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Enzymatic 1-*N*-Acetylation of Paromomycin by an Actinomycete Strain #8 with Multiple Aminoglycoside Resistance and Paromomycin Sensitivity

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An actinomycete strain #8 with multiple aminoglycoside (AG) resistance and paromomycin (PRM) sensitivity was examined for its capability of enzymatic modification of AGs. Cell free extracts from the strain converted all of the examined AGs including PRM in the presence of acetyl CoA. PRM was completely modified to at least two products (major and minor spots upon TLC) without significant reduction of the antibiotic activity of the reaction mixture. The structure determination and antibiotic assay of the purified major product revealed 1-*N*-acetylPRM and its antibiotic activity (12% activity of PRM), indicating the existence of AAC(1). It was thus obvious that the 1-*N*-acetylation of PRM did not cause PRM resistance. Apramycin, the substrate of the known AAC(1), was not readily acetylated, suggesting that the AAC(1) of strain #8 is a new type. Two diacetylated products (1,2′-di-*N*-acetylPRM and 1,6‴-di-*N*-acetylPRM) were found in the minor spot, suggesting the existence of additional AACs.

Enzymatic modification has been the major biochemical basis for the aminoglycoside (AG) resistance in clinical bacteria[1~4]. It involves *O*-phosphorylation by aminoglycoside phosphotransferases (APH), *O*-adenylylation by adenylyltransferases (AAD), *N*-acetylation by acetyltransferases (AAC) and both *O*-phosphorylation and *N*-acetylation by a bifunctional enzyme, AAC(6′)/APH(2″)[5~7]. According to recent reports[8~10], AACs and AAC(6′)/APH(2″) have been increasing their importance in clinically-occurring AG resistant bacteria such as *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis*.

HOTTA *et al.* have been interested in the emergence of resistance to arbekacin[4,11] (ABK), which is an anti-MRSA agent in Japan widely used since its approval in 1990, because AAC(6′)/APH(2″)-dependent ABK resistance is major[12,13] although ABK possesses the modification sites for AAC(3), AAC(2′) and AAC(6′). Therefore, they have studied about the possible emergence of AAC-dependent ABK resistance using AAC(3)[14] and AAC(2′)[15] of *Streptomyces* origin. Consequently, it turned out that ABK was readily converted to 3″-*N*-acetylABK and 2′-*N*-acetylABK by the AAC(3) and AAC(2′), respectively. Interestingly, both acetylated products retained substantial antibiotic activity so that neither AAC(3) nor AAC(2′) contributed to ABK resistance.

Recently, we isolated a soil actinomycete strain designated #8 as an ABK resistant and cloned a gene segment coding for an AAC(6′) capable of converting ABK to 6′-*N*-acetylABK with a weak antibiotic activity[12,16]. Strain #8 showed a novel multiple AG resistance profile; *i.e.* resistant to wide varieties of AGs with the 6′-NH_2 including neomycin (NM) but sensitive to paromomycin (PRM) with the 6′-OH that is closely related in structure with NM. Usually, NM-resistant bacteria show the cross resistance to PRM. In this sense, strain #8 that is resistant to NM but sensitive to PRM is very unusual and therefore an unknown biochemical event is suggested to be involved in the AG resistance phenotype. Then we carried out enzymatic modification of AGs using cell free extracts from

strain #8. It turned out that not only the AGs with the 6′-NH_2 but also PRM with the 6′-OH were completely converted to their acetylated products. Interestingly, as in the cases of ABK acetylations[14,15] by AAC(3) and AAC(2′) of *Streptomyces* origin, the incubated reaction mixture of PRM apparently kept high antibiotic activity. In this paper we describe the formation of 1-*N*-acetylPRM and its little contribution to PRM resistance.

Materials and Methods

General

FABMS spectra were measured on a Jeol JMS-SX102 mass spectrometer. ^1H and ^{13}C NMR spectra in D_2O at pD 1.5~2.2 were taken on Jeol JNX-EX400 and JMN-A500 spectrometers. ^1H NMR spectra were recorded using HOD (δ=4.80) as an internal standard, and ^{13}C NMR spectra using 1,4-dioxane (δ=67.4) as an internal standard. High-voltage paper electrophoresis[17] (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 and Rydon-Smith reagents. The relative mobility (Rm) to alanine was calculated. TLC was performed on a silica gel plate (E. Merck, Art. 5715 or 5721) developed with 5% KH_2PO_4 or MeOH/18% NH_4OH/$CHCl_3$ (4:3:1). The plates were dried and sprayed with ninhydrin reagent. AGs as the sulfate were obtained from the antibiotic collection at National Institute of Infectious Diseases. The other chemicals were commercially available.

