Acetylation of Aminoglycoside Antibiotics with 6'-Methylamino Group, Istamycin B and Micronomicin, by a Novel Aminoglycoside 6'-Acetyltransferase of Actinomycete Origin

ATSUKO SUNADA, YOKO IKEDA†, SHINICHI KONDO† and KUNIMOTO HOTTA*

Department of Bioactive Molecules,
National Institute of Infectious Diseases,
1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
† Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku,
Tokyo 141-0021, Japan

(Received for publication July 11, 2000)

An aminoglycoside (AG) 6'-acetyltransferase, AAC(6'), of actinomycete origin[1] characterized by the capability of acetylating astromicin (ASTM) as well as amikacin (AMK) was examined for acetylation of istamycin B[2] (ISMB; an ASTM group AG) and micronomicin (MCR; a gentamicin (GM) group AG) that possess 6'-$NHCH_3$ known to be refractory to some AAC(6')s of clinical origin[3~5]. Consequently it turned out that the actinomycete AAC(6') rather readily acetylated ISMB followed by MCR, correlating with resistance level to these AGs of *Streptomyces lividans* TK21 containing the cloned *aac(6')* gene. The structure of acetylation product of ISMB was analyzed and determined to be 6'-*N*-acetylISMB.

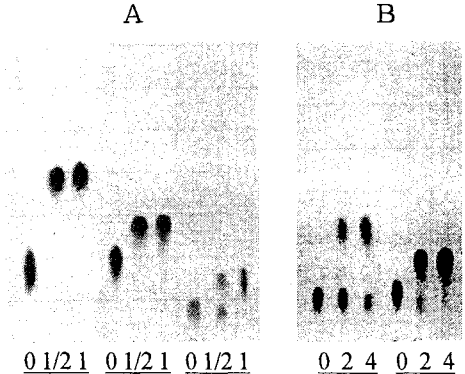
Two types (I containing varieties of subtypes and II) of AAC(6') have been reported among clinical AG-resistant bacteria[6]. They are distinguishable from each other on the basis of difference in substrate specificity to GM and AMK. The type I enzymes are capable of conferring resistance to AMK, but not to GM, whereas the type II enzymes *vice versa*. None of clinically-occurring AAC(6')s reported so far is capable of conferring resistance to both AMK and GM. In addition, none of the known AAC(6')s except for AAC(6')-Ie that was artificially derived from a bifunctional AG-modifying enzyme, AAC(6')/APH(2")[6,7] has been known to be capable of acetylating ASTM. In such a background, we discovered that a rare actinomycete strain #8 possessed a novel AAC(6') showing the same substrate specificity as that of AAC(6')-Ie[1]. Cloning and characterization of *aac(6')* gene designated *rac* revealed that the putative amino acid sequence deduced from the ORF of *rac-aac(6')* gene showed similarity with those of two AAC(6') subfamilies containing AAC(6')-Ie (unpublished).

Another point to note for rac-AAC(6') was its capability of acetylating AGs with 6'-methylamino group such as ISMB and MCR that have been known to be refractory to some AAC(6') enzymes[1~4]. In the present report, we therefore examined the rac-AAC(6') for acetylation of these AGs in relation with the resistance level of *S. lividans* TK21/pANT-S2 containing the cloned *rac-aac(6')* gene.

For acetylation reaction, cell free extracts were prepared from *S. lividans* TK21/pANT-S2 containing the *rac-aac(6')* gene[1] and incubated at 37°C for 3 hours with AGs in the following reaction mixture; 500 μg/ml AG, 0.1 M phosphate buffer (pH 7.0), 1% (v/v) cell free extract and 5 mM acetylCoA (sodium salt; Sigma) in a 50 μl of the reaction mixture. Formation of acetylated compounds was monitored by ninhydrin reaction after TLC using a silica gel plate (E. Merk Art. 5715) and 5% KH_2PO_4 as the developing agent.

Fig. 1-A shows acetylation of ISMB and MCR as well as ASTM. It was obvious that ISMB and MCR, both having 6'-methylamino group, were acetylated by rac-AAC(6'). The acetylation rate was relatively fast with ISMB and ASTM and slow with MCR. Another experiment (Fig. 1-B) indicated that ISMB was acetylated faster than ASTM. The reaction mixtures after complete acetylation did not show substantial antibiotic activity (data not shown). Since it was shown that *S. lividans* TK21/pANT-S2 was resistant to

Fig. 1. Acetylation of ASTM, ISMB and MCR by cell free extracts from *S. lividans* TK21/pANT-S2.



Reaction mixtures containing 0.5 mg/ml of AGs were incubated at 37°C for 1/2~4 hours.

200 μg/ml of ISMB, 100 μg/ml of ASTM and 10 μg/ml of MCR[1], the acetylation rate of ISMB, ASTM and MCR turned out to correlate with the resistance level to these AGs of *S. lividans* TK21/pANT-S2.

