Enzymatic 2′-*N*-Acetylation of Arbekacin and Antibiotic Activity of Its Product†

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Aminoglycoside antibiotics (AGs) with a free 2′-amino group were subjected to enzymatic *N*-acetylation using a cell free extract that contained an aminoglycoside 2′-*N*-acetyltransferase, AAC (2′), derived from a kasugamycin-producing strain of *Streptomyces kasugaensis*. TLC and antibiotic assay of the incubated reaction mixtures revealed that a modified compound retaining substantial antibiotic activity was formed from arbekacin (ABK), while modification of the other AGs resulted in the marked decrease in antibiotic activity. Structure determination following isolation from a large scale reaction mixture showed the modified ABK to be 2′-*N*-acetyl ABK. In addition, 2′,6′-di-*N*-acetyl ABK was formed as a minor product. The same conversion also occurred with dibekacin (DKB) resulting in the formation of 2′-*N*-acetyl DKB and 2′,6′-di-*N*-acetyl DKB. MIC determination showed antibacterial activity (1.56～3.13 μg/ml) of 2′-*N*-acetyl ABK against a variety of organisms. By contrast, 2′-*N*-acetyl DKB showed no substantial antibiotic activity. We believe 2′-*N*-acetyl ABK has the highest and broadest antibacterial activity, compared with known *N*-acetylated AGs.

Arbekacin (ABK)[1～3], a semisynthetic aminoglycoside antibiotic (AG), is one of the two anti-MRSA agents (the other is vancomycin) used clinically in Japan. Emergence of MRSA (methicillin-resistant *Staphylococcus aureus*) that caused a serious problem in clinics in the 1980s has led to increased interest in ABK. Because of its activity against MRSA strains including ones with AG resistance[4～7], ABK was specifically approved in 1990 as an MRSA-targeted drug. ABK shows excellent activity against a wide variety of known AG resistant bacteria including MRSA with AG modifying enzymes[8,9], although ABK contains amino and hydroxyl groups that may be modified by these enzymes. Like amikacin (AMK), the side chain [(*S*)-4-amino-2-hydroxybutyric acid; AHB] connected to 1-amino group of ABK is believed to play a critical role in preventing the access of AG modifying (or inactivating) enzymes. Only a small number of MRSA strains with a moderate level (12.5～25 μg/ml) of ABK resistance due to a bifunctional enzyme (2″-*O*-phosphorylation and 6′-*N*-acetylation) have been reported so far[8]. However, one cannot rule out the possibility of emergence in the future of new AG-resistant strains with AG-modifying enzymes that may inactivate ABK. Such enzymes[2] may include AAC (3), AAC (2′), AAC (6′) and AAD (2″).

Usually one has to wait for the emergence of new resistant bacteria to confirm such possibilities. We have developed an alternative approach; *i.e.* exposure of ABK to novel AG modifying enzymes available from actinomycetes such as the AG producers. AG producers have been suggested as the origin of resistance determinants[10] and evidence showing biochemical and genetic linkages in AG modification between clinical bacteria and AG-producing streptomycetes has been obtained[11,12].

We have been studying AG-producing strains of *Streptomyces* in terms of multiple AG resistance[13] and have identified resistance determinants that are not involved in self-resistance[14]. They include the cryptic kanamycin resistance gene in a streptomycin (SM)-producing strain of *S. griseus* and the istamycin resistance gene in kasugamycin (KSM)-producing *S. kasugaensis*

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MB273. The former is due to a *kan* gene coding for AAC (3) and the latter to an *ist* gene coding for AAC (2') that acetylates the 1- or 2'-amino group of astromicin (ASTM) family AGs depending on the configuration of 1-amino group; acetylation occurs at 2'-amino group in ASTM with axial 1-NH_2 and at 1-amino group in istamycin B with equatorial 1-NH_2[14].

