution of each antibiotic.

1-N-Ethylsorbistin A_1 (2a) and 1,4-Di-N-ethylsorbistin A_1 (3a)——A mixture of **1** (1.0 g) in 80% CH_3CHO (2 ml), water (20 ml) MeOH (10 ml) and 1 N NaOH (2 ml) was allowed to stand at room temperature for 50 min. The solvent was evaporated *in vacuo* to dryness. To the residue was added 80% CH_3CHO (2 ml) and MeOH (10 ml); the solution was warmed at 50° for 5 min and then cooled. $NaBH_4$ (0.4 g) was added in small amounts to the solution. The reaction mixture was allowed to stand overnight at room temperature and the solvent was evaporated to dryness. The residue was dissolved in water, and the solution was passed through an Amberlite CG-50 (NH_4) column (30 ml). The column was eluted with 0.04 N NH_4OH (100 ml). The eluate was concentrated to a sirup and the sirup was lyophilized to give 1-N-ethyl derivative (**2a**, 125 mg). *Anal.* Calcd. for $C_{17}H_{35}N_3O_9 \cdot 3/2H_2O$: C, 45.01; H, 7.62; N, 8.30. Found: C, 45.12; H, 7.91; N, 8.57.

Then, the column was eluted with 0.05 N NH_4OH (50 ml). The eluate was evaporated to a sirup and the residual sirup was lyophilized to give 1,4-di-N-ethyl derivative (**3a**, 67 mg). *Anal.* Calcd. for $C_{19}H_{39}N_3O_9 \cdot 1/2H_2O$: C, 49.33; H, 8.72; N, 9.08. Found: C, 49.10; H, 8.52; N, 8.95.

1-N-*p*-Chlorobenzylsorbistin A_1 (2b) and 1,4-Di-N-*p*-chlorobenzylsorbistin A_1 (3b)——A mixture of **1** (0.5 g), *p*-chlorobenzaldehyde (0.2 g), water (10 ml), MeOH (7 ml) and 1 N NaOH (1 ml) was stirred for 30 min at room temperature and $NaBH_4$ (0.2 g) was added in small portions to the reaction mixture, which was allowed to stand overnight. The solvent was evaporated to dryness, and the aqueous solution (10 ml) of the residue was passed through an Amberlite CG-50 (NH_4) column (30 ml). The column was eluted with 0.015 N NH_4OH (200 ml) and the eluate was lyophilized to give **2b** (324 mg). *Anal.* Calcd. for $C_{22}H_{36}ClN_3O_9 \cdot 1/2H_2O$: C, 49.76; H, 7.02; Cl, 6.68; N, 7.91. Found: C, 49.78; H, 6.70; Cl, 6.70; N, 7.70.

The column was then eluted with 0.02 N NH_4OH (100 ml) and the eluate was concentrated and the residual sirup was lyophilized to give **3b** (200 mg). *Anal.* Calcd. for $C_{29}H_{41}Cl_2N_3O_9 \cdot H_2O$: C, 52.41; H, 6.52; Cl, 10.67; N, 6.32. Found: C, 52.68; H, 6.27; Cl, 10.49; N, 6.29.

1-N-Isobornyloxycarbonylsorbistin A_1 (4)——To a mixture of **1** (4.2 g), MeOH (60 ml), water (20 ml) and Et_3N (3 g) was added iBoc-Cl (2.8 g) in MeOH (20 ml) in 5 min. The reaction mixture was stirred at room temperature for 3 hr; then was added with iBoc-Cl (0.22 g). After stirring for 1 hr, the solvent was evaporated and the aqueous solution of the residue was extracted with EtOAc. The aqueous layer was passed through an Amberlite CG-50 (NH_4) column (400 ml) and the column was eluted with 0.05 N NH_4OH (600 ml). The eluate was concentrated and the residual sirup was lyophilized to give **4** (2.1 g). *Anal.* Calcd. for $C_{26}H_{47}N_3O_{11} \cdot 2H_2O$: C, 50.88; H, 8.38; N, 6.85. Found: C, 50.66; H, 7.97; N, 6.62.

