Communications to the Editor

ACETYLATION OF ASTROMICIN BY THE AMINOGLYCOSIDE 6′-*N*-ACETYLTRANSFERASE OF A GENTAMICIN-RESISTANT *S. AUREUS*

Sir:

Astromicin (ASTM, fortimicin A) exhibited a broad spectrum of *in vitro* and *in vivo* antibacterial activities against many species of bacteria. ASTM has a greater activity against aminoglycoside-resistant strains of Gram-positive and Gram-negative bacteria, which can produce aminoglycoside phosphotransferases [APH(3′), APH(3″)], aminoglycoside adenylyltransferases [AAD(4′), AAD (2″), AAD(6)], and aminoglycoside acetyltransferases [AAC(6′), AAC(2′), AAC(3)-3]. However, ASTM is not active against the strains that produce the AAC(3)-1[1,2]. Therefore, ASTM is effective against bacteria capable of producing various aminoglycoside-inactivating enzymes except for AAC(3)-1. We have been interested in ASTM-inactivating enzymes, *i. e.*, AAC(3)-1 and so far unknown enzymes.

In this paper, we report the isolation and structural determination of the inactivated ASTM resulting from enzymatic reaction with a gentamicin-resistant (GM^r) transductant in *Staphylococcus aureus* MS15009.

In 1982~1983, about 20 to 30% of *S. aureus* strains isolated from hospitals in Japan were resistant to aminoglycoside antibiotics (AGAC). Most of these strains were resistant to all the commercially available AGAC including neomycin (NM), paromomycin (PM), lividomycin (LV), ribostamycin (RB), kanamycin (KM) A, B and C, dibekacin (DKB), gentamicin (GM), sisomicin (SS), amikacin (AK), netilmicin (NT), ASTM and/or streptomycin (SM). Three clinical isolates of *S. aureus* resistant to AGAC have been used for transductional analysis. As shown in Table 1, AGAC resistance was transduced to a lysozyme-sensitive *S. aureus* mutant MS15009 at a frequency of 10^{-6} to 10^{-7}. Resistance patterns of the transductants were examined. All of the transductants were resistant to GM, SS, NT, KM-A, DKB and AK but sensitive to LV and/or SM. The levels of drug resistance (MIC, μg/ml) were GM (50), SS (50), NT (1.6), ASTM (>100), KM-A (>100), DKB (50), AK (6,3) and LV (0.8). Strain MS15009 is AGAC sensitive and the MICs of AGAC were GM (0.2), SS (0.1), NT (0.1), ASTM (0.8), KM-A (0.8), DKB (0.2), AK (1.6) and LV (0.8).

We prepared crude cell-free extracts from a lysozyme-sensitive and GM^r transductant of *S. aureus* MS15009. The lysozyme sensitive mutant of *S. aureus* is sufficiently available for the isolation of macromolecules such as aminoglycoside-inactivating enzymes from the cell lysate after treatment with lysozyme[3].

The reaction mixture was incubated at 37°C for 12 hours and was stopped by heating at 100°C for 5 minutes. Then the mixture was centrifuged at 10,000×*g* for 30 minutes. The residual ASTM activity in the supernatant fluid was bioassayed with *Bacillus subtilis* ATCC 6633 as the test organism. It was found that ASTM was completely inactivated. The supernatant fluid was passed through a column of Amberlite CG-50 (NH_4^+ form, 50 ml), and the column was washed with distilled H_2O. Then the inactivated ASTM was eluted with 0.15 N NH_4OH. The eluted

Table 1. Transduction of aminoglycoside resistances to *S. aureus* MS15009.

Donor	Selective drug[a]	Transduction frequency[b]	No. of colonies tested	Resistance patterns of transductants
MS12567	GM	5.1×10^{-6}	50	GM, SS, NT, ASTM, KM-A, DKB, AK (50/50)
	KM	5.7×10^{-6}	50	
MS12580	GM	9.7×10^{-7}	50	GM, SS, NT, ASTM, KM-A, DKB, AK (50/50)
	KM	3.1×10^{-6}	50	
MS12582	GM	4.9×10^{-6}	50	GM, SS, NT, ASTM, KM-A, DKB, AK (50/50)
	KM	7.7×10^{-6}	50	

[a] GM (3.1 μg/ml), KM (25 μg/ml).