In this paper, AGs with 2'-amino group were subjected to enzymatic acetylation using a cell free extract of *S. lividans* TK21 containing the AAC (2') gene cloned from kasugamycin-producing *S. kasugaensis* MB273 and found that all were acetylated, but that the acetylation product (2'-*N*-acetyl ABK) of ABK showed substantial antibiotic activity against a variety of Gram-positive and -negative bacteria.

Materials and Methods

Organisms

Streptomyces lividans TK21 containing pANT12 that carries an AAC (2') gene from *S. kasugaensis* MB273 cloned into pIJ702 was used[14]. Test organisms for MIC determination were available from the culture collection of Institute of Microbial Chemistry. Fifty clinical strains of MRSA[9] from a hospital in Osaka in 1986~1990 were obtained from Takeda Analytical Research Laboratories, Ltd.

Chemicals

Arbekacin (ABK), dibekacin (DKB), kanamycin (KM) and ribostamycin (RSM) as sulfate or free base were obtained from Meiji Seika Kaisha, Ltd. The other reagents were commercially available.

Preparation of Cell Free Extract

S. lividans TK21 containing the AAC (2') gene was grown at 27°C for 2 days in Bacto Tryptic Soy Broth (Difco) supplemented with 10 μg/ml each of RSM and thiostrepton on a rotary shaker. The mycelium was harvested and washed once with deionized water followed by a buffer (10 mM Tris-HCl, pH 7.8, 60 mM NH_4Cl, 10 mM magnesium acetate and 1 mM dithiothreitol) and centrifuged. The packed mycelium was disrupted by grinding with aluminum oxide together with deoxyribonuclease I and then extracted with the above buffer. The extract was centrifuged at 30,000 × *g* for 30 minutes and the resulting supernatant (S30) was stored at −20°C.

Antibiotic Resistance

Aerial mycelium of *S. lividans* TK21/pANT12 grown at 27°C to maturation on ISP No. 4 medium (Inorganic salts-starch agar; Difco) containing 10 μg/ml each of RSM and thiostrepton was scraped off and streaked on ISP No. 2 medium plates containing different concentrations of one of the antibiotics tested. Growth was scored after 1 week incubation at 27°C.

Acetylation Reaction

In a small scale experiment, a 100 μl reaction mixture consisting of 100 μg/ml of an aminoglycoside antibiotic, 5 mM acetyl-CoA (sodium salt; Sigma), 100 mM potassium phosphate buffer (pH 7.0), and 20 μl of the cell free extract (23 mg protein/ml) was incubated at 37°C for 60 minutes. For a large scale reaction (10 or 20 ml), concentrations of an antibiotic and acetyl-CoA were changed to 1 mg (as free base)/ml and 36 mM, respectively, and the incubation was carried out overnight.

Detection of Modification Products

Modification products of ABK and DKB were monitored by TLC and HVPE (high-voltage paper electrophoresis). For TLC, 5 μl of samples (reaction mixtures after incubation, and eluate fractions after chromatography) were spotted on a silica gel plate (E. Merck, Art. 5715) which was then developed with a solvent system consisting of $CHCl_3$-MeOH-conc. NH_4OH-H_2O (1:4:2:1) and stained with ninhydrin reagent, unless otherwise described. ABK and DKB showed Rf 0.19 and 0.48, respectively. HVPE was carried out on a CAMAG HVE system at 3,300 V for 10 minutes, using HCOOH-CH_3COOH-H_2O (25:75:900, pH 1.8) as an electrolyte solution[15]. After electrophoresis, the paper was air-dried and stained with ninhydrin and Rydon-Smith reagents. Rm (relative mobility to alanine) values of ABK and DKB were 2.03 and 2.06, respectively.

