STRUCTURES OF ENZYMATICALLY MODIFIED PRODUCTS OF ARBEKACIN BY METHICILLIN-RESISTANT *Staphylococcus aureus*

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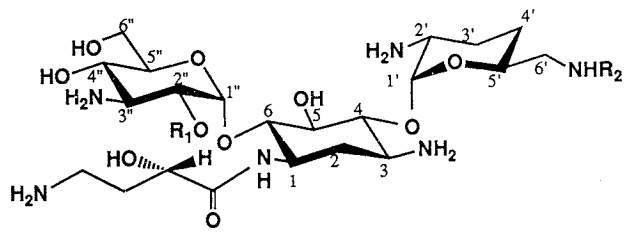
Only a limited number of strains of methicillin-resistant *Staphylococcus aureus* (MRSA) moderately resistant to arbekacin (ABK) have been isolated clinically. Three inactivated products of ABK have been obtained by reaction with excess amounts of a crude enzyme preparation extracted from an ABK-resistant MRSA strain (MIC, 25 μg/ml). The 2″-*O*-phosphate was the major product together with small amounts of the 6′-*N*-acetate and the double modification product. The structures of these modification products were determined by MS and NMR spectral analyses.

Our studies on the resistance mechanism and chemical modifications of aminoglycoside antibiotics led to the synthesis of arbekacin (ABK) (Fig. 1) by the 1-*N*-acylation of dibekacin with (*S*)-4-amino-2-hydroxybutyric acid.[1,2] ABK was refractory to most aminoglycoside-modifying enzymes in resistant bacteria, and inhibited not only Gram-negative bacteria including pseudomonas, but also staphylococci.[3,4] UBUKATA *et al.*[5] reported that ABK is active against all aminoglycoside-resistant strains of *Staphylococcus aureus* and *S. epidermidis* producing the three enzymes, APH (3′), APH (2″)/AAC(6′) and/or AAD(4′,4″). Thereafter, the potent antimicrobial effects of ABK on methicillin-resistant *Staphylococcus aureus* (MRSA) were demonstrated by clinical studies.[6] At the end of 1990 ABK was introduced in Japan as a useful chemotherapeutic agent for the treatment of infections caused by MRSA.

In early 1991～middle 1992, few MRSA strains moderately resistant to ABK (MIC, 12.5～25 μg/ml) were isolated clinically, but highly resistant strains were not. We have examined the inactivation of aminoglycoside antibiotics in four clinical isolates of aminoglycoside-resistant *S. aureus*. Among them,

Fig. 1. Structures of arbekacin and its inactivated products.



Arbekacin (ABK)	$R_1=H$	$R_2=H$
Arbekacin 2″-phosphate (ABK-P)	$R_1=PO(OH)_2$	$R_2=H$
6′-*N*-Acetylarbekacin (ABK-A)	$R_1=H$	$R_2=COCH_3$
6′-*N*-Acetylarbekacin 2″-phosphate (ABK-PA)	$R_1=PO(OH)_2$	$R_2=COCH_3$

two strains were susceptible to ABK, but the other two were moderately resistant (12.5 and 25 μg/ml).

In this paper, we present the profile of inactivation of aminoglycoside antibiotics by crude enzyme extracts from these strains. Furthermore, we have isolated three inactivation products of arbekacin (Fig. 1); arbekacin 2″-phosphate (ABK-P), 6′-*N*-acetylarbekacin (ABK-A) and 6′-*N*-acetylarbekacin 2″-phosphate (ABK-PA), and elucidated their structures by spectral analyses.

Enzymatic Inactivation of Aminoglycoside Antibiotics

Four aminoglycoside-resistant strains of *S. aureus* were used; MS16459 and MS16526 are highly resistant to methicillin (>400 μg/ml); MS16486 and MS16502 are moderately susceptible (3.13 μg/ml). As shown in Table 1, all four strains were highly resistant to kanamycin (KM), tobramycin (TOB) and gentamicin (GM). Among them, MS16459 and MS16486 were susceptible to ABK and moderately resistant to amikacin (AMK). MS16502 and MS16526 were moderately resistant to ABK. From these MIC profiles,[5,7] it was suggested that all strains are of type 2b MRSA, producing two aminoglycoside-modifying enzymes, APH(2″)/AAC(6′)[8,9] and ADD(4′,4″).[10] The MIC values of aminoglycoside antibiotics correlated with inactivation by crude enzyme preparations of the four strains (Table 2). MS16526 strain with MIC 25 μg/ml to ABK was used for subsequent studies on the isolation of inactivated products of ABK.