4-N-Ethylsorbistin A_1 (5)——A mixture of **4** (500 mg) in 80% CH_3CHO (2 ml), water (20 ml), MeOH (10 ml) and 1 N NaOH (2 ml) was allowed to stand at room temperature for 50 min. The solvent was evaporated *in vacuo* to dryness. The residue was dissolved in a mixture of 80% CH_3CHO (2 ml) and MeOH (10 ml) and the solution was warmed at 50° for 5 min, then cooled to room temperature. $NaBH_4$ (0.4 g) was added in small portions to the solution. The reaction mixture was allowed to stand overnight at room temperature, and poured into water. The mixture was extracted with EtOAc. The EtOAc layer was mixed with aqueous HCl and the aqueous layer was neutralized and re-extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was dissolved in CF_3COOH (6 ml) and the solution was allowed to stand for 30 min at room temperature. The solution was diluted with ether and the resulting precipitate was collected. The precipitate was dissolved in water (50 ml) and the solution was passed through an Amberlite CG-50 (NH_4) column (30 ml). The column was eluted with 0.1 N NH_4OH (50 ml) and the eluate was concentrated and the sirup was lyophilized to afford **5** (95 mg). *Anal.* Calcd. for $C_{17}H_{35}N_3O_9 \cdot 3/2H_2O$: C, 45.01; H, 7.62; N, 8.30. Found: C, 45.20; H, 7.75; N, 8.20.

1,4-N,N,N′,N′-Tetrabenzyl-4′-aminosorbistin A_1 (7)——To a stirred mixture of **1** (20 g), $NaHCO_3$ (250 g) and water (500 ml) was added dropwise benzylchloride (150 ml) in EtOH (500 ml) at room temperature during 30 min. After refluxing for 6 hr, the solvent was evaporated *in vacuo*. The residue was extracted with EtOAc. The EtOAc solution was mixed with 1 N HCl. The aqueous layer was concentrated *in vacuo* and crystals precipitated were collected and recrystallized from dilute aqueous HCl to afford dihydrochloride of **6** (23.5 g) as colorless crystals, mp 157—158°. *Anal.* Calcd. for $C_{43}H_{55}N_3O_9 \cdot 2HCl \cdot 4H_2O$: C, 57.21; H, 7.21; Cl, 7.87; N, 4.66. Found: C, 56.77; H, 6.80; Cl, 7.99; N, 4.70.

A mixture of **6** (20 g), KOH (50 g), MeOH (500 ml) and water (500 ml) was refluxed for 3 hr. After the solvent were evaporated *in vacuo*, the residue was extracted with EtOAc. The EtOAc solution was dried over Na_2SO_4 and the solvent was evaporated to give **7** (13 g). *Anal.* Calcd. for $C_{40}H_{51}N_3O_8 \cdot 1/2H_2O$: C, 67.58; H, 7.23; N, 5.91. Found: C, 67.40; H, 7.36; N, 5.75.

4′-N-Ethylsorbistin A_1 (8a)——A mixture of **7** (1.0 g), 80% CH_3CHO (2 ml), 1 N NaOH (2 ml) and MeOH (10 ml) was stirred for 1 hr at room temperature. The solvent was evaporated *in vacuo* and to the residue was added a mixture of 80% CH_3CHO (2 ml) and MeOH (10 ml). The solution was stirred for 1 hr at room temperature. After this procedure was repeated 5 times, the residue was dissolved in MeOH (20 ml). To the solution was added $NaBH_4$ (500 mg) with stirring at room temperature. The reaction mixture was allowed to stand overnight, diluted with water, and extracted with EtOAc. The EtOAc layer was mixed with dilute aqueous HCl and the acidic aqueous layer was neutralized with aqueous NaOH and re-extracted with EtOAc. The EtOAc layer was washed with water, dried over Na_2SO_4 and the solvent was evaporated.

NII-Electronic Library Service

The residue was dissolved in 70% MeOH and the pH of the solution was adjusted to 3—4; then the solution was hydrogenated over 5% Pd/C (100 mg) at room temperature under atmospheric pressure for 3 hr. The mixture was filtered and the filtrate was evaporated *in vacuo*. The aqueous solution (10 ml) of the residue was passed through an Amberlite CG-50 (NH_4) column (30 ml). The column was eluted with 0.15 N NH_4OH (75 ml) and the eluate was concentrated to a sirup; the sirup was lyophilized to give 4′-N-ethyl derivative of **1** (**8a**, 80 mg). *Anal.* Calcd. for $C_{14}H_{31}N_3O_8 \cdot H_2O$: C, 43.40; H, 8.58; N, 10.85. Found: C, 43.59; H, 8.27; N, 10.90.