Isolation of Modification Products

After incubation, the large scale reaction mixture (20 ml) containing ABK (20.8 mg) was diluted to 50 ml with H_2O and loaded on a column of Amberlite CG-50 (NH_4^+, 20 ml). The column was then washed with H_2O (20 ml) and eluted with a 0~2% linear gradient of aqueous ammonia (100 ml). The eluate was collected as approximately 2 ml fractions which were monitored for the modified compound by both ninhydrin and Rydon-Smith reactions following HVPE. Since two different modified compounds were detected, fractions containing either one of them were pooled and concentrated to yield two different crude powders. The crude powder (16.3 mg) containing the major modified compound with Rm 1.73 was obtained from fractions 41~45. The other crude powder (8.4 mg) was obtained from fractions 39 and 40 and contained the minor modified compound with Rm 1.45. Subsequently, these crude powders were rechromatographed on a column of Amberlite CG-50 (NH_4^+, 10 ml) by eluting with 120 ml of aqueous ammonia with a linear gradient (0.2%~0.8% for the major crude powder and 0.1~0.6% for the minor crude powder) and the eluates were collected as 1 ml fractions. Fractions containing the major (designated as compound **A**) and the minor (designated compound **B**) components were

pooled and concentrated. Consequently, the purified compounds **A** (14.9 mg) and **B** (1.7 mg) were obtained as colorless powders.

Isolation of the modification products of DKB followed the above procedures with a slight modifications; *e.g.* use of a 0~1% linear gradient of aqueous ammonia for the elution of the modified products from the column. Two products were obtained from the DKB (19 mg) reaction mixture; one (4.6 mg) from fractions 50~53 containing the major modified compound with Rm 1.83 (designated compound **C**) and the other (10 mg) from fractions 47~49 containing the minor modified compound with Rm 1.55 (designated compound **D**). For purification, **C** and **D** products were rechromatographed on an Amberlite CG50 column (NH_4^+, 10 ml) by eluting with a 0.1~0.7% linear gradient of aqueous ammonia. Consequently, **C** (4.5 mg) and **D** (1.7 mg) were obtained as colorless powders.

Physicochemical Analysis

MP was determined with a Electrothermal IA9100 digital melting point apparatus and data were uncorrected. FAB-MS was measured on a JEOL JMX-SX102 spectrometer. ^1H and ^{13}C NMR spectra in D_2O at pD 1.5~2.0 were recorded on JEOL JNM-EX400 and JNM-A500 spectrometers.

Antibiotic Activity

Antibiotic activity of the reaction mixture was monitored by the paper disc method using *Bacillus subtilis* PCI 219 as the test organism on Mycin Assay Agar Arei (Kyoei Pharmaceutical Co.). MICs were determined by the serial two-fold agar dilution method in Bacto Müller-Hinton Medium (Difco) after incubation at 37°C for 17 hours.

Results

Acetylation of Aminoglycoside Antibiotics with a Free 2′-Amino Group

A variety of 2′-NH_2 containing aminoglycoside antibiotics (AGs) were subjected to resistance and *in vitro* acetylation tests with *S. lividans* TK21/pANT12 containing the AAC (2′) gene cloned from kasugamycin-producing *S. kasugaensis* MB273 (Table 1). The organism showed relatively low levels (5~25 μg/ml) of resistance to ASTM, DKB, gentamicin C complex (GM), neomycin (NM) and paromomycin (PRM), and a high level (200 μg/ml) of resistance to RSM, but was sensitive to 2.5 μg/ml of ABK as well as 2′-NH_2-lacking KM. When these antibiotics were incubated in the presence of acetyl-CoA with a cell free extract from the organism, complete modification of all the antibiotics but KM was confirmed by TLC analysis (data not shown). No modification was detected with KM. Paper disc antibiotic assay against *B. subtilis* of the reaction mixtures revealed substantial inactivation with DKB, ASTM, GM, PRM, RSM (hitherto >90%) and neomycin (40~50%). However, no apparent inactivation was observed with ABK although it was completely acetylated.

