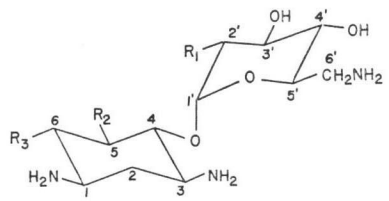
AMINOGLYCOSIDE 6′-N-ACETYLTRANSFERASE OF *PSEUDOMONAS AERUGINOSA*: STRUCTURAL REQUIREMENTS OF SUBSTRATE

Sir:

As reported previously,[1-5] aminoglycoside 6′-N-acetyltransferases [AAC(6′)] in R factor-carrying *Escherichia coli* and *Pseudomonas aeruginosa* are involved in the mechanism of resistance to several aminoglycoside antibiotics such as kanamycin, kanamycin B, 3′,4′-dideoxykanamycin B (DKB) and ribostamycin. Gentamicin C_{1a} and sisomicin are acetylated by these enzymes[6,7]. In this paper, we report the structural requirements of the substrate for the action of AAC(6′) prepared from a strain of *P. aeruginosa*.



	R_1	R_2	R_3
Kanamycin	OH	OH	3-Amino-3-deoxy-α-D-glucopyranosyl
Kanamycin B	NH_2	OH	3-Amino-3-deoxy-α-D-glucopyranosyl
Neamine	NH_2	OH	OH
Ribostamycin	NH_2	β-D-Ribofuranosyl	OH

The supernatant (S-100) of 100,000 *g* centrifugation of disrupted cells of *P. aeruginosa* GN315 was obtained by the method reported previously[8]. Ten ml of the S-100 (60.2 mg protein per ml, total 42.1 U; 1 U was defined as the acetylation of 1 μmole of DKB per minute) was applied to an affinity column[8] (DKB-Sepharose 4B, 10 ml) and AAC(6′) was eluted with a linear gradient of sodium chloride from 0 to 1.0 M. The enzyme appeared in the fraction eluted with 0.65 M sodium chloride (28.5 ml, 307 μg protein per ml, total 17.5 U), and 29.2-fold purification was achieved.

Activity of the enzyme was determined by the following procedure. The reaction mixture (300 μl) contained 6.25~50 nmoles of antibiotic, 400 nmoles of acetyl coenzyme A, 66 nCi of acetyl-1-^{14}C-acetyl coenzyme A (56.2 μCi/μmole, New England Nuclear, Boston, Mass.), 3 μmoles of magnesium acetate, 18 μmoles of potassium chloride, 3 μmoles of 1, 4-dithiothreitol, 30 μl of 100 mM phosphate buffer (pH 7.2) and 50 μl of the enzyme solution. After incubation for 15 minutes at 37°C, the reaction was stopped by heating in a boiling water bath for 3 minutes, and the reaction mixture was diluted with 7 ml of water. The solution thus obtained was passed through a column of Amberlite CG-50 resin (NH_4^+ form, 1 ml), and the column was washed with 10 ml of water. The acetylated antibiotic adsorbed on the column was eluted with 3 ml of 4 N aqueous ammonia directly into a scintillation vial. Radioactivity (dpm) of the acetylated antibiotic was counted by a liquid scintillation system (Aloka LSC-653) with 8 ml of BRAY's scintillator. In the case of methyl 2,6-diamino-2,6-dideoxy-α-D-glucopyranoside (Me-2, 6-AG) as substrate, Dowex 1-X4 resin (OH^- form) was employed for the examination of the reaction mixture.

As shown in Table 1, AAC(6′) of *P. aeruginosa* GN315 transferred the acetyl to the 6′-amino group of kanamycin, 4′-deoxykanamycin, amikacin, kanamycin B, tobramycin, DKB, neamine, ribostamycin, butirosins A and B, and gentamicin C_{1a}. These antibiotics showed no inhibition of the growth of *P. aeruginosa* GN315 at 100 μg/ml. 6′-N-Alkyl compounds such as 6′-N-methylamikacin, 6′-N-methyltobramycin and 6′-N-methyl-DKB were hardly acetylated, and inhibited the growth of this strain (MIC of 6.25~25 μg/ml). Acylation of the 1-amino group of kanamycin and DKB with (*S*)-4-amino-2-hydroxybutyric acid slightly decreased the 6′-N-acetylation, however, the 1-N-acylation of kanamycin B and neamine increased susceptibility to the 6′-N-acetylation. Thus, the 1-amino group may be involved in the reaction of AAC(6′). It is noticeable that 6′-N-alkylation with 1-N-acylation prevents the 6′-N-acetylation. 6′-N-Methylamikacin, 6′-N-ethylamikacin, 1-N-[(*S*)-4-amino-2-hydroxybutyryl]-6′-N-methyl-DKB, 1-N-(DL-isoseryl)-6′-N-methyl-DKB and 1-N-[(*S*)-5-amino-2-hydroxy-*n*-valeryl]-6′-N-methyl-DKB were hardly acetylated. However, 6′-N-ethylation decreased the antibacterial activity against both sensitive strains and *P. aeruginosa*[9]. Acylation of the 3-amino group (3-N-[(*S*)-4-amino-2-hydroxybutyryl]-DKB and 3-N-