Antibiotic Resistance

Strain #8 is a soil actinomycete isolated as an ABK resistant[16]. Aerial mycelium of the strain grown on ISP No. 2 or ISP No. 4 agar medium (Difco) were streaked on ISP No. 2 medium plates containing antibiotics at different concentrations ranging 2.5~200 μg/ml and incubated for 1 week at 28°C to check their growth.

Preparation of Cell Free Extract

Strain #8 was grown at 27°C for 2~3 days in 100 ml of Tryptic Soy Broth (Difco) or Nutrient Broth (Difco) supplemented with 5~10 μg/ml of ABK on a rotary shaker. Procedures hereafter were carried out at 0°C. Mycelia were harvested by centrifugation and washed twice with 30 ml of water followed by 30 ml of the following buffer: 10 mM Tris-HCl, pH 7.8, 40 mM NH_4Cl, 10 mM $Mg(AcO)_2$ and 1 mM DTT. The washed and packed mycelium was disrupted by grinding with 3 volumes of Al_2O_3 together with a small amount of deoxyribonuclease I (Sigma) powder and then extracted with 2 ml of the above buffer. The Al_2O_3 was removed by centrifugation and the supernatant was then centrifuged at 30,000×*g* for 30 minutes and the resulting supernatant (S30) as cell free extract was stored at −30°C until use.

Enzymatic Reaction

For acetylation reaction, a 50 μl reaction mixture consisted of 250 μg/ml of an AG, 0.1 M phosphate buffer (pH 7.0), 10% (v/v) cell free extract and 4 mM acetyl CoA, and was incubated at 37°C for a few hours. To check phosphorylation or adenylylation, 4 mM ATP was substituted for 4 mM acetyl CoA.

Isolation of Acetylated Products of PRM

A large scale enzymatic reaction was carried out in a mixture (10 ml) consisting of PRM sulfate (703 μg/mg, 14.2 mg, 16.2 μmol), 50 mM acetyl CoA (Na salt, Sigma), 10% (v/v) of the cell free extract of strain #8 and 0.1 M phosphate buffer (pH 7.0). After incubation at 37°C for 22 hours, the mixture was passed through a column of Amberlite CG-50 (NH_4^+, 18 ml) that was then washed with H_2O (20 ml), and eluted with a gradient solution of 0 to 0.475 N ammonia (250 ml). Approximately 2-ml fractions of the eluate were collected. The eluate fractions No. 92~97 (*ca.* 0.3 N ammonia fraction, 12 ml) containing acetylated products were concentrated to yield a solid (14.5 mg). The solid was loaded on a preparative TLC plate developed with 5% KH_2PO_4. The major product band with Rf 0.16 was eluted, concentrated and subjected Sephadex LH-20 column (80% MeOH) to yield 1-*N*-acetylPRM (6.8 mg) as the pure major acetylated product. From the minor product band with Rf 0.40, a mixture (2.2 mg) of two diacetylated products, 1,2′- and 1,6‴-di-*N*-acetylPRM, was obtained.

Antibiotic Activity

Regular paper disc method was employed for antibiotic assay using Mycin Assay Agar Arei (Mikuni Kagaku Ltd.) seeded with *Bacillus subtilis* ATCC6633 as the test organism. The inhibition zone was measured after incubation at 37°C overnight.

Results

AG Resistance Profile of Strain #8

As shown in Table 1, strain #8 showed resistance (25~200 μg/ml) to wide varieties of AGs; *i.e.* kanamycin (KM),

Table 1. Resistance to and acetylation of aminoglycoside antibiotics by strain #8.