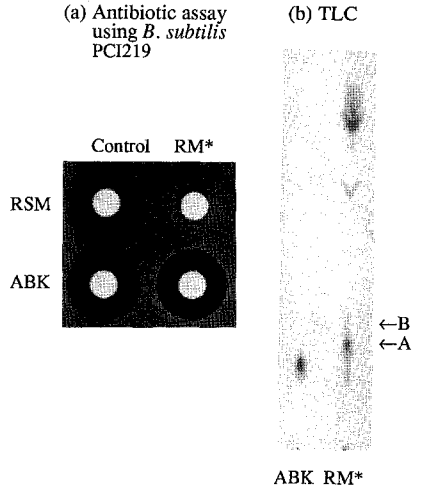
Fig. 1 clearly shows that RSM was inactivated, whereas ABK was not. There existed, however, a clear difference in the contour of the inhibition zone between the unincubated ABK reaction mixture (control) and the incubated one; being ill-defined with the former and clear with the latter. Since this suggested that modification of ABK occurred, the incubated ABK reaction mixture was submitted to TLC analysis (Fig. 1). Consequently, it

Table 1. Resistance of *S. lividans* TK21/pANT12 to aminoglycoside antibiotics with 2′-amino group and their acetylation and inactivation.

Antibiotic	Resistance	Acetylation*	Inactivation*
Arbekacin	< 2.5 μg/ml	100 %	0 %
Dibekacin	10	100	> 90
Gentamicin	5	100	> 90
Astromicin	25	100	> 95
Neomycin	10	100	40~50
Paromomycin	25	80~90	80~90
Ribostamycin	200	100	100
Kanamycin	< 2.5	0	0

* Antibiotics (100 μ/ml) were subjected to *in vitro* acetylation reaction with a cell free extract from *S. lividans* TK21/pANT12. Acetylation rate was estimated on the basis of ninhydrin positive spots upon TLC. Inactivation rate was estimated by the paper disc assay against *B. subtilis* PCI219 of the reaction mixtures.

Fig. 1. Antibiotic activity and modified compound formation.



Control: Unincubated reaction mixture.
RM*: Incubated reaction mixture.

turned out that ABK (Rf 0.19) was almost completely converted to a modified compound with Rf 0.25 (compound **A**) and a small amount of a compound with Rf 0.32 (compound **B**).

Isolation of Modified Arbekacin and Dibekacin

In order to isolate the modified ABK compounds, we carried out a large scale acetylation reaction of ABK (20.8 mg) followed by a column chromatography using Amberlite CG-50 (NH_4^+). Two distinctive compounds **A** and **B** were obtained; **A** as the major compound (14.9 mg) and **B** as the minor compound (1.7 mg). As summarized in Table 2, **A** showed Rm 1.73 (HVPE) and Rf 0.25 (TLC). Antibiotic assay by a cup assay method using *B. subtilis* PCI219 as the test organism and ABK as the standard indicated that the antibiotic activity of **A** was 42% of that of ABK. **B** exhibited Rm 1.33 (HVPE), Rf 0.32 (TLC) and no substantial antibiotic activity (<5% of ABK).

On the other hand, when DKB, the precursor of ABK was subjected to the same modification, the antibiotic activity of the DKB (19 mg) reaction mixture was eliminated almost completely. Isolation procedures revealed that DKB (Rf 0.48) was also converted to two different modified compounds as in the case of ABK; the major one (4.5 mg) with Rm 1.83 and Rf 0.63 (designated as **C**) and the minor one (1.7 mg) with Rm 1.44 and Rf 0.76 (designated as compound **D**) as summarized in Table 2.

Structures of Acetylated Products of ABK and DKB

As shown in Table 2, FAB-MS (positive, MH^+) of **A** an **B** provided *m/z* 595 and 637, respectively, indicating monoacetylated and diacetylated products of ABK, respectively. Compounds **C** and **D** provided *m/z* 494 and 536 which was regarded as monoacetylated and diacetylated products of DKB, respectively.

The ^1H and ^{13}C NMR spectra of these acetylated products were compared with those of ABK and DKB in D_2O at pD 1.5~2.0, as their characteristic signals are

Table 2. Physico-chemical properties of modification products of ABK and DKB.