Table 1. Acetylation of aminoglycoside antibiotics by AAC(6′) and their minimal inhibitory concentrations against *P. aeruginosa* GN315 and A3.

Antibiotics	Acetylated product (nmole)	MIC (μg/ml)	
		GN315	A3
Kanamycin	7.64	>100	50
6′-N-Methylkanamycin[12]	5.23	>100	>100
1-AHB-Kanamycin[13] (amikacin)	6.36	100	3.12
6′-N-Methylamikacin[9]	0.72	25	6.25
6′-N-Ethylamikacin[9]	0.56	100	25
4′-Deoxykanamycin[14]	4.32	>100	1.56
Kanamycin B	4.89	>100	50
1-AHB-Kanamycin B[15]	6.22	50	6.25
3′-Deoxykanamycin B[16] (tobramycin)	4.25	>100	0.78
6′-N-Methyltobramycin[12]	3.78	6.25	1.56
DKB[17]	11.53	>100	1.56
6′-N-Methyl-DKB[12]	2.35	12.5	3.12
1-AHB-DKB[15]	6.44	25	6.25
1-IS-6′-N-Methyl-DKB[18]	6.58	12.5	3.12
1-AHB-6′-N-Methyl-DKB[18]	2.88	6.25	3.12
1-AHV-6′-N-Methyl-DKB[18]	0.49	12.5	3.12
3-AHB-DKB[19]	1.43	>100	>100
2′-AHB-DKB[19]	6.08	>100	100
3″-AHB-DKB[19]	13.62	>100	>100
6′-AHB-DKB[19]	1.47	>100	25
Neamine	5.58	>100	>100
1-AHB-Neamine	10.29	>100	3.12
3′,4′-Dideoxyneamine[20]	8.74	>100	50
Ribostamycin	9.13	>100	>100
3-N-Acetylribostamycin[21]	3.56	inactive	
Butirosin A	7.45	>100	6.25
Butirosin B	6.39	>100	6.25
Gentamicin C_{1a}	15.98	100	3.12
Gentamicin C_2	2.07	6.25	6.25
Gentamicin C_1	0.57	12.5	12.5
Paromamine	0.12	>100	>100
Kanamycin C	0.37	>100	>100
Me-2,6-AG	0.00	inactive	

Reaction mixture contained 50 nmoles of an antibiotic. MIC: minimal inhibitory concentrations were determined by agar dilution streak method (nutrient agar, 37°C, 18 hours).
AHB: N-[(*S*)-4-amino-2-hydroxybutyryl] IS: N-(DL-isoseryl)
AHV: N-[(*S*)-5-amino-2-hydroxy-*n*-valeryl]

acetylribostamycin) also prevented the 6′-N-acetylation. This modification decreased the antibacterial activity. A 6′-N-acylated compound, 6′-N-[(*S*)-4-amino-2-hydroxybutyryl]-DKB, was not susceptible to the 6′-N-acetylation, but it has little antibacterial activity. Acylation of the 2′-amino group of DKB decreased the 6′-N-acetylation, and also decreased the antibacterial activity. Acylation of the 3″-amino group of DKB had no effect on the 6′-N-acetylation, and markedly decreased the antibacterial activity. From the fact that neamine is acetylated rapidly but Me-2,6-AG is not acetylated, the presence of 2-deoxystreptamine was suggested to be necessary for this enzyme reaction. Deoxygenation

Table 2. Susceptibility of aminoglycoside antibiotics to AAC(6′) of *P. aeruginosa* GN315.