4′-N-Propylsorbistin A_1 (8b)——A mixture of **7** (1.0 g), propionaldehyde (2 ml), 1 N NaOH (2 ml) and MeOH (10 ml) was worked-up as described above to afford **8b** (170 mg). *Anal.* Calcd. for $C_{15}H_{33}N_3O_8 \cdot H_2O$: C, 44.88; H, 8.79; N, 10.47. Found: C, 44.85; H, 9.01; N, 10.55.

4′-N,N-Dimethylsorbistin A_1 (9)——To a mixture of **7** (1.0 g), 37% HCHO (2 ml), CH_3CN (10 ml) and tetrahydrofuran (THF) (4 ml) was added in small portions $NaBH_3CN$ (0.2 g) with stirring at room temperature. After stirring for 3 hr, the mixture was neutralized with AcOH and the solvent was evaporated. The residue was dissolved in water and extracted with EtOAc. The EtOAc layer was washed with 1 N NaOH. The EtOAc solution was mixed with dilute aqueous HCl and acidic aqueous layer was neutralized with 1 N NaOH and re-extracted with EtOAc. The solvent was evaporated and the residue was dissolved in 50% MeOH (30 ml). The pH of the solution was adjusted to 3—4; then the solution was hydrogenated over 5% Pd-C (200 mg) at 50° for 3 hr. The mixture was filtered and the filtrate was evaporated *in vacuo*. The aqueous solution of the residue was passed through an Amberlite CG-50 (NH_4) column (20 ml). The column was eluted with 0.1 N NH_4OH (50 ml) and the eluate was concentrated to a sirup, which was lyophilized to give **9** (240 mg). *Anal.* Calcd. for $C_{14}H_{31}N_3O_8 \cdot H_2O$: C, 43.40; H, 8.58; N, 10.85. Found: C, 43.12; H, 8.39; N, 10.72.

3,6′,4‴-Tri-N-Cbz-butirosin A (13)——i) Synthesis from 3,6′,4‴-Tri-N-Cbz-butirosin A 3′-phosphate[19] (12): A mixture of 3,6′,4‴-tri-N-Cbz-butirosin A 3′-phosphate (1.0 g), water (50 ml), BuOH (50 ml) and AcOH (0.5 ml) was kept at 90° for 7 hr and then at 100° for 15 hr. To the reaction mixture was added water and BuOH, and the pH of the mixture was adjusted to 2—3 with dilute aqueous HCl. The BuOH layer was washed with water and the solvent was evaporated. To the residue was added MeOH and the solution was heated and the insoluble material was filtered off. The filtrate was concentrated to about 1 ml and the concentrate was kept standing in a refrigerator to deposit white prisms of **13** (350 mg). mp 205—207°. *Anal.* Calcd. for $C_{45}H_{59}N_5O_{18}$: C, 56.42; H, 6.21; N, 7.31. Found: C, 56.22; H, 6.04; N, 7.20.

ii) Synthesis from Butirosin A (**10**): To a stirred mixture of **10** (10 g), water (200 ml) and THF (100 ml) was added HONB-Cbz (16.0 g) in THF (100 ml) under ice-cooling during 10 min while keeping the pH at 7 with saturated sodium carbonate. After 2 hr, the solvent was evaporated and the aqueous solution (200 ml) of the residue was adjusted to pH 9—10 with 0.1 N NaOH, and extracted with BuOH (500 ml). The BuOH layer was washed with water and the solvent was evaporated. The crystallization from MeOH (15 ml) gave white crystals of **13** (6.8 g). mp 205—207°. This product was identified with that obtained by the procedure of i) by the comparison of the IR and PMR spectra and mixed mp.

