Chemical Modification of Aminoglycoside Antibiotics. Some N-Alkyl Derivatives of Sorbistin A₁ (P-2563 P) and Butirosin A

KIYOSHI NARA, KOUICHI YOSHIOKA, and MAKOTO KIDA

Central Research Division, Takeda Chemical Industries, Ltd.¹⁾

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Some N-alkyl derivatives of sorbistin A₁ (P-2563 P) (1) and butirosin A (10) were prepared by reductive alkylation with aldehyde and NaBH₄ or NaBH₃CN. Among them, 4'-N-propylsorbistin A₁ (8b) showed antimicrobial activity against gram-positive bacteria comparable to sorbistin A₁ (1) and showed broader spectrum of activity against gram-negative bacteria including some of the sorbistin A₁-resistant strains (*K. pneumoniae*, *Ps. aeruginosa*) than sorbistin A₁ (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 A₁; 2'-N-alkylbutirosin A; sorbistin A₁-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 mechanism²⁾ 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-methylkanamycin⁴⁾ and 1-N-ethylsisomicin⁵⁾ 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 A₁)⁷⁾ (1), and butirosin A⁸⁾ (10), expecting to obtain new compounds of improved therapeutic properties.

Preparation of N-Alkyl Derivatives

1,4 and 4'-N-Alkylsorbistin A₁ and 2'-N-alkylbutirosin A were prepared by reductive alkylation with aldehyde and sodium borohydride (NaBH₄) or sodium cyanoborohydride (NaBH₃CN).

1. 1-N-Alkylsorbistin A₁ (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 pK_a values and steric factors. It was previously demonstrated⁶⁾ that 1-N-(*p*-methoxybenzyl) derivative of 1 was exclusively

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obtained by reductive alkylation of **1** with *p*-methoxybenzaldehyde by NaBH_4 . Further, when the proton magnetic resonance (PMR) spectrum of a monoacetate⁶⁾ 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

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 NaBH₄ 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 A₁ (5)**—4-N-Ethylsorbistin A₁ (**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 CH₃CHO and NaBH₄, followed by removal of the iBoc with trifluoroacetic acid (CF₃COOH) to afford **5**.

3. **4'-N-Alkylsorbistin A₁ (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 CH₃CHO or propionaldehyde and the reaction product was reduced with NaBH₄ 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 NaBH₃CN,¹¹⁾ followed by removal of the protecting groups. NaBH₃CN 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)**—Haskell¹²⁾ 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 NaBH₄ 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 NaBH₃CN followed by removal of the Cbz (Chart 2). The ¹³C-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

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*) 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.

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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.

Chart 2

TABLE I. ¹³C-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

Results and Discussion

Minimum inhibitory concentrations (MICs) of sorbistin A₁ 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 A₁ (**1**) (Table II). In order to compare

TABLE II. Antimicrobial Activities of N-Alkyl Derivatives of Sorbistin A₁ (**1**)

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

the antimicrobial activity of 4'-N-propylsorbistin A₁ (**8b**) with sorbistin A₁ (**1**) against gram-negative bacteria of clinical isolates, susceptibility of 100 strains of *Klebsiella pneumoniae* and 98 strains of *Pseudomonas aeruginosa* to sorbistin A₁ (**1**) and 4'-N-propylsorbistin A₁ (**8b**) were tested. The susceptibility patterns of these strains are shown in Fig. 1. The 4'-N-propylsorbistin A₁ (**8b**) was almost the same activity against *K. pneumoniae* as sorbistin A₁ (**1**),

Fig. 1. Sensitivity Distribution of Clinical Isolates

Sorbistin A₁ (1)
 —●—: *Klebsiella pneumoniae* (100 strains).
 —○—: *Pseudomonas aeruginosa* (98 strains).
 4'-N-Propylsorbistin A₁ (8b)
 ---●---: *Klebsiella pneumoniae* (100 strains)
 ---○---: *Pseudomonas aeruginosa* (98 strains).

but was approximately two-folded less susceptible to *Ps. aeruginosa* than sorbistin A₁ (1). However, the antimicrobial activity of 4'-N-propylsorbistin A₁ (8b) against sorbistin A₁-resistant strains of *K. pneumoniae* which were not inhibited at concentrations higher than 400 μg/ml of sorbistin A₁ (1), was found more potent than that of sorbistin A₁ (1) (Table III).