Antibiotics	K_m ($\times 10^{-6}$M)	V_{max} (nmole/min.)	V_{max}/K_m ($\times 10^{-3}$)
Kanamycin	181.8	1.43	7.9
6′-N-Methylkanamycin	2,000.0	1.43	0.7
Amikacin	21.5	0.63	29.3
6′-N-Methylamikacin	1,000.0	0.40	0.4
6′-N-Ethylamikacin	>2,000.0	<0.02	0.0
4′-Deoxykanamycin	32.8	0.69	21.0
Kanamycin B	38.1	0.42	11.0
1-AHB-Kanamycin B	35.9	0.63	17.5
Tobramycin	55.6	1.25	22.5
6′-N-Methyltobramycin	1,067.0	0.23	0.2
DKB	200.0	5.00	25.0
6′-N-Methyl-DKB	1,667.0	1.33	0.8
1-AHB-DKB	67.0	1.25	18.7
1-IS-6′-N-Methyl-DKB	181.8	0.95	5.2
I-AHB-6′-N-Methyl-DKB	310.0	0.23	0.7
1-AHV-6′-N-Methyl-DKB	2,000.0	0.33	0.2
Gentamicin C_{1a}	80.0	1.82	22.8
Gentamicin C_2	44.4	0.22	5.0
Gentamicin C_1	>2,000.0	<0.02	0.0
Neamine	44.4	0.95	21.4
Ribostamycin	285.7	6.66	23.3
Butirosin B	23.8	0.63	26.5

K_m and V_{max} were determined from LINEWEAVER-BURK plots. The enzyme concentration and the reaction time are constant in all cases.

of the 4′-hydroxyl group of kanamycin decreased the 6′-N-acetylation, and this hydroxyl group is probably involved in the reaction. However, dideoxygenation of the 3′- and 4′-hydroxyl groups (3′,4′-dideoxyneamine and DKB) increased the rate of the 6′-N-acetylation.

The kinetic data are shown in Table 2. The values of V_{max}/K_m for the antibiotics indicate the degree of susceptibility to the 6′-N-acetylation. 6′-N-Ethylamikacin, 1-N-[(*S*)-5-amino-2-hydroxy-*n*-valeryl]-6′-N-methyl-DKB and gentamicin C_1 showed the lowest affinity ($K_m \geqq 2\times 10^{-3}$M) to the enzyme, and these antibiotics were hardly acetylated. 6′-N-Methylamikacin, 6′-N-methyl-DKB, 6′-N-methyltobramycin and 6′-N-methylkanamycin showed a low affinity (K_m $1\sim 2\times 10^{-3}$M), and they were acetylated slowly. 1-N-(DL-Isoseryl)-6′-N-methyl-DKB, 1-N-[(*S*)-4-amino-2-hydroxybutyryl]-6′-N-methyl-DKB and gentamicin C_2 showed relatively high affinity (K_m $4.4\sim 31\times 10^{-5}$ M), but low V_{max}. It is noticeable that amikacin, kanamycin B, tobramycin, DKB, neamine and ribostamycin over 83 μM exhibit substrate inhibition against the reaction of AAC(6′). Kanamycin C and paromamine which do not have the 6′-amino group were non-competitive inhibitors, and K_i values were 8.3 and 69.8×10^{-6}M, respectively.

The data described above indicate that concurrent 6′-N-alkylation and 1-N-acylation yield active derivatives unsusceptible to the enzyme. Depending on the sources, 6′-N-acetyltransferases are different in their substrate requirements[10,11]. The AAC(6′) here studied one which has a low activity in acetylating 6′-N-methyl derivative.