Isolation of Inactivated Products of ABK

Reactions were carried out in a mixture of ABK (50 μmol) with excess amounts of the crude enzyme solution (260 mg protein), acetyl CoA (0.2 mmol) and ATP (0.2 mmol), at 37°C for 18 hours. The inactivated products in the reaction mixture were purified by column chromatography on Amberlite GC-50 resin. Rechromatography gave mainly ABK-P (32.1 μmol), together with ABK-A (6.6 μmol) and ABK-PA (5.8 μmol) in good yields.

Table 1. MICs (μg/ml) of aminoglycoside antibiotics against clinically isolated *S. aureus*.

S. aureus	ABK	KM	TOB	GM	AMK
MS16459	6.25	>100	>100	>100	50
MS16486	1.56	>100	>100	>100	12.5
MS16502	12.5	>100	>100	>100	100
MS16526	25	>100	>100	>100	>100

Table 2. Inactivation rate (%) of aminoglycoside antibiotics by the crude enzyme preparations.

S. aureus	Enzyme (protein mg/ml)	ABK	TOB	GM	NTL
MS16459	0.38	77	100	100	100
	0.19	39	100	94	89
	0.10	nt	100	66	57
MS16486	0.38	54	100	97	98
	0.19	16	100	61	56
	0.10	nt	65	45	22
MS16502	0.38	50	100	95	97
	0.19	20	100	61	59
	0.10	nt	74	46	25
MS16526	0.38	97	nt	nt	nt
	0.19	58	100	100	100
	0.10	33	100	98	86

nt: Not tested.

Table 3. ^{1}H NMR spectral data.