Group	Antibiotic	Resistance[a] (μg/ml)	Acetylation	Activity[b] (%)
Kanamycin	KM	100	+	0
	DKB[c]	100	+ (2 spots)[d]	tr
	AMK[c]	25	+	5
	ABK[c]	25	+	40
Gentamicin	GM	≦2.5	+	30
	SISO	25	nt	nt
	MCR	25	+	tr
	ISP[c]	100	+	tr
	NTL[c]	≦50	nt	nt
Astromicin	ASTM	50	+	tr
	ISM-B	100	+	tr
Neomycin-	RSM	200	+	0
Paromomycin	NM	25	+ (2 spots)[d]	70
	PRM	<2.5	+ (2 spots)[d]	100

[a] ISP No.2 agar medium was used. [b] Antibiotic activity (*B. subtilis* ATCC6633) of the enzymatic reaction mixture relative to that of the control without acetyl CoA was measured. [c] Semisynthetic antibiotics. [d] Two conversion spots were observed upon TLC. nt: not tested, tr: trace.

dibekacin (DKB), amikacin (AMK), ABK, sisomicin (SISO), micronomicin (MCR), isepamicin (ISP), netilmicin (NTL), astromicin (ASTM), istamycin B (ISM-B), ribostamycin (RSM), and NM. However, the strain was specifically sensitive to 2.5 μg/ml of GM and PRM.

Enzymatic Modification of AGs

When incubated in the presence of acetyl CoA with cell free extract from strain #8, all the tested AGs including PRM were converted (Fig. 1 and Table 1). PRM was completely converted to at least two products (major and minor spots upon TLC). The reaction mixtures of DKB and NM also gave two clear spots upon TLC. When the antibiotic activity of the PRM reaction mixture was checked after incubation, no apparent reduction was observed (Table 1). The reaction mixtures of ABK, GM and NM also showed antibiotic activity after incubation. The other reaction mixtures showed no activity. No detectable conversion of PRM and other AGs tested were observed in the presence of ATP (data not shown).

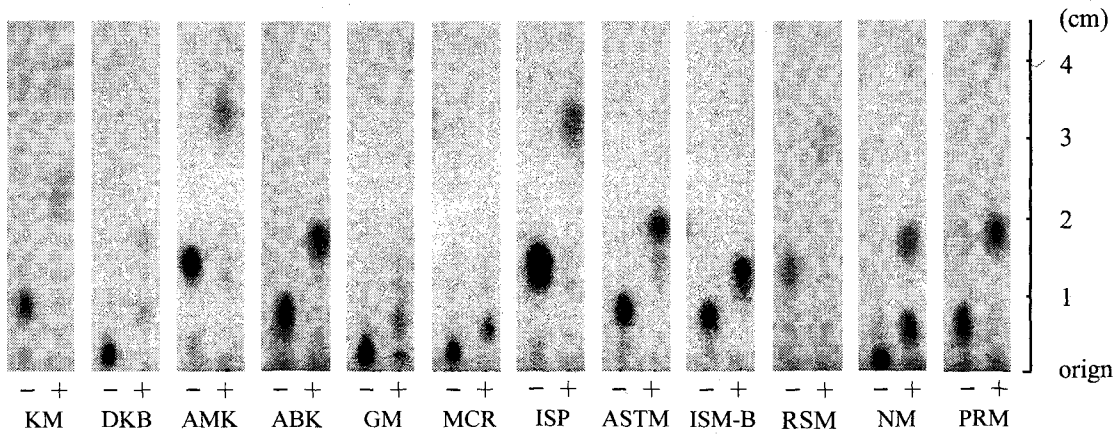
1-*N*-AcetylPRM

The major product, 1-*N*-acetylPRM (Fig. 2) was isolated as a colorless solid; FABMS (pos.), *m/z* 658 $(M+H)^+$ ($C_{25}H_{47}N_5O_{15}$, mol. wt. 657); HVPE Rm 1.49 (PRM: 1.74); TLC (MeOH/18% $NH_4OH/CHCl_3$, 4 : 3 : 1) Rf 0.47 (PRM: 0.34); TLC (5% KH_2PO_4) Rf 0.16 (PRM: 0.11); ^1H and ^{13}C NMR data in D_2O at pD 2.0, see Table 2. ^1H and ^{13}C chemical shift assignments were made by ^1H-^1H-COSY and HMQC experiments. The 1-H signal at δ 3.90 shifted to lower field than that of PRM and the C-2 (δ 30.8) and C-6 (δ 74.8) signals showed β-carbon shifts due to the *N*-acetylation. Based on these, the structure of the major product was determined to be 1-*N*-acetylPRM. The enzyme that catalyzed the 1-*N*-acetylation of PRM was thus regarded as AAC(1). The antibacterial activity against *Bacillus subtilis* PCI219 by a paper disc method showed 12% activity of PRM.