Compound	Powder	MP (dec.)	Rm (HVPE)	Rf (TLC)	FAB-MS (positive, MH^+)	Remarks
A	colorless	164~169°C	1.73	0.25	*m/z* 595	Monoacetyl ABK
B	colorless	nd*	1.33	0.32	*m/z* 637	Diacetyl ABK
C	colorless	149~155°C	1.83	0.61	*m/z* 494	Monoacetyl DKB
D	colorless	nd*	1.44	0.76	*m/z* 536	Diacetyl DKB

* Not determined.

Table 3. ^1H NMR spectral data ABK, DKB and their monoacetyl and diacetyl derivatives.

Proton	Chemical shift[a], δ ppm (*J* Hz)					
	ABK	**A**[b]	**B**	DKB	**C**	**D**[b]
1'-H	5.80 d (3.4)	5.53 d (4.0)	5.22 d (3.4)	5.82 d (3.4)	5.54 d (3.4)	5.27 d (3.2)
2'-H	3.59 ddd (9.6, 6.9, 3.1)	4.00 ddd (12.0, 5.2, 4.2)	3.98 m	3.61 m	3.99 m	4.02 m
6'-H_2	3.15 dd (13.7, 6.8)	3.15 dd (14.0, 6.4)	3.27 dd (14.2, 5.4)	3.13 dd (13.7, 6.8)	3.13 dd (13.7, 5.9)	3.28 dd (14.0, 6.4)
	3.29 dd (13.7, 3.1)	3.27 dd (14.0, 4.0)	3.39 dd (14.2, 4.2)	3.29 dd (13.7, 3.4)	3.26 dd (13.7, 3.4)	3.42 dd (14.0, 4.4)
1"-H	5.20 d (3.9)	5.19 d (4.0)	5.15 d (3.4)	5.14 d (3.4)	5.11 d (3.4)	5.12 d (3.6)
$COCH_3$		2.02 s	1.97 s		2.00 s	2.00 s

[a] δ ppm from TSP (0 ppm) in D_2O (pD 1.5~2.0) at 400 MHz (JEOL JNM-EX400).

[b] At 500 MHz (JNM-A500): all protons of **A** were assigned by ^1H–^1H COSY, NMQC and HMBC experiments.

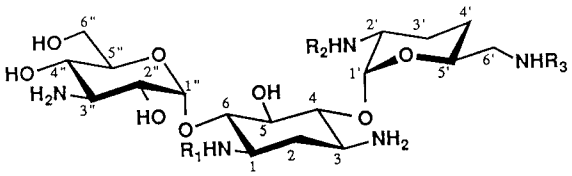
shown in Tables 3 and 4. The ^1H NMR spectra of **A** and **C** provided an acetyl CH_3 signal at δ 2.02 and 2.00, respectively. The 2′-H signals at δ 4.00 and 3.99 of **A** and **C**, respectively, were found to shift to lower field than those of the respective parent antibiotics (δ 3.59 for ABK and δ 3.61 for DKB). On the other hand, the ^{13}C NMR spectra of **A** and **C** yielded acetyl CO signals (at δ 174.4 and 174.5, respectively) as well as an acetyl CH_3 signal at δ 22.8. In addition, the β-carbons (C-1′ and C-3′) of the acetamide group shifted to lower field in both **A** and **C**. Based on these data, the structures of **A** and **C** were determined as 2′-*N*-acetyl ABK and 2′-*N*-acetyl DKB, respectively (Fig. 2).

On the other hand, diacetylated products (**B** and **D**) provided lower field shifts of 2′-H and 6′-H_2 in their ^1H NMR spectra (Table 3) and β-carbon shifts at C-1′, C-3′ and C-5′ in their ^{13}C NMR spectra (Table 4). Based on these data, the structures of **B** and **D** were deduced to be 2′,6′-di-*N*-acetyl ABK and 2′,6′-di-*N*-acetyl DKB, respectively (Fig. 2).