Proton	ABK		ABK-P		ABK-A		ABK-PA	
	δ ppm	(J Hz)	δ ppm	(J Hz)	δ ppm	(J Hz)	δ ppm	(J Hz)
1-H	4.06 ddd	(12.6, 9.5, 4.4)	4.09 m		4.16 ddd	(12.6, 10.5, 4.6)	4.08 m	
2-Hax	1.77 ddd	(13.0, 12.6, 12.6)	1.92 ddd	(12.7, 12.7, 12.7)	1.88 ddd	(12.8, 12.8, 12.6)	1.90 ddd	(12.8, 12.8, 12.8)
2-Heq	2.11 ddd	(13.0, 4.4, 4.1)	2.33 ddd	(12.7, 4.3, 4.3)	2.30 ddd	(12.8, 4.6, 4.1)	2.32 ddd	(12.8, 4.3, 4.3)
3-H	3.31 ddd	(12.6, 9.7, 4.1)	3.55 ddd	(12.7, 10.0, 4.3)	3.55 ddd	(12.8, 10.3, 4.1)	3.54 m	
4-H	3.84 dd	(9.7, 9.0)	3.99 dd	(10.9, 9.5)	3.93 dd	(10.3, 9.5)	3.87[a] m	
5-H	3.78 m		3.90 m		3.85 dd	(9.5, 9.5)	3.89[a] m	
6-H	3.83 dd	(9.5, 8.7)	3.91 m		3.93 dd	(10.5, 9.5)	3.91[a] m	
1′-H	5.78 d	(3.6)	5.78 d	(3.8)	5.60 d	(3.5)	5.55 d	(3.6)
2′-H	3.49 ddd	(10.8, 6.2, 3.6)	3.59 m		3.60 ddd	(10.8, 4.5, 3.5)	3.56 m	
3′-H_2	1.99 m		2.06 m		2.06 m		2.01 m	
4′-Hax	1.54 dddd	(12.7, 12.7, 11.3, 5.1)	1.65 m		1.60 m		1.57 m	
4′-Heq	1.89 m		1.96 m		1.94 m		1.90 m	
5′-H	4.16 dddd	(11.3, 8.5, 3.1, 2.5)	4.22 m		4.07 m		4.06 m	
6′-H_2	3.03 dd	(13.3, 8.5),	3.14 dd	(13.5, 7.0),	3.34 dd	(14.1, 5.6),	3.31 dd	(14.1, 5.4),
	3.20 dd	(13.3, 3.1)	3.29 dd	(13.5, 3.2)	3.45 dd	(14.1, 5.4)	3.41 dd	(14.1, 5.1)
1″-H	5.15 d	(3.8)	5.37 d	(3.5)	5.22 d	(3.8)	5.36 d	(3.6)
2″-H	3.73 dd	(10.8, 3.8)	4.26 ddd	(11.0, 8.5, 3.5)	3.83 dd	(11.0, 3.8)	4.26 ddd	(11.0, 8.7, 3.6)
3″-H	3.37 dd	(10.8, 10.3)	3.55 dd	(11.0, 10.0)	3.46 dd	(11.0, 10.0)	3.55 dd	(11.0, 10.0)
4″-H	3.62 dd	(10.3, 10.0)	3.76 dd	(10.0, 9.8)	3.74 dd	(10.0, 10.0)	3.77 dd	(10.0, 10.0)
5″-H	4.02 ddd	(10.0, 3.2, 3.2)	4.04 ddd	(9.8, 5.8, 2.1)	4.09 ddd	(10.0, 3.8, 2.6)	4.04 m	
6″-H_2	3.79 m		3.79 dd	(12.3, 5.8),	3.84 m		3.79 dd	(12.3, 5.6),
			3.92 dd	(12.3, 2.1)	3.86 m		3.91 dd	(12.3, 2.6)
2‴-H	4.25 dd	(9.4, 3.8)	4.32 dd	(10.0, 3.5)	4.33 dd	(9.5, 3.8)	4.32 dd	(10.0, 3.6)
3‴-H_2	1.94 ddt	(14.6, 9.4, 7.3),	2.05 ddt	(14.5, 10.0, 7.1),	2.02 m		2.06 ddt	(14.5, 10.0, 7.1),
	2.14 ddt	(14.6, 7.3, 3.8)	2.21 ddt	(14.5, 7.1, 3.5)	2.23 ddt	(14.4, 7.2, 3.8)	2.20 ddt	(14.5, 7.1, 3.5)
4‴-H_2	3.14 t	(7.3)	3.22 t	(7.1)	3.22 t	(7.2)	3.22 t	(7.1)
$NCOCH_3$					2.06 s		2.03 s	

δ: ppm from TSP (0 ppm) in D_2O (pD 2) at 400 MHz.

[a] Chemical shifts were assigned by ^{1}H–^{1}H and ^{1}H–^{13}C COSY.

Table 4. ^{13}C NMR spectral data.

Carbon	ABK δ ppm	ABK-P δ ppm	ABK-A δ ppm	ABK-PA δ ppm	Carbon	ABK δ ppm	ABK-P δ ppm	ABK-A δ ppm	ABK-PA δ ppm
C-1	49.7 d	48.7 d	49.5 d	48.7 d	C-1″	98.7 d	97.8 d	98.9 d	97.8 d
C-2	32.3 t	30.6 t	31.1 t	30.6 t	C-2″	69.0 d	72.0 d	68.8 d	72.0 d
C-3	49.7 d	49.6 d	49.8 d	49.7 d	C-3″	55.8 d	54.7 d	56.0 d	54.7 d
C-4	79.2 d	78.7 d	80.3 d	80.2 d	C-4″	66.7 d	66.6 d	66.3 d	66.6 d
C-5	75.8 d	76.1 d	75.3 d	75.8 d	C-5″	72.8 d	73.6 d	72.9 d	73.5 d
C-6	80.8 d	83.5 d	80.8 d	83.4 d	C-6″	60.6 t	60.8 t	60.5 t	60.8 t
C-1′	95.3 d	96.3 d	96.8 d	97.2 d	C-1‴	176.2 s	176.5 s	176.3 s	176.5 s
C-2′	49.6 d	49.6 d	49.8 d	49.8 d	C-2‴	70.3 d	70.5 d	70.4 d	70.5 d
C-3′	21.6 t	21.3 t	21.9 t	21.9 t	C-3‴	31.6 t	31.3 t	31.6 t	31.3 t
C-4′	26.5 t	26.3 t	26.0 t	26.2 t	C-4‴	37.7 t	37.9 t	37.8 t	37.9 t
C-5′	66.6 d	66.7 d	69.6 d	69.4 d	Ac-CO			175.4 s	175.4 s
C-6′	43.5 t	43.4 t	43.1 t	43.3 t	Ac-CH_3			22.7 q	22.7 q

δ: ppm from TMS in D_2O (pD 2) using dioxane (67.4 ppm) as the internal reference at 100 MHz.