The Minor Di-*N*-acetyl Products

A mixture of two minor diacetylated products, 1,2′- and

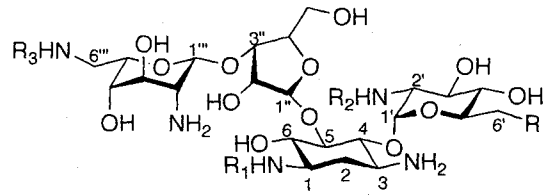
Fig. 1. Silica gel TLC of the enzymatic reaction mixtures.



After incubation for 4 hours, silica gel plates were spotted with 2 μl of the reaction mixtures and developed with 5% KH_2PO_4. −: without acetyl CoA (control), +: with acetyl CoA.

1,6‴-di-*N*-acetylPRM (Fig. 2) at a ratio of 5:2, was obtained as a colorless solid; FABMS (pos.), *m/z* 700 $(M+H)^+$ ($C_{27}H_{49}N_5O_{16}$, mol. wt. 699); HVPE Rm 1.25; TLC (MeOH/18% $NH_4OH/CHCl_3$, 4:3:1) Rf 0.56; TLC (5% KH_2PO_4); Rf 0.40. The structures of the two minor products (1,2′- and 1,6‴-di-*N*-acetylPRM) were deduced from low-field shifts of 1-H, 2′-H and 6‴-H, and high-field shifts of 2-H, 1′-H and 5‴-H in ^1H NMR (500 MHz, pD 1.6); 1,2′-di-*N*-acetyl: δ 3.89 (1-H), 1.66 and 2.26 (2-H), 5.70 (1′-H), 4.05 (2′-H), 2.03 and 2.08 ($COCH_3$); 1,6‴-di-*N*-acetyl: δ 3.89 (1-H), 1.67 and 2.27 (2-H), 4.17 (5‴-H), 3.43 and 3.60 (6‴-H), 5.70 (1′-H), 2.03 and 2.05 ($COCH_3$).

Fig. 2. Structures of paromomycin and its acetyl products.



Neomycin B (NM) R = NH_2, R_1 = H, R_2 = H, R_3 = H
Paromomycin I (PRM) R = OH, R_1 = H, R_2 = H, R_3 = H
1-*N*-AcetylPRM R = OH, R_1 = Ac, R_2 = H, R_3 = H
1,2'-Di-*N*-acetylPRM R = OH, R_1 = Ac, R_2 = Ac, R_3 = H
1,6'''-Di-*N*-acetylPRM R = OH, R_1 = Ac, R_2 = H, R_3 = Ac

Difference in Substrate Specificity between the AAC(1) and the Known One

In order to know whether the difference in substrate specificity, if any, between the putative AAC(1) of strain #8 and the known one is present, we examined enzymatic acetylation of apramycin (APR) that is a good substrate of the known AAC(1)[18]. Consequently, as shown in Fig. 3, it turned out that APR was very slowly acetylated by the cell free extract of strain #8, compared to the acetylation rate of PRM, suggesting the difference in substrate specificity between both AAC(1) enzymes.

Discussion

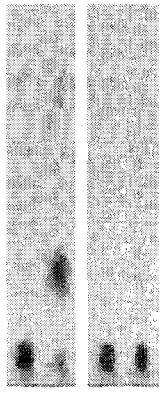
Strain #8 shows multiple resistance to wide varieties of AGs with 6′-NH_2. The strain is however sensitive to PRM lacking 6′-NH_2. In a separate paper[16], we cloned a gene segment (0.9 kb) coding for AAC(6′) that conferred multiple resistance to AGs with 6′-NH_2 except ABK and NM of which the 6′-*N*-acetyl products exhibited weak antibiotic activities. Since strain #8 was sensitive to PRM lacking 6′-NH_2 so that PRM is free from 6′-*N*-acetylation, we have thought that strain #8 should not have any AAC capable of modifying PRM. However, PRM was unexpectedly acetylated and the resulting product, 1-*N*-

Table 2. NMR spectral data of paromomycin and 1-*N*-acetylparomomycin.