MORIMASA YAGISAWA
SHINICHI KONDO
TOMIO TAKEUCHI
HAMAO UMEZAWA

Institute of Microbial Chemistry
Kamiosaki, Shinagawa-ku, Tokyo, Japan

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References

1) OKANISHI, M.; S. KONDO, Y. SUZUKI, S. OKAMOTO & H. UMEZAWA: Studies on inactivation of kanamycin and resistances of *E. coli*. J. Antibiotics, Ser. A 20: 132~135, 1967
2. UMEZAWA, H.; M. OKANISHI, R. UTAHARA, K. MAEDA & S. KONDO: Isolation and structure of kanamycin inactivated by a cell free system of kanamycin-resistant *E. coli*. J. Antibiotics, Ser. A 20: 136~141, 1967
3) YAGISAWA, M.; H. NAGANAWA, S. KONDO, T. TAKEUCHI & H. UMEZAWA: 6′-N-Acetylation of 3′,4′-dideoxykanamycin B by an enzyme in a resistant strain of *Pseudomonas aeruginosa*. J. Antibiotics 25: 495~496, 1972
4) YAMAMOTO, H.; M. YAGISAWA, H. NAGANAWA, S. KONDO, T. TAKEUCHI & H. UMEZAWA: Kanamycin 6′-acetate and ribostamycin 6′-acetate, enzymatically inactivated products by *Pseudomonas aeruginosa*. J. Antibiotics 25: 746~747, 1972
5) UMEZAWA, H.: "Advances in Carbohydrate Chemistry and Biochemistry" Vol. 30, pp. 183~225, Academic Press, New York, N.Y., 1974
6) BENVENISTE, R. & J. DAVIES: Enzymatic acetylation of aminoglycoside antibiotics by *Escherichia coli* carrying an R factor. Biochemistry 10: 1787~1796, 1971
7) O'HARA, K.; M. KONO & S. MITSUHASHI: Structure of enzymatically acetylated sisomicin by *Pseudomonas aeruginosa*. J. Antibiotics 27: 349~351, 1974
8) UMEZAWA, H.; H. YAMAMOTO, M. YAGISAWA, S. KONDO, T. TAKEUCHI & Y.A. CHABBERT: Kanamycin phosphotransferase I: Mechanism of cross resistance between kanamycin and lividomycin. J. Antibiotics 26: 407~411, 1973
9) UMEZAWA, H.; K. IINUMA, S. KONDO &. K. MAEDA: Synthesis and antibacterial activity of 6′-N-alkyl derivatives of 1-N-[(*S*)-4-amino-2-hydroxybutyryl]-kanamycin. J. Antibiotics 28: 483~485, 1975
10) KAWABE, H.; S. KONDO, H. UMEZAWA & S. MITSUHASHI: R-Factor mediated aminoglycoside antibiotics resistance in *Pseudomonas aeruginosa*: a new aminoglycoside 6′-N-acetyltransferase. Antimicr. Agents & Chemoth. (in press)
11) LEGOFFIC, F. & A. MARTEL: Resistance to aminosides produced by an isozyme, kanamycin acetyltransferase. Biochimie 56: 893~898, 1974
12) UMEZAWA, H.; Y. NISHIMURA, T. TSUCHIYA & S. UMEZAWA: Syntheses of 6′-N-methylkanamycin and 3′,4′-dideoxy-6′-N-methylkanamycin B active against resistant strains having 6′-N-acetylating enzymes. J. Antibiotics 25: 743~745, 1972
13) KAWAGUCHI, H.; T. NAITO, S. NAKAGAWA & K. FUJISAWA: BB-K8, a new semisynthetic aminoglycoside antibiotic. J. Antibiotics 25: 695~708, 1972
14) UMEZAWA, S.; Y. NISHIMURA, Y. HATA, T. TSUCHIYA, M. YAGISAWA & H. UMEZAWA: Synthesis of 4′-deoxykanamycin and its resistance to kanamycin phosphotransferase II. J. Antibiotics 27: 722~725, 1974
15) KONDO, S.; K. IINUMA, H. YAMAMOTO, K. MAEDA & H. UMEZAWA: Syntheses of 1-N-{(*S*)-4-amino-2-hydroxybutyryl}-kanamycin B and -3′,4′-dideoxykanamycin B active against kanamycin-resistant bacteria. J. Antibiotics 26: 412~415, 1973
16) TAKAGI, Y.; T. MIYAKE, T. TSUCHIYA, S. UMEZAWA & H. UMEZAWA: Synthesis of 3′-deoxykanamycin B. J. Antibiotics 26: 403~406, 1973
17) UMEZAWA, H.; S. UMEZAWA, T. TSUCHIYA & Y. OKAZAKI: 3′, 4′-Dideoxykanamycin B active against kanamycin-resistant *Escherichia coli* and *Pseudomonas aeruginosa*. J. Antibiotics 24: 485~487, 1971
18) UMEZAWA, H.; K. IINUMA, S. KONDO, M. HAMADA & K. MAEDA: Synthesis of 1-N-acyl derivatives of 3′, 4′-dideoxy-6′-N-methylkanamycin B and their antibacterial activities. J. Antibiotics 28: 340~343, 1975
19) KONDO, S.; K. IINUMA, H. YAMAMOTO, Y. IKEDA, K. MAEDA & H. UMEZAWA: Syntheses of (*S*)-4-amino-2-hydroxybutyryl derivatives of 3′,4′-dideoxykanamycin B and their antibacterical activities. J. Antibiotics 26: 705~707, 1973
20) UMEZAWA, S.; T. TSUCHIYA, T. JIKIHARA & H. UMEZAWA: Synthesis of 3′, 4′-dideoxyneamine active against kanamycin-resistant *E. coli* and *P. aeruginosa*. J. Antibiotics 24: 711~712, 1971
21) KOJIMA, M.; N. EZAKI, S. AMANO, S. INOUYE & T. NIIDA: Bioconversion of ribostamycin (SF-733). II. Isolation and structure of 3-N-acetylribostamycin, a microbiologically inactive product of ribostamycin produced by *Streptomyces ribosidificus*. J. Antibiotics 28: 42~47, 1975