[b] Transduction frequency was expressed as the number of transductants per the number of input phages.

Fig. 1. 6′-*N*-Acetylastromicin and some of its mass spectral degradation productions.

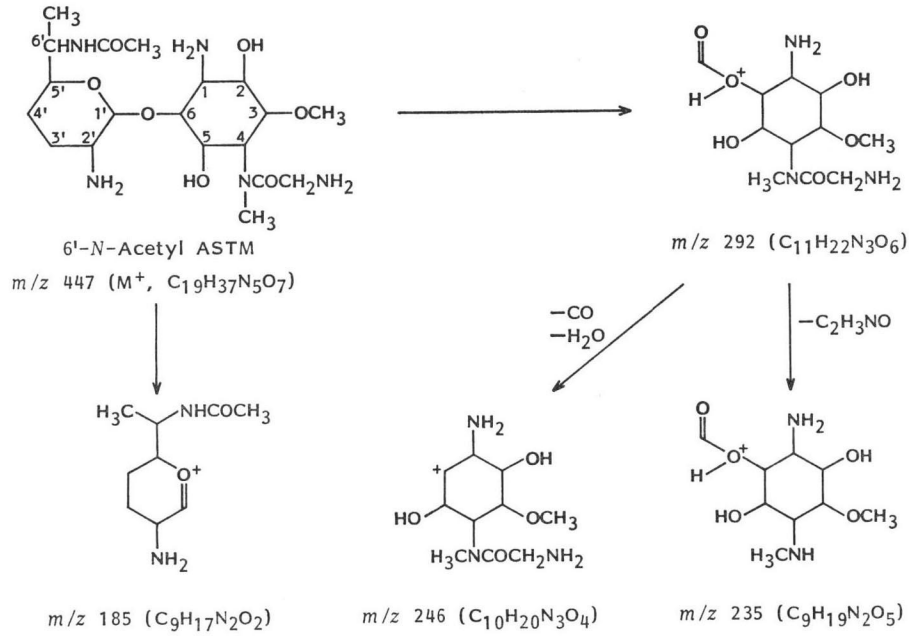

Table 2. Assignment of ^{13}C NMR spectrum of the inactivated astromicin and astromicin.

	Inactivated ASTM			ASTM		
	pD 9.5	pD 1.0	β-Shift	pD 11.5	pD 1.0	β-Shift
C-1	55.4	54.2		55.4	54.2	
C-2	71.0	66.3	4.7	71.1	66.2	4.9
C-3	72.1	72.6		73.7	72.4	
C-4	52.6	52.0		52.5	51.8	
C-5	72.9	71.5		72.9	71.4	
C-6	78.6	74.7	3.9	78.4	74.4	4.0
3-OCH_3	56.4	56.8		56.4	56.8	
4-NCH_3	32.3	32.1		32.2	32.0	
$COCH_2NH_2$	174.4	168.8	5.8	176.4	168.7	7.7
$COCH_2NH_2$	43.1	41.3		43.4	41.4	
C-1′	99.4	96.0	3.4	100.0	95.3	4.7
C-2′	50.0	49.7		50.2	49.4	
C-3′	25.8	21.2	4.6	26.9	21.6	5.3
C-4′	27.1	25.5		27.4	26.3	
C-5′	72.2	72.0		74.9	70.8	4.1
C-6′	49.0	48.6		50.6	51.8	
6′-CH_3	17.0	16.3		18.5	15.3	3.2
$COCH_3$	174.4	174.5				
$COCH_3$	22.8	22.8				

The reaction mixture was incubated at 37°C for 12 hours and was stopped by heating at 100°C for 5 minutes. Then the mixture was centrifuged at 10,000×*g* for 30 minutes. The residual ASTM activity in the supernatant fluid was bioassayed with *B. subtilis* ATCC 6633 as the test organism. It was found that ASTM was completely inactivated. The supernatant fluid was passed through a column of Amberlite CG-50 (NH_4^+ form, 50 ml), and the column was washed with distilled H_2O. Then the inactivated ASTM was eluted with 0.15 N NH_4OH, collected and concentrated to dryness. After apply to a TLC on silica gel (solvent; 2-PrOH - $CHCl_3$ - 25% NH_4OH, 2: 1: 1), the spot which gave positive ninhydrin reaction was raked up, and extracted with 0.15 N NH_4OH and lyophilized. The product (20 mg) showed a single spot of Rf 0.46 on TLC using silica gel and above mentioned solvent system, while the Rf values of ASTM and 1-*N*-acetylated ASTM were 0.43 and 0.51, respectively, under the same conditions.