Antibacterial Activity of 2′-*N*-Acetyl ABK

Since pure monoacetylated products (**A** and **C**) of ABK and DKB were obtained in significant amounts, the

Fig. 2. Structures of dibekacin, arbekacin and their acetylation products by an ACC (2′) derived from kasugamycin-producing *S. kasugaensis* MB273.



	R_1	R_2	R_3
ABK (Arbekacin)	AHB	H	H
2′-*N*-Acetyl ABK (**A**)	AHB	CH_3CO	H
2′,6′-Di-*N*-acetyl ABK (**B**)	AHB	CH_3CO	CH_3CO
DKB (Dibekacin)	H	H	H
2′-*N*-Acetyl DKB (**C**)	H	CH_3CO	H
2′,6′-Di-*N*-acetyl DKB (**D**)	H	CH_3CO	CH_3CO

AHB =

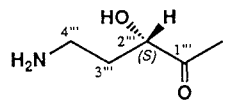


Table 4. ^{13}C NMR spectral data of ABK, DKB and their monoacetyl and diacetyl derivatives.

Carbon	Chemical shift[a], δ ppm					
	ABK	A[b]	B	DKB	C	D[b]
C-1′	95.9	97.8	99.2	95.9	97.8	99.2
C-2′	49.5	48.8	49.1	49.2	48.8	49.1
C-3′	21.3	22.6	22.6	21.2	22.5	22.7
C-5′	66.7	66.7	69.6	66.7	66.7	69.6
C-6′	43.3	43.4	43.4	43.3	43.4	43.5
Ac-CO		174.4	174.3		174.5	174.4
			175.4			175.4
Ac-CH_3		22.8	22.7		22.8	22.8
			23.1			23.1

[a] δ ppm from TMS in D_2O (pD 1.5~2.0) using dioxane (67.4 ppm) as the internal reference at 100 MHz.
[b] At 125 MHz: all carbons of **A** were assigned by HMBC and HMQC experiments.

Table 5. Minimum inhibitory concentrations of 2′-*N*-acetyl ABK (**A**) and 2′-*N*-acetyl DKB (**C**).

Test organism	Aminoglycoside modifyin enzyme	MIC (μg/ml)				
		ABK	A	DKB	C	AMK
Staphylococcus aureus FDA209P		0.20	1.56	≤0.20	100	0.39
S. aureus Smith		≤0.20	1.56	≤0.20	50	≤0.20
S. aureus Ap01	AAD(4′,4″)	0.78	25	1.56	>100	>100
S. epidermidis 109	AAD(4′,4″)	≤0.20	6.25	1.56	>100	3.13
Bacillus anthracis		≤0.20	1.56	≤0.20	50	0.39
B. subtilis PCI219		≤0.20	3.13	0.78	25	≤0.20
Escherichia coli NIHJ		0.39	1.56	≤0.20	25	≤0.20
E. coli K-12		0.20	1.56	0.39	50	0.39
E. coli K-12 ML1629		0.78	6.25	0.78	100	0.78
E. coli JR66/W677	APH(3′)-II, AAD(2″)	3.13	12.5	50	>100	1.56
Shigella dysenteriae JS11910		3.13	12.5	0.78	100	3.13
Salmonella typhi T-63		0.39	3.13	0.39	50	0.78
Providencia sp. Pv16	AAC(2′)	0.78	3.13	12.5	>100	0.78
Serratia marcescens		6.25	25	12.5	>100	6.25
Pseudomonas aeruginosa A3		0.78	50	0.39	100	0.39
P. aeruginosa TI-13	APH(3′)-I	1.56	50	0.78	>100	1.56
P. aeruginosa GN315	AAC(6′)-4	6.25	>100	100	>100	25
P. aeruginosa 99	AAC(3)-I	6.25	>100	3.13	>100	6.25
P. aeruginosa 21-75	APH(3′)-III	25	>100	>100	>100	6.25
P. aeruginosa PST1	AAC(3)-III	3.13	100	>100	>100	25

Table 6. Anti-MRSA activity of ABK and 2′-*N*-acetyl ABK (**A**).