General Procedure for Preparing 2′-N-Substituted Butirosin A (14a—14e)——To a mixture of 13 (500 mg), MeOH (20 ml), BuOH (20 ml) and 1 N NaOH (2 ml) was added an aldehyde (37% HCHO, 80% CH_3CHO, others, 100%) (1 ml) and the mixture was evaporated at 5°, then, to the residue was added MeOH (20 ml) and aldehyde (1 ml) and, again, the mixture was evaporated at 50°. After the same procedure was repeated three times, MeOH (50 ml) and $NaBH_4$ (400 mg) were added to the residue and the mixture was allowed to stand overnight. The reaction mixture was evaporated and the residue was dissolved in water (100 ml). The solution was adjusted to pH 3 with 0.1 N HCl and the impurities were extracted with EtOAc. The aqueous layer was extracted with BuOH and the solvent was evaporated. To the residue was added MeOH (30 ml), water (20 ml) and 5% Pd-C (200 mg) and the mixture was adjusted to pH 3, and stirred for 3 hr under hydrogen atmosphere at room temperature. After the reaction, the catalyst was filtered off and the filtrate was evaporated. The aqueous solution (10 ml) of the residue was adjusted to pH 5.5—6.0, and the solution was passed through an Amberlite CG-50 (NH_4) column (20 ml). The column was eluted with 0.3 N NH_4OH (50 ml) and the eluate was concentrated *in vacuo* to a sirup, which was lyophilized to give a desired product.

2′-N-Methylbutirosin A (**14a**) (19 mg). *Anal.* Calcd. for $C_{22}H_{43}N_5O_{12} \cdot H_2CO_3 \cdot 1/2H_2O$: C, 43.12; H, 7.24; N, 10.93. Found: C, 42.95; H, 7.48; N, 10.82.

2′-N-Ethylbutirosin A (**14b**) (83 mg). *Anal.* Calcd. for $C_{23}H_{45}N_5O_{12} \cdot H_2CO_3 \cdot 1/2H_2O$: C, 44.03; H, 7.39; N, 10.70. Found: C, 43.88; H, 7.13; N, 10.61.

2′-N-Propylbutirosin A (**14c**) (21 mg). *Anal.* Calcd. for $C_{24}H_{47}N_5O_{12} \cdot H_2CO_3 \cdot H_2O$: C, 44.31; H, 7.59; N, 10.33. Found: C, 44.51; H, 7.11; N, 10.10.

2′-N-Benzylbutirosin A (**14d**) (47 mg). *Anal.* Calcd. for $C_{28}H_{47}N_5O_{12} \cdot H_2CO_3 \cdot 3H_2O$: C, 45.72; H, 7.28; N, 9.19. Found: C, 45.67; H, 6.43; N, 9.11.

---

19) K. Yoshioka, S. Yamamoto, H. Mabuchi, and K. Hiraga, *Chem. Pharm. Bull.* (Tokyo), in preparation.

NII-Electronic Library Service

2′-N-(*p*-Chloro)benzylbutirosin A (**14e**) (72 mg). *Anal.* Calcd. for $C_{28}H_{46}ClN_5O_{12} \cdot H_2CO_3 \cdot H_2O$: C, 45.82; H, 6.63; Cl, 4.66; N, 9.21. Found: C, 45.80; H, 6.45; Cl, 4.41; N, 9.10.

2′-N,N-Dimethylbutirosin A (15)——To a mixture of **13** (500 mg), 37% HCHO (1 ml), acetonitrile (20 ml) and THF (5 ml) was added $NaBH_3CN$ (50 mg) while keeping the solution neutral with AcOH and the solution was stirred for 2 hr. The reaction mixture was evaporated and to the residue was added 1 N NaOH and BuOH. The BuOH layer was evaporated and to the residue was added MeOH, water and 5% Pd-C. The mixture was adjusted to pH 3, and stirred for 4 hr at room temperature under hydrogen atmosphere. The catalyst was filtered off and the filtrate was evaporated and the aqueous solution (10 ml) of the residue was adjusted to pH 5.5—6.0. The solution was passed through an Amberlite CG-50 (NH_4) (20 ml) column. The column was eluted with 0.3 N NH_4OH (500 ml). The eluate was concentrated to a sirup, which was lyophilized to give **15** (107 mg). *Anal.* Calcd. for $C_{23}H_{45}N_5O_{12} \cdot H_2CO_3 \cdot H_2O$: C, 43.43; H, 7.44; N, 10.55. Found: C, 43.55; H, 6.93; N, 10.12.