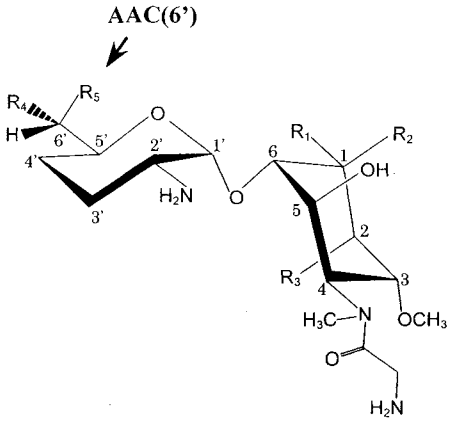
Among the acetylated AG derivatives by rac-AAC(6′), the acetylated ISMB was chosen for isolation and strucutre determination, because the acetylation of ISMB by AAC(6′) has never been reported. A reaction mixture (10 ml) consisting of 10 mg ISMB sulfate (2.13 mM), 0.1 M phosphate buffer (pH 7.0), 3% (v/v) cell free extract and 5 mM acetylCoA was incubated at 37°C for 6 hours. After complete acetylation was confirmed by TLC, the reaction mixture was passed through a column of Amberlite CG-50 (NH_4^+, 5 ml) which was then washed with 10 ml of H_2O. Subsequently, the column was eluted with 0.4% aqueous ammonia and the resulting eluate solution was collected as 0.8 ml fractions. Fractions showing positive reaction with ninhydrin (yellow) and Rydon-Smith reagents after high-voltage papar electrophoresis (HVPE)[8] were rechromatographed on a column of Amberlite CG-50 (NH_4^+, 10 ml). The column was eluted with 50 ml of 0.1% aqueous ammonia and collected as 1.4 ml fractions. From the fractions showing positive reaction upon ninhydrin as well as Rydon-Smith reactions, 5.1 mg of pure acetylated ISMB was obtained as a colorless powder. FABMS spectra were measured using a Jeol JMX-SX102 mass spectrometer. ^1H and ^{13}C NMR spectra ware measured in D_2O at pD1.9 using JMN-A500 spectrometer.

Table 1. NMR spectral data of istamycin B and its acetylation product by rac-AAC(6′).

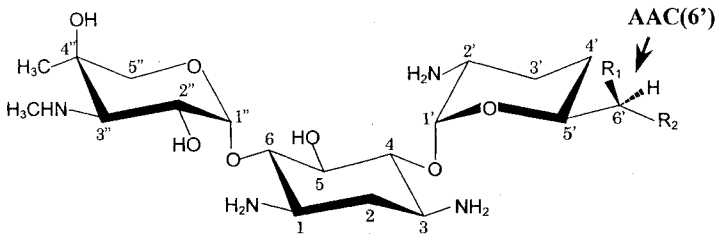
Position	Chemical shift (δ ppm [a, b])			
	ISM B		Acetylated ISM B	
	^{13}C	^1H	^{13}C	^1H
1	47.0	3.88ddd	47.2	3.83ddd
2	29.2	1.84ddd 2.62ddd	29.1	1.79m 2.62m
3	71.8	4.04m	71.8	4.04m
4	56.5	4.37dd	56.6	4.33dd
5	68.2	4.40dd	68.0 (67.7) [c]	4.38m
6	73.3	4.01dd	73.3 (72.9) [c]	4.04m
1'	92.8	5.45d	92.7 (92.1) [c]	5.39d (5.46d) [c]
2'	49.5	3.62ddd	49.5	3.62m
3'	21.2	2.05m	22.0 (21.9) [c]	1.97m
		2.10m		2.07m
4'	26.6	1.60dddd 1.96dddd	26.6	1.57m 1.91m
5'	66.4	4.17m	69.3	4.04m
6'	52.9	3.08dd 3.16dd	52.3	3.41dd 3.56m
3-OCH_3	56.4	3.45s	56.4	3.45s
4-NCH_3	31.8	3.08s	31.8	3.09s
6'-NCH_3	34.2	2.75s	39.2 (35.7) [c]	3.11s (2.95s) [c]
Gly-CO	168.6		168.6 (168.7) [c]	
Gly-CH_2	41.1	4.07ABq	41.1	4.08ABq
Ac-CO			175.5 (175.9) [c]	
Ac-CH_3			21.7 (21.4) [c]	2.12s (2.15s) [c]

a: Measured in D_2O (pD 1.9). b: Assigned by DEPT, ^1H-^1H COSY and ^{13}C-^1H COSY experiments. c: Minor signals (ratio of approximately 2 : 1) of isomeric forms due to *N*-acetylation of the methylamino group are shown in parentheses.

Fig. 2. Structures of 6′-*N*-acetylated derivatives of ASTM, ISMB and MCR.