MRSA*	Isolates tested	MIC (μg/ml)			
		Compound	Range	50 %	90 %
Total isolates	50	ABK	<0.20~3.13	0.39	12.5
		A	1.56~>100	12.5	100
Isolates with APH(2")/AAC(6')	31	ABK	0.39~3.13	0.78	1.56
		A	6.25~>100	25	100
Isolates without APH(2")/AAC(6')	19	ABK	<0.20~0.78	<0.20	0.39
		A	1.56~6.25	6.25	6.25

* Fifty strains of MRSA isolated at a hospital in Osaka in 1986~1990 were used.

MIC of both compounds were determined. As shown in Table 5, **A** exhibited substantial antibiotic activities (MIC: 1.56~3.13 μg/ml) against bacterial strains such as *Staphylococcus aureus* FDA209P, *Bacillus subtilis* PCI219, *Escherichia coli* NIHJ, *Providencia* sp. PV16 containing an AAC(2′) and *Salmonella typhi* T-63, although MIC levels were 1/4~1/8 of those of ABK and AMK (Table 5). However, the activity of **A** against MRSA strains was markedly lower, compared with the excellent activity of ABK (Table 6). By contrast, **C** exhibited no significant antibiotic activity against the organisms tested (Table 5). Furthermore, the two di-acetylated products (**B** and **D**) were inactive (<5% of the activities of ABK and DKB). The good antibacterial activity of 2′-*N*-acetyl ABK was unexpected.

Discussion

S. lividans TK21/pANT12 containing an AAC (2′) gene was resistant to a variety of 2′-NH_2-containing AGs. However, the strain was sensitive to ABK at 2.5 μg/ml (the lowest level tested; Table 1). *In vitro* acetylation reaction using the cell free extract from the organism indicated that no apparent inactivation of ABK occurred when the activity of the incubated reaction mixture was tested with *B. subtilis*. We first considered that these results should be due to the protecting effect by the side chain, AHB, on AAC (2′). To our surprise, however, TLC analysis revealed that ABK was readily converted to 2′-*N*-acetyl ABK yet retained antibiotic activity. The antibiotic activity of 2′-*N*-acetyl ABK was confirmed by the MIC determination experiment (MIC range 1.56~3.13 μg/ml against a variety of bacteria). It has been known that most of AGs markedly lose their antibiotic activities by enzymatic modification such as acetylation, adenylylation and phosphorylation. Accordingly, we did not observe any significant antibiotic activity of 2′-*N*-acetyl derivatives of DKB, RSM and ASTM that we examined. The acetylation product of NM by AAC (2′) retained some activity, however it was much weaker than the 2′-*N*-acetylation product of ABK. Therefore, we believe that the antibiotic activity (MIC range 1.56~3.13 μg/ml) of 2′-*N*-acetyl ABK is exceptionally high among the *N*-acetylated AGs.

It has been shown that ABK is highly active against a wide variety of bacteria including MRSA strains that have AG-modifying enzymes[4~9]. ABK has been clinically used for about 5 years as an MRSA-targeted drug, but the emergence of ABK-resistant strains still remains very low. In fact, only a few MRSA strains with moderate levels (12.5~25 μg/ml) of ABK resistance due to a bifunctional enzyme APH (2″)/AAC (6′) have been reported to date[8,9]. We believe that the lack of 3′ and 4′ hydroxyl groups and the introduction of the AHB side chain play key roles in the activity of ABK to exhibit activity against a wide variety of bacteria with AG-modifing enzymes. Our present study reveals a novel aspect of ABK; 2′-*N*-acetylation did not result in the total loss of its antibiotic activity; the resulting 2′-*N*-acetyl ABK retained substantial antibiotic activity against a variety of bacteria including a *Providencia* strain expressing AAC (2′). It should also be noted that not only this *Providencia* strain but also *S. lividans* TK21 containing the cloned *Streptomyces* AAC (2′) were sensitive to low levels of ABK, although ABK is likely to be 2′-*N*-acetylated in these organisms. Therefore, this novel aspect may be an advantage in the clinical use of ABK.