Preparation of 3′-Phosphotransferase II from *E. coli* JR 66/W 67——*E. coli* JR 66/W 677 was grown in 40 ml of a medium consisting of meat extract 0.5%, polypepton 0.5%, yeast extract 0.5%, glycerol 0.5% (pH 7.2) in a 200 ml Erlenmeyer flask at 37° for 24 hr on a rotary shaker. Forty ml of the culture fluid was transferred into 360 ml of the same medium in a 1 l Erlenmeyer flask. The cultivation was carried out at 37° for 6 hr on the rotary shaker. Cells were harvested by centrifugation at $8000 \times \boldsymbol{g}$ for 10 min and washed twice with 0.02 M phosphate buffer (pH 7.0). The washed cells were suspended in 0.02 M phosphate buffer (pH 7.0), and treated with a Kubota Model 200 M sonic oscillator (9kHz) under ice-cooling for 10 min. The broken cell suspension was centrifuged at $10000 \times \boldsymbol{g}$ for 20 min, and the supernatant solution was further purified by ammonium sulfate fractionation, affinity chromatography on butirosin A-Sepharose 4B and gel filtration on Sephadex G-100.[20] Ten ml of the purified enzyme (44 U/ml, specific activity; 114 U/mg protein) were obtained in 10% yield.

Phosphorylation of Butirosin A (10) and 2′-N-Propylbutirosin A (14c)——To an aqueous solution containing 50 μmol of potassium phosphate buffer (pH 7.0), 40 μmol of ATP, 20 μmol of magnesium acetate, and 0.1 ml of the purified enzyme in a total volume of 1 ml, butirosin A (**10**) (10 mg) or 2′-N-propylbutirosin A (**14c**) (10 mg) was added. The reaction mixture was incubated at 37°. Aliquots were heated at 80° for 10 min. The reaction products were detected by TLC [silica gel plate (Merck); Upper phase ($CHCl_3$–MeOH–NH_4OH–water=4:3:2:1)–MeOH=5:3; detected by ninhydrin]. The phosphorylation patterns are shown as TLC chromatograms in Fig. 2.

Sorbistin A_1-Inactivation Experiment by Cell Free Extracts from Sorbistin A_1-Resistant Strains——*K. pneumoniae* GN 7555, *K. pneumoniae* GN 7558, *Ps. aeruginosa* GN 8551, and *Ps. aeruginosa* GN 8786 were grown in 250 ml of a medium consisting of Trypticase Soy Broth (BBL) in a 1 l Erlenmeyer flask at 37° for 16 hr on the rotary shaker. Cells were harvested by centrifugation at $8000 \times \boldsymbol{g}$ for 10 min and washed twice with 0.05 M phosphate buffer (pH 7.0). The washed cells were sonicated with Kubota Model 200 M sonic oscillator (9 KHz) (wet weight 1 g/3 ml 0.05 M phosphate buffer). The broken cell suspension was centrifuged at $10000 \times \boldsymbol{g}$ for 20 min. The supernatant thus obtained was used for the experiments.

To an aqueous solution containing 50 μmol of potassium phosphate buffer (pH 7.0), 40 μmol of ATP, 20 μmol of magnesium acetate, 2 μmol of coenzyme A, and 0.1 ml of the supernatant of a total volume of 1 ml, sorbistin A_1 (**1**) (10 mg) was added. The reaction mixture was incubated at 37° for 20 hr. The remaining sorbistin A_1 (**1**) was determined by microbioassay using *E. coli* IFO 3044 and TLC method (silica gel, solvent; propanol–pyridine–AcOH–water=15:10:3:12, detected by ninhydrin). Sorbistin A_1-inactivation was not observed with any of the 4 resistant strains.

Acknowledgement The authors would like to thank Drs. E. Ohmura, M. Isono, K. Morita and Y. Nakao of this division for their advice and encouragement. Thanks are also due to Messrs. H. Suide and H. Shiotani for technical assistance.

20) K. Nakahama, H. Shirafuji, I. Nogami, M. Kida, and M. Yoneda, *Agric. Biol. Chem.*, 41, 2437 (1977).

NII-Electronic Library Service