Sorbistin A₁-inactivation by ATP with cell free extracts prepared from these resistant strains was examined. The remaining sorbistin A₁ was determined by microbioassay and thin-layer chromatography (TLC) method. Sorbistin A₁-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 A₁ (8b) against Resistant Organisms

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

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TABLE IV. Antimicrobial Activities of 2'-N-Alkylbutirosin A

Organism		MIC ($\mu\text{g/ml}$)			
		Butirosin A	14a	14b	14c
<i>Staphylococcus aureus</i>	FDA 209 P	1.56	6.25	6.25	12.5
<i>Escherichia coli</i>	NIHJ JC 2	6.25	12.5	12.5	12.5
<i>Escherichia coli</i>	O-111	3.13	12.5	6.25	6.25
<i>Escherichia coli</i>	ML 1410 R 5	25	25	100	100
<i>Klebsiella pneumoniae</i>	DT	0.78	1.56	0.78	1.56
<i>Klebsiella pneumoniae</i>	GN 3853	3.13	6.25	6.25	6.25
<i>Klebsiella pneumoniae</i>	TN 802	25	100	100	>100
<i>Pseudomonas aeruginosa</i>	IFO 3080	3.13	12.5	25	25
<i>Pseudomonas aeruginosa</i>	TI-13	6.25	25	12.5	50
<i>Pseudomonas aeruginosa</i>	GN 3347	25	50	50	>100
<i>Proteus vulgaris</i>	GN 4413	50	50	12.5	25
<i>Proteus morganii</i>	IFO 3168	12.5	50	50	100
<i>Proteus morganii</i>	GN 4381	100	100	50	>100
<i>Proteus rettgeri</i>	GN 4425	12.5	12.5	12.5	25
<i>Proteus rettgeri</i>	GN 4424	50	25	12.5	50
<i>Proteus rettgeri</i>	GN 4427	100	100	50	100
<i>Proteus inconstans</i>	TN 800	100	12.5	3.13	25
<i>Serratia marcescens</i>	IFO 12648	50	50	25	50
<i>Serratia marcescens</i>	TN 24	50	50	25	50
<i>Citrobacter freundii</i>	GN 99	25	100	25	100
<i>Citrobacter freundii</i>	GN 726	100	50	12.5	25
<i>Enterobacter cloacae</i>	TN 581	3.13	6.25	6.25	6.25
<i>Enterobacter cloacae</i>	TN 594	12.5	25	12.5	25
<i>Salmonella typhosa</i>	58	3.13	6.25	3.13	6.25
<i>Shigella flexneri</i>	EW 10	6.25	12.5	12.5	12.5
<i>Shigella sonnei</i>	EW 33	6.25	25	12.5	25

TABLE V. Antimicrobial Activities of 2'-N-Alkylbutirosin A against Resistant Organisms

Organism		MIC ($\mu\text{g/ml}$)			
		Butirosin A	14a	14b	14c
<i>Escherichia coli</i>	JR 66/W 677	>100	>100	>100	100
<i>Escherichia coli</i>	GN 3464	>100	>100	100	50
<i>Pseudomonas aeruginosa</i>	GN 315	>100	>100	>100	>100
<i>Bacillus brevis</i>	IFO 12334	>100	>100	>100	>100
<i>Proteus morganii</i>	GN 4392	>100	100	50	100
<i>Proteus rettgeri</i>	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 A₁ (p-2563 P) (1) and those of butirosin A (10) by reductive alkylation with aldehyde and NaBH₄ or NaBH₃CN. The N-alkylation of the amino group, particularly 4'-amino group in sorbistin A₁ (1) and 2'-amino group in butirosin A (10) led to some improved antimicrobial activity. In case of the N-alkyl derivatives of sorbistin A₁, it was found that 4'-N-propylsorbistin A₁ (8b) showed antimicrobial activity against gram-positive and gram-negative bacteria comparable to sorbistin A₁ (1), and also showed antimicrobial activity against some of the sorbistin A₁-resistant bacteria found in the clinical isolates. In case of the 2'-N-alkyl derivatives of

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'-P 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

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

1-N-Ethylsorbistin A₁ (2a) and 1,4-Di-N-ethylsorbistin A₁ (3a)—A mixture of **1** (1.0 g) in 80% CH₃CHO (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% CH₃CHO (2 ml) and MeOH (10 ml); the solution was warmed at 50° for 5 min and then cooled. NaBH₄ (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 (NH₄) column (30 ml). The column was eluted with 0.04 N NH₄OH (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 C₁₇H₃₅N₃O₉·3/2H₂O: 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 NH₄OH (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 C₁₉H₃₉N₃O₉·1/2H₂O: C, 49.33; H, 8.72; N, 9.08. Found: C, 49.10; H, 8.52; N, 8.95.