Based on these considerations, we would predict that the emergence of ABK-resistant strains with AG-modifying enzymes will remain of low incidence. It will be of interest if ABK-resistant strains with AG-modifying enzymes other than APH (2″)/AAC (6′) emerge in the future.

Finally it should be noted that a small amount of inactive 2′,6′-di-*N*-acetyl ABK was formed under the conditions we used. It is not known if this compound is produced by the same enzyme AAC (2′). This question is being investigated.

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References

1) KONDO, S.; K. IINUMA, H. YAMAMOTO, K. MAEDA & H. UMEZAWA: Synthesis 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

2) UMEZAWA, H. & S. KONDO: Mechanism of resistance to aminoglycoside antibiotics. *In* Aminoglycoside Antibi-

otics. *Ed.* H. UMEZAWA & I. R. HOOPER, pp. 267～292, Springer Verlag, Berlin, Heidelberg, New York, 1982

3) KONDO, S.: Development of arbekacin and synthesis of new derivatives stable to enzymatic modifications by methicillin-resistant *Staphylococcus aureus*. Jpn. J. Antibiotics 47: 561～574, 1994
4) OKAMOTO, R.; S. IYOBE & S. MITSUHASHI: Antibacterial activity of HBK. Chemotherapy (Tokyo) 34 (Suppl. 1): 1～10, 1986
5) YAMASHITA, N.; K. UBUKATA, R. NONOGUCHI, A. GOTOH, M. MATSUSHITA & M. KONNO: Antibacterial activity of HBK against staphylococci resistant to aminoglycosides. Chemotherapy (Tokyo) 34(Suppl. 1): 33～40, 1986
6) UBUKATA, K.; N. YAMASHITA, A. GOTOH & M. KONNO: Purification and characterization of aminoglycoside modifying enzymes from *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. 25: 754～759, 1984
7) WADA, K.; H. TAKEDA, M. ARAKAWA, K. OZAKI & M. TAKANO: Studies on multiple-resistant *Staphylococcus aureus*. Chemotherapy (Tokyo) 35: 213～218, 1987
8) KONDO, S.; A. TAMURA, S. GOMI, Y. IKEDA, T. TAKEUCHI & S. MITSUHASHI: Structures of enzymatically modified products of arbekacin by methicillin-resistant *Staphylococcus aureus*. J. Antibiotics 46: 310～315, 1993
9) KONDO, S.; Y. IKEDA, S. HATTORI, M. HAMADA & T. TAKEUCHI: Susceptibility of methicillin-resistant *Staphylococcus aureus* to various antibiotics. Classification by aminoglycoside-modifying enzymes and antibiotics active against MRSA. Jpn. J. Antibiotics 44: 1211～1215, 1991
10) BENVENISTE, R. & J. DAVIES: Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Nat. Acad. Sci. U.S.A., 70: 2276～2280, 1973
11) DAVIES, J.: Another look at antibiotic resistance. J. Gen. Microbiol. 138: 1553～1559, 1992
12) SHAW, K. J.; P. N. RATHER, R. S. HARE & G. H. MILLER: Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. 57: 138～163, 1993
13) HOTTA, K.; A. TAKAHASHI, N. SAITO, Y. OKAMI & H. UMEZAWA: Multiple resistance to aminoglycoside antibiotics in actinomycetes. J. Antibiotics 36: 1748～1754, 1983
14) HOTTA, K.; J. ISHIKAWA, T. OGATA & S. MIZUNO: Secondary aminoglycoside resistance in aminoglycoside-producing strains of *Streptomyces*. Gene, 115: 113～117, 1992
15) UMEZAWA, H. & S. KONDO: Electrophoresis of antibiotics. *In* Methods in Enzymology. Vol. 43. Antibiotics. *Ed.*, J. H. HASH, pp. 279～290, Academic Press, New York, 1975