Table 3. The substrate profiles of aminoglycoside-inactivation enzymes isolated from *S. aureus* MS15009 GM^r.

Substrate	Radioactivity (cpm)		Substrate	Radioactivity (cpm)	
	1-^{14}C	γ-^{32}P		1-^{14}C	γ-^{32}P
Gentamicin C_1	0	25,672	Tobramycin	23,897	10,240
Gentamicin C_{1a}	17,741	25,400	Amikacin	14,654	1,412
Gentamicin C_2	4,197	25,797	Ribostamycin	18,832	0
Sisomicin	8,979	22,679	Butirosin A	2,369	0
Netilmicin	2,993	5,704	Neomycin C	19,764	0
Astromicin A	7,369	0	Paromomycin	0	0
Kanamycin A	24,392	10,341	Lividomycin A	0	0
Kanamycin B	23,563	9,976	Apramycin	0	0
Kanamycin C	0	17,227	Dihydrostreptomycin	0	0
3′,4′-Dideoxykanamycin B	20,134	12,138			

The cell was disrupted sonically at 20 kHz for 15 minutes in ice water bath and then centrifuged at 30,000×*g* for 60 minutes at 4°C. The supernatant thus obtained (S-30 fraction) was used as the crude enzyme solution. The preparative inactivation mixture for ASTM (total volume, 300 ml) contained 60 ml of S-30 fraction (5.1 mg of protein per ml), 220 mg of ASTM, 7.26 g of disodium ATP (neutralized with $NaHCO_3$), 3.18 g of $Mg(CH_3COO)_2 \cdot 4H_2O$, 50 mg of Co-enzyme A, 10 ml of 180 mM 2-mercaptoethanol, 30 ml of 600 mM KCl, 100 ml of 0.2 M Tris-maleate buffer (pH 7.0), and distilled H_2O. Protein content was determined by the method of LOWRY *et al.*

Incorporation of ^{14}C from [8-^{14}C]ATP (adenylylation) was not observed.

fractions which showed a positive ninhydrin reaction were collected and concentrated to dryness. After thin-layer chromatography (TLC) on silica gel (solvent; 2-PrOH - $CHCl_3$ - 25% NH_4OH, 2: 1: 1), the spot which gave a positive ninhydrin reaction was scraped off, extracted with 0.15 N NH_4OH, and lyophilized. The product (20 mg) showed a single spot of Rf 0.46 on TLC using silica gel in the above mentioned solvent system, while the Rf values of ASTM and 1-*N*-acetylated ASTM were 0.43 and 0.51, respectively, under the same conditions.

Major mass fragmentations (prominant peaks at *m/z* 447, 246, 235 and 185, and weak peak at *m/z* 292) of the inactivated ASTM are shown in Fig. 1. The molecular ion of the compound appeared at *m/z* 447, consistent with a mono-*N*-acetylastromicin. Peaks in the mass spectrum due to the fortamine and glycine moieties appeared at *m/z* 292, 246 and 235[4,5]. The intense peak corresponding to the diaminosugar (6′-*epi*-purpurosamine B) appeared at *m/z* 185, however, 42 mass units higher than in the parent antibiotic[4,5], indicating that acetylation had occurred on the diaminosugar moiety. The ^{1}H NMR spectrum (free base in D_2O) of the compound was similar to that of ASTM with the exception of an additional acetyl signal at 1.99 ppm. These results are consistent with a 2′-*N*-acetyl or 6′-*N*-acetyl ASTM.

The specific position of the acetyl group was deduced from the ^{13}C NMR spectrum (Table 2). All the carbons were observed in the inactivated ASTM including the two extra carbons of the *N*-acetyl group at 174.4 and 22.8 ppm. When the ^{13}C NMR spectrum was measured in acidic media, significant β-shifts were observed for the C-2, C-6, carbonyl carbon of the glycyl group, C-1′ and C-3′, but were not observed for the C-5′ and the carbon of 5′-CH_3, indicating that the amino group on C-6′ was not free.