1-N-p-Chlorobenzylsorbistin A₁ (2b) and 1,4-Di-N-p-chlorobenzylsorbistin A₁ (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 NaBH₄ (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 (NH₄) column (30 ml). The column was eluted with 0.015 N NH₄OH (200 ml) and the eluate was lyophilized to give **2b** (324 mg). *Anal.* Calcd. for C₂₂H₃₆ClN₃O₉·1/2H₂O: 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 NH₄OH (100 ml) and the eluate was concentrated and the residual sirup was lyophilized to give **3b** (200 mg). *Anal.* Calcd. for C₂₉H₄₁Cl₂N₃O₉·H₂O: 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 A₁ (4)—To a mixture of **1** (4.2 g), MeOH (60 ml), water (20 ml) and Et₃N (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 (NH₄) column (400 ml) and the column was eluted with 0.05 N NH₄OH (600 ml). The eluate was concentrated and the residual sirup was lyophilized to give **4** (2.1 g). *Anal.* Calcd. for C₂₆H₄₇N₃O₁₁·2H₂O: C, 50.88; H, 8.38; N, 6.85. Found: C, 50.66; H, 7.97; N, 6.62.

4-N-Ethylsorbistin A₁ (5)—A mixture of **4** (500 mg) in 80% CH₃CHO (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% CH₃CHO (2 ml) and MeOH (10 ml) and the solution was warmed at 50° for 5 min, then cooled to room temperature. NaBH₄ (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 Na₂SO₄ and the solvent was evaporated. The residue was dissolved in CF₃COOH (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 (NH₄) column (30 ml). The column was eluted with 0.1 N NH₄OH (50 ml) and the eluate was concentrated and the sirup was lyophilized to afford **5** (95 mg). *Anal.* Calcd. for C₁₇H₃₅N₃O₉·3/2H₂O: 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 A₁ (7)—To a stirred mixture of **1** (20 g), NaHCO₃ (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 C₄₃H₅₅N₃O₉·2HCl·4H₂O: 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 Na₂SO₄ and the solvent was evaporated to give **7** (13 g). *Anal.* Calcd. for C₄₀H₅₁N₃O₈·1/2H₂O: C, 67.58; H, 7.23; N, 5.91. Found: C, 67.40; H, 7.36; N, 5.75.

4'-N-Ethylsorbistin A₁ (8a)—A mixture of **7** (1.0 g), 80% CH₃CHO (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% CH₃CHO (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 NaBH₄ (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 Na₂SO₄ and the solvent was evaporated.

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 (NH₄) column (30 ml). The column was eluted with 0.15 N NH₄OH (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 C₁₄H₃₁N₃O₈·H₂O: C, 43.40; H, 8.58; N, 10.85. Found: C, 43.59; H, 8.27; N, 10.90.

4'-N-Propylsorbistin A₁ (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 C₁₅H₃₃N₃O₈·H₂O: C, 44.88; H, 8.79; N, 10.47. Found: C, 44.85; H, 9.01; N, 10.55.

4'-N,N-Dimethylsorbistin A₁ (9)—To a mixture of 7 (1.0 g), 37% HCHO (2 ml), CH₃CN (10 ml) and tetrahydrofuran (THF) (4 ml) was added in small portions NaBH₃CN (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 (NH₄) column (20 ml). The column was eluted with 0.1 N NH₄OH (50 ml) and the eluate was concentrated to a sirup, which was lyophilized to give 9 (240 mg). *Anal.* Calcd. for C₁₄H₃₁N₃O₈·H₂O: 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'-phosphate¹⁹⁾ (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 C₄₅H₅₉N₅O₁₈: 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% CH₃CHO, 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 NaBH₄ (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 (NH₄) column (20 ml). The column was eluted with 0.3 N NH₄OH (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 C₂₂H₄₃N₅O₁₂·H₂CO₃·1/2H₂O: 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 C₂₃H₄₅N₅O₁₂·H₂CO₃·1/2H₂O: 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 C₂₄H₄₇N₅O₁₂·H₂CO₃·H₂O: 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 C₂₈H₄₇N₅O₁₂·H₂CO₃·3H₂O: 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.

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 $8000 \times g$ 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 $10000 \times g$ 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 μ mol of potassium phosphate buffer (pH 7.0), 40 μ mol of ATP, 20 μ mol 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 $8000 \times g$ 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 $10000 \times g$ for 20 min. The supernatant thus obtained was used for the experiments.

To an aqueous solution containing 50 μ mol of potassium phosphate buffer (pH 7.0), 40 μ mol of ATP, 20 μ mol of magnesium acetate, 2 μ mol 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.

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