	R_1	R_2	R_3	R_4	R_5
Astromicin (ASTM)	H	NH_2	OH	CH_3	NH_2
Istamycin B (ISMB)	NH_2	H	H	H	$NHCH_3$
6′-*N*-AcetylASTM	H	NH_2	H	CH_3	$NHCOCH_3$
6′-*N*-AcetylISMB	NH_2	H	H	H	$N(CH_3)COCH_3$



	R_1	R_2
Gentamicin (GM) C_1	CH_3	$NHCH_3$
C_{1a}	H	NH_2
C_2	CH_3	NH_2
Micronomicin (MCR)	H	$NHCH_3$
6′-*N*-AcetylMCR	H	$N(CH_3)COCH_3$

Consequently, the following data were obtained; FABMS (positive) *m/z* 432 (MH^+), HVPE Rm 1.61 (ISMB 1.85), and TLC (MeOH : 17% NH_4OH : $CHCl_3$=4 : 3 : 1) Rf 0.88 (ISMB 0.71). The ^1H and ^{13}C NMR spectra were shown in Table 1. The 6′-H signals (δ 3.41 and 3.56) and the 6′-NCH_3 signal (δ 3.11) shifted to lower field than those of ISMB. The β-carbon shift at C-5′ (δ 69.3) was also observed. Some doubly signals based on isomeric forms of the *N*-acetyl-*N*-methylamino group were observed[9]. Based on these, the acetylated ISMB was determined to be 6′-*N*-acetylISMB (Fig. 2). It showed no substantial antibiotic activity (<0.2% of the activity of ISMB) upon antibiotic asaay (regular cup assay) against *B. subtilis* ATCC 6633 (data not shown).

In this context, we demonstrated that this enzyme acetylated ASTM at 6′-NH_2[1]. MCR will also be acetylated at 6′-NH_2, although structure was not determined.

The rac-AAC(6′) is distinctive from clinically-occuring AAC(6′)s in terms of capability of acetyalting ASTM group AGs although similarities at the amino acid level were observed between rac-AAC(6′) and AAC(6′)-I species[10] such as Im and In (unpublished data). Only AAC(6′)-Ie derived artificially from a bifunctional AG-modifying enzyme AAC(6′)/APH(2″)[6,7] is exceptionally similar to rac-AAC(6′) in substrate specificity. Similarity was also found in their ORF at the amino acid level but not at the nucleotide level (unpublished data). These findings may therefore allow us to categorize rac-AAC(6′) and AAC(6′)-Ie as type III AAC(6′) separating from type I and II AAC(6′)s .

The *rac-aac(6′)* did not confer resistance to GM in spite of its capability of acetylating 6′-$NHCH_3$. This will be due to that GM-C_1 (a component of GM) having both CH_3 and $NHCH_3$ at 6′-position is refractory to rac-AAC(6′) as previously reported[1].

References

1) Zhu, C. B.; A. Sunada, J. Ishikawa, Y. Ikeda, S. Kondo & K. Hotta: Role of aminoglycoside 6′-acetyltransferase in a novel multiple aminoglycoside resistance of an actinomycete strain #8: inactivation of aminoglycosides with 6′-amino group except for arbekacin and neomycin. J. Antibiotics 52: 889~894, 1999

2) Okami, Y.; K. Hotta, M. Yoshida, D. Ikeda, S. Kondo & H. Umezawa: New aminoglycoside antibiotics, istamycins A and B. J. Antibiotics 32: 964~966, 1979

3) Hotta, K.; M. Yoshida, M. Hamada & Y. Okami: Studies on new aminoglycoside antibiotics, istamycins, from an actinomycete isolated from a marine environment III. Nutritional effects on istamycin production and additional chemical and biological properties of istamycins. J. Antibiotics 33: 1515~1520, 1990

4) Okachi, R.; I. Kawamoto, S. Takasawa, M. Yamamoto,

S. SATO, T. SATO & T. NARA: A new antibiotic XK-62-2 (Sagamicin) I. Isolation, physicochemical and antibacterial properties. J. Antibiotics 27: 793~800, 1974

5) OHKOSHI, M.; K. OKADA & N. KAWAMURA: Micronomicin. Jpn. J. Antibiotics 35: 691~703, 1982

6) SHAW, K. J.; P. N. RATHER, R. S. HARE & G. H. MILLER: Molecular genetics of aminoglycosideresistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev. 57: 138~163, 1993

7) FERRETTI, J. J.; K. S. GILMORE & P. COURVALIN: Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J. Bacteriol. 167: 631~638, 1986

8) UMEZAWA, H. & S. KONDO: Electrophoresis of antibiotics. *In* Methods ijn Enzymology, Vol. 43, Antibiotics. *Ed.*, J. H. HASH, pp. 279~290, Academic Press, New York, 1975

9) THOMAS, W. A. & M. K. WILLIAMS: ^{13}C Nuclear magnetic resonance spectroscopy and *cis/trans* isomerism in dipeptides containing proline. J. C. S. Chem. Comm. 1972: 994, 1997

10) WU, H. G.; G. H. MILLER, M. G. BLANCO, R. S. HARE & K. J. SHAW: Cloning and characterization of an aminoglycoside 6′-*N*-acetyltransferase gene from *Citrobacter freundii* which confers an altered resistance profile. Antimicrob. Agents Chemother. 41: 2439~2447, 1972