Structure Determination of Inactivated Products of ABK

By MS analysis, the structures of three inactivated products were suggested to be the acetate (ABK-A, MW 594), phosphate (ABK-P, MW 632) and doubly modified product (ABK-PA, MW 674), respectively. The ^1H and ^{13}C NMR spectral signals of ABK were completely assigned by ^1H-^1H COSY, ^1H-^{13}C COSY and differential NOE experiments, and compared with those of inactivated products (Tables 3 and 4).

The ^1H NMR spectrum of ABK-A showed an acetyl CH_3 signal at δ 2.06 and the 6′-methylene signals shifted to lower field than those of ABK. The ^{13}C NMR spectrum showed an acetyl CO and CH_3 signals at δ 175.4 and 22.7, respectively, and the β-carbon (C-5′) of the acetamido group shifted to lower field (δ 69.6). Thus, the structure of ABK-A was determined to be 6′-*N*-acetylarbekacin.

In the ^1H NMR spectra of ABK-P, the 2″-H showed lower-field shift (δ 4.04) and ddd multiplicity having P-O-C-H coupling (J=8.5 Hz). The C-2″ signal at δ 72.0 shifted to lower field than that of ABK (δ 69.0), and two-bond or three-bond couplings between C-2″ or C-3″ and phosphorus were observed. Therefore, ABK-P is arbekacin 2″-phosphate.

From ^1H and ^{13}C NMR spectral data, the doubly modified form of ABK-PA was deduced to be 6′-*N*-acetylarbekacin 2″-phosphate. Although the enzymes were not purified, the isolation of the doubly modified product suggested that ABK was inactivated by a bifunctional enzyme, APH(2″)/AAC(6′) reported by LeGoffic[8,9] and Ubukata.[5] Dual modification by *O*-phosphorylation and *N*-acetylation by an aminoglycoside-modifying enzyme is a new finding. However, the mechanism of formation is not clear and the question remains whether it is produced simultaneously or sequentially.

Thus, complete structure assignments of three new inactivated products of ABK were obtained by MS and NMR spectral analyses.[2] The structural studies on the enzymatic inactivation of ABK will provide new impetus to chemical modifications of aminoglycoside antibiotics.

Experimental

General

MP's were determined with an Electrothermal IA9100 digital melting point apparatus and were uncorrected. FAB-MS was measured on a JEOL JMX-SX mass spectrometer. ^1H and ^{13}C NMR spectra in D_2O at pD 2 were recorded on a JEOL JNX-GSX400 spectrometer. High-voltage paper electrophoresis[11]

(HVPE) was carried out on a CAMAG HVE system at 3,300 V for 15 minutes, using HCOOH - CH_3COOH - H_2O (25 : 75 : 900, pH 1.8) as an electrolyte solution, and stained with ninhydrin reagent. The relative mobilities (Rm) to alanine were calculated and ABK showed Rm 1.87. TLC was carried out on a silica gel plate (E. Merck, Art. 5715) developed with $CHCl_3$ - MeOH - 25% aqueous ammonia (2 : 2 : 1) and ABK showed Rf 0.10. Minimum inhibitory concentrations (MICs) were determined by the serial 2-fold agar dilution method in Bacto Mueller-Hinton Medium (Difco) after incubation at 37°C for 17 hours.

Antibiotics and Resistant Strains

Aminoglycoside antibiotics were obtained as follows: ABK sulfate and AMK sulfate from Meiji Seika Kaisha, Ltd.; GM sulfate, SIGMA Chemical Co.; TOB, Shionogi & Co., Ltd.; netilmicin (NTL) sulfate; Sankyo Co., Ltd. Four clinically isolated aminoglycoside-resistant strains, *S. aureus* MS16459, MS16486, MS16502 and MS16526 were from the collection of the Episome Institute.