The substrate specificity of the S-30 fractions was examined by radio isotope assay. As shown in Table 3, the enzyme acetylated GM-C_{1a}, GM-C_2, SS, NT, ASTM, KM-A, KM-B, DKB, tobramycin (TB), AK, RB, butirosin (BT)-A and NM-C, but did not acetylate GM-C_1, KM-C, PM and LV, suggesting that *S. aureus* MS15009 GM^r contained a 6′-*N*-acetylating enzyme. The enzyme phosphorylated GM-C group, SS, NT, KM group, DKB, TB and AK with the exception of ASTM, BT-A, NM-C, PM, LV, apramycin and SM, suggesting that the strain MS15009 GM^r produces a APH(2″)[6,7].

Recently JONES *et al.*[8] have reported that Staphylococci producing both AAC(6′) and APH(2″) were resistant to ASTM. However, the inactivation site of ASTM has not yet been eluci-

dated. From our results, the inactivated ASTM was concluded to be 6′-*N*-acetylastromicin. Comparing with our previous results, this enzyme was classified as AAC(6′)-V[9].

TOYOJI OKUBO
MATSUHISA INOUE
MASAO NAGASHIMA
MITSUYOSHI OKII†
TAKAO IIDA†
SUSUMU MITSUHASHI

Laboratory of Drug Resistance
in Bacteria,
Gunma University School of Medicine,
Maebashi, Japan
†Tokyo Research Laboratory,
Kyowa Hakko Kogyo Co., Ltd.
Tokyo, Japan

(Received October 18, 1984)

References

1) OHASHI, Y.; H. KAWABE, K. SATO, N. NAKAMURA, S. KURASHIGE & S. MITSUHASHI: *In vitro* and *in vivo* antibacterial activity of KW1070, a new aminoglycoside antibiotic. Antimicrob. Agents Chemother. 17: 138～143, 1980
2) SATO, S.; T. IIDA, R. OKACHI, K. SHIRAHATA & T. NARA: Enzymatic acetylation of fortimicin A and seldomycin factor 5 by aminoglycoside 3-acetyltransferase I: [AAC(3)-I] of *E. coli* KY 8348. J. Antibiotics 30: 1025～1027, 1977
3) INOUE, M.; T. OKUBO, H. OSHIMA, T. SAITO, M. KATO & S. MITSUHASHI: Isolation and characterization of lysozyme-sensitive mutants of *Staphylococcus aureus*. J. Bacteriol. 144: 1186～1189, 1980
4) EGAN, R. S.; R. S. STANASZEK, M. CIROVIC, S. L. MUELLER, J. TADANIER, J. R. MARTIN, P. COLLUM, A. W. GOLDSTEIN, R. L. DE VAULT, A. C. SINCLAIR, E. E. FAGER & L. A. MITSCHER: Fortimicins A and B, new aminoglycoside antibiotics. III. Structural identification. J. Antibiotics 30: 552～563, 1977
5) IIDA, T.; M. SATO, I. MATSUBARA, Y. MORI & K. SHIRAHATA: The structures of fortimicins C, D, and KE. J. Antibiotics 32: 1273～1279, 1979
6) KAWABE, H.; H. NAGANAWA, S. KONDO, H. UMEZAWA & S. MITSUHASHI: New plasmid-mediated phosphorylation of gentamicin C in *Staphylococcus aureus*. Microbiol. Immunol. 22: 515～521, 1978
7) LE GOFFIC, F.; A. MARTEL, N. MOREAU, M. L. CAPMAU, C. J. SOUSSY & J. DUVAL: 2″-*O*-Phosphorylation of gentamicin components by a *Staphylococcus aureus* strain carrying a plasmid. Antimicrob. Agents Chemother. 12: 26～30, 1977
8) JONES, R. N.; A. L. BARRY, P. C. FUCHS, T. L. GAVAN, H. M. SOMMERS & E. H. GERLACH: Fortimicin A: Collaborative *in vitro* susceptibility comparison with amikacin and gentamicin against 11,840 clinical bacterial isolates. Antimicrob. Agents Chemother. 16: 823～838, 1979
9) MITSUHASHI, S.: Proposal for a rational nomenclature for phenotype, genotype, and aminoglycoside-aminocyclitol modifying enzymes. *In* Drug Action and Drug-resistance in Bacteria. 2. Aminoglycoside Antibiotics. *Ed.* S. MITSUHASHI, pp. 267～270, Univ. Tokyo Press, 1975