	Chemical shift δ ppm			
	PRM		1-*N*-AcetylPRM	
Position	^{13}C	^{1}H	^{13}C	^{1}H
1	50.4	3.38 m	49.6	**3.90** m
2	28.7	1.88 ddd	**30.8**	1.68 ddd
		2.51 ddd		2.28 ddd
3	49.5	3.60 m	50.1	3.54 m
4	77.9	4.07 dd	78.6	4.00 dd
5	84.9	3.95 dd	85.8	3.90 m
6	72.9	3.75 dd	**74.8**	3.58 dd
1'	96.7	5.82 d	96.9	5.80 d
2'	54.4	3.45 dd	54.7	3.44 dd
3'	69.5	3.91 m	69.7	3.95 m
4'	69.8	3.51 dd	70.1	3.52 dd
5'	74.5	3.78 m	74.6	3.80 m
6'	60.7[a]	3.76 m	60.7	3.80 m
		3.91 m		3.94 m
1"	110.7	5.40 d	110.8	5.40 d
2"	74.0	4.42 dd	74.2	4.41 dd
3"	75.7	4.56 dd	75.8	4.58 dd
4"	81.9	4.22 ddd	81.8	4.23 ddd
5"	60.9[a]	3.77 m	61.1	3.80 m
		3.91 m		3.94 m
1"'	95.9	5.32 d	96.0	5.32 d
2"'	51.5	3.60 m	51.7	3.61 dd
3"'	68.3	4.24 dd	68.4	4.26 dd
4"'	67.9	3.84 m	68.1	3.85 m
5"'	70.8	4.34 ddd	71.0	4.35 ddd
6"'	41.1	3.40 m	41.2	3.40 dd
		3.44 m		3.45 dd
Ac-CO			175.1	
Ac-CH_3			22.9	2.03 s

Chemical shifts (δ, ppm) were measured in D_2O at pD 2.0 with a Jeol JNM-A500 spectrometer. Bold letters show β-carbon shifts in ^{13}C spectrum and lower-field shift in ^{1}H spectrum due to the *N*-acetylation.
[a] exchangeable.

Fig. 3. Enzymatic acetylation of paromomycin and apramycin.



Acetylation reaction was performed for 4 hours in the presence (+) and absence (−) of acetyl CoA.

acetylPRM, showed a weak but clear antibiotic activity (12% of PRM activity). To our knowledge, the formation and antibiotic activity of 1-*N*-acetylPRM has never been shown. It was thus obvious that the 1-*N*-acetylation of PRM did not cause PRM resistance.

The AAC that catalyzes the 1-*N*-acetylation of PRM was putatively regarded as an AAC(1) enzyme. The substrate specificity of this enzyme seems to be different from that of the known AAC(1)[18], because apramycin (APR) (a good substrate of the latter) was only slowly acetylated by the enzyme of strain #8. Therefore, the enzyme may be designated AAC(1)-II. Cloning of this AAC gene is now under way.

Concerning the antibiotic activity of the reaction mixture, those of ABK, GM and NM also showed antibiotic activity after incubation. In the case of ABK and NM, it seemed very likely that 6'-*N*-acetylABK[4,16] and 6'-*N*-acetylNM[19] that have antibiotic activity were formed and remained. Therefore, one should be aware of that acetylation does not always result in inactivation of AGs. In the case of GM consisting of three components GM-C_1, -C_2 and -C_{1a}, there was an unconverted spot upon TLC (Fig. 1) that could be regarded as GM-C_1 (having both 6'-*C*- and *N*-methyl groups) known to be refractory to AAC(6')[2].

Another point to note was the reaction mixtures of DKB, NM and PRM for the two conversion spots upon TLC. Actually, the mixture of 1,2'-di-*N*-acetyl and 1,6"'-di-*N*-acetyl products was obtained as the minor products from the PRM reaction mixture. It seemed likely that these diacetylated products resulted from slow acetylation of 1-*N*-acetylPRM by the putative AAC(2') and AAC(6"'). The spots with higher Rf upon TLC of the reaction mixtures of DKB and NM may also be diacetylation products. Based on these findings, it will be conclusive that strain #8 contains some different or multifunctional AACs in addition to AAC(6') and AAC(1).

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