Preparation of the Crude Enzyme Solution[12)]

S. aureus MS16526 was inoculated into a medium (100 ml) containing Bacto Tryptone (Difco) 1.0%, yeast extract 0.5%, NaCl 0.5% and glucose 0.1% (pH 7.2) in a Sakaguchi flask and grown at 37°C for 14 hours on a rotary shaker. A 5 ml portion was transferred into the same medium (2 flasks, 200 ml) and grown for 5 hours; these cells were collected by centrifugation at 8,000 × *g* for 20 minutes, washed with 50 mM Tris-HCl - 10 mM $MgCl_2$ buffer (pH 7.8) and resuspended in the same buffer (8 ml). To the cell suspension, lysostaphin (SIGMA) was added at a concentration of 25 μg/ml. The mixture was kept at 37°C for 30 minutes and the cells were disrupted by sonication. The supernatant was obtained by centrifugation at 30,000 × *g* for 30 minutes and used as the crude enzyme solution. The protein content was determined to be 26 mg/ml by Lowry method.

The protein contents of cell extracts from *S. aureus* MS16459, MS16486 and MS16502 strains were 25, 25 and 26 mg/ml, respectively.

Enzymatic Inactivation of Aminoglycoside Antibiotics

Reactions were carried out in a mixture (0.5 ml) containing an aminoglycoside antibiotic (500 μg/ml) 0.05 ml, 0.02 M ATP (SIGMA) 0.05 ml, 0.5 mM acetyl CoA (SIGMA) 0.05 ml, 0.02 M $Mg(AcO)_2$ 0.05 ml and the crude enzyme solution (0.30 ml) diluted (Table 2) with 0.2 M Tris-malate buffer (pH 7.0) at 37°C for 3 hours. Residual antibiotic activity in the reaction mixture was determined by bioassay using *Bacillus subtilis* ATCC6633 as a test organism, and the inactivation % were calculated. The results are shown in Table 2.

Large Scale Inactivation of ABK

To a mixture consisting of 50 mM ABK (27.6 mg/ml H_2O) 1 ml, 40 mM ATP (pH 7.0) 5 ml, 40 mM acetyl CoA in 0.1 M potassium phosphate buffer (pH 7.0, PB) 5 ml, 20 mM $Mg(AcO)_2$ 2 ml and 0.5 M PB 3 ml, the crude enzyme solution (protein content 25 mg/ml, prepared from *S. aureus* MS16526 as described above) 10 ml and 0.1 M PB 10 ml were added. The reaction mixture was incubated at 37°C for 18 hours and heated at 100°C for 2 minutes. The supernatant (33 ml) was obtained by centrifugation at 8,000 × *g* for 10 minutes and did not show any antibiotic activity.

Isolation of Inactivated Products

The supernatant was diluted to 80 ml with H_2O and passed through a column of Amberlite CG-50 (NH_4^+ - H^+ 7 : 3, 20 ml). After washing with H_2O (100 ml), the column was eluted with 2% aqueous ammonia and ninhydrin-positive eluate (20 ml) was concentrated to dryness (31.2 mg). The residue was rechromatographed on a column of Amberlite CG-50 (NH_4^+, 20 ml). The column was eluted with H_2O (40 ml), 0.1% (250 ml) and 0.5% aqueous ammonia (100 ml), successively, and fractions of 3 ml were collected.

ABK-PA (3.9 mg, 5.8 μmol) was obtained by concentration of fractions 2 ~ 4 showing Rm 1.34 (HVPE) and Rf 0.08 (TLC). MP 164 ~ 186°C (dec); FAB-MS (positive) *m/z* 675, (negative) *m/z* 673.

ABK-P (20.3 mg, 32.1 μmol) was obtained from fractions 27 ~ 33 showing Rm 1.68 and Rf 0.07. MP

182~195°C (dec); FAB-MS (positive) *m/z* 633, (negative) *m/z* 631.

ABK-A (3.9 mg, 6.6 μmol) was obtained from fractions 76~85 showing Rm 1.68 and Rf 0.14. MP 152~161°C (dec); FAB-MS (positive) *m/z* 595.

The ^{1}H and ^{13}C NMR spectra of ABK and its inactivated products are shown in Tables 3 and 4, respectively.

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