ENZYMATIC CONVERSION OF CEPHAMYCIN C BY D-AMINO ACID OXIDASE FROM *TRIGONOPSIS VARIABILIS*

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(Received for publication March 4, 1980)

D-Amino acid oxidase (EC 1.4.3.3) systems from *Trigonopsis variabilis* SANK 59963 were found to catalyze cephamycin C. The reaction products were assigned to 7β-(5-carboxy-5-oxovalerylamido)-7α-methoxy-3-carbamoyl-3-cephem-4-carboxylic acid, and 7β-(4-carboxybutyrylamido)-7α-methoxy-3-carbamoyl-3-cephem-4-carboxylic acid, respectively. D-Amino acid oxidase from hog kidney was not able to catalyze cephamycin C.

In 1950, penicillin acylase activity in *Penicillium chrysogenum* Q176 was first reported by SAKAGUCHI and MURAO[1]. They reported that the mycelium of this strain was capable of degrading benzylpenicillin into phenylacetic acid and a compound named 'penicin'. The matter rested for some years until BATCHELOR and his coworkers[2] reported that 6-aminopenicillanic acid (6-APA) was detected in a culture broth of *P. chrysogenum* W51.20 to which no side chain precursors had been added; the identity of this 6-APA was proved by these workers by virtue of the fact that, on phenylacetylation, benzylpenicillin was produced.

On the other hand, cephalosporin C which differs from the penicillins by possessing 7-aminocephalosporanic acid (7-ACA) as its nucleus is produced in the culture broth of *Cephalosporium acremonium* (ABRAHAM and NEWTON[3]). Furthermore, cephamycin C was isolated from the culture broth of several strain of streptomycetes[4,5], characterized as having a methoxy group in addition to D-α-aminoadipic acid on the 7-position of the cephem nucleus and found to be much more resistant to β-lactamase[6] than cephalosporin C.

In the penicillin biosynthesis by *P. chrysogenum*, 6-APA with L-α-aminoadipoyl side chain (isopenicillin N) has been thought to be an intermediate for the deacylation or transacylation pathway.

However, no acylase has yet been reported to be capable of removing the D-α-aminoadipoyl chain from naturally occurring cephamycin C or cephalosporin C. Discovery of such an enzyme would greatly facilitate the production of the semi-synthetic cephamycins and cephalosporins, and it is therefore expected that such an acylase will be found in the near future.

However, in 1972, deamination of cephalosporin C was achieved using D-amino acid oxidase from yeast and pig kidney[7,8] to produce 7-ACA with glutaryl side chain which in turn became a feasible substrate for the deacylation by penicillin acylase[9].

The present paper describes enzymatic conversion of cephamycin C by D-amino acid oxidase from *Trigonopsis variabilis* in respect to its reaction product as well as its enzymatic kinetics.

Materials and Methods

Chemicals

DL-Methionine, DL-alanine, D-alanine, DL-α-aminoadipic acid, *o*-dianisidine and D-amino acid oxidase (hog kidney) were purchased from Sigma Chemical Co., Ltd., U.S.A., and Ajinomoto Co., Ltd., Japan. Cephalosporin C and cephamycin C were prepared from the culture broths of *Cephalosporium acremonium* and *Streptomyces jumonjinensis*[5], respectively.

Microorganism

A strain of *Trigonopsis variabilis* SANK 59963 (IAM 4443), a D-amino acid oxidase producing strain[10], was used in this study.

Fermentation

The seed medium used was the basal medium according to SENTHESHANMUGANATHAN and NICKERSON[10] modified by addition of 0.3% DL-alanine. For the fermentation medium, 0.25% DL-methionine in the seed medium was replaced by 0.3% DL-alanine. The seed culture were carried out for 3 days at 28°C on a reciprocal shaker at 120 r.p.m. in 500-ml SAKAGUCHI flasks, each containing 100 ml of the media. Two ml of the seed culture was transferred into a 500-ml SAKAGUCHI flask containing 100 ml of fermentation medium. Fermentation was carried out under the same conditions as those for the seed culture. The cells were harvested by centrifugation of the culture broth at 5,000$\times g$ for 10 minutes at 4°C.

Enzyme preparation

Activation of D-amino acid oxidase in *T. variabilis* was carried out as follows; the cells (20 g in a pasty cake form) were suspended in 0.1 M pyrophosphate buffer (1,000 ml), pH 8.1, added toluene (10 ml), and the mixture was incubated with continuous stirring for 4 hours at 28°C. The activated enzyme preparations (catalase involved) were obtained after centrifugation at 5,000$\times g$ for 10 minutes at 4°C. To determine substrate specificity of D-amino acid oxidase, solubilized enzyme was prepared as follows; the cells of *T. variabilis* were suspended in 0.1 M pyrophosphate buffer, pH 8.1, sonicated for 15 minutes under ice cooling in a sonic oscillator (9 kHz, Chōonpa Kōgyō Co., Ltd., Tokyo) and centrifuged at 10,000$\times g$ for 10 minutes at 4°C. The supernatant fluid was used for the assay of substrate specificity of D-amino acid oxidase.

Assay of D-amino acid oxidase activity

D-Amino acid oxidase activity was determined spectrophotometrically by formation of hydrogen peroxide using *o*-dianisidine as hydrogen donor and indicator.

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on a cellulose sheet (Eastman Chromagram sheet 6065) using the solvent system of *n*-butanol - acetic acid - water (4: 1: 2, v/v). The position of cephamycins on the chromatogram was detected by irradiation with ultraviolet-light or by spraying with ninhydrin or 2,4-DNP reagent.

Determination of organic acid component of C-II

A Shimadzu gas chromatograph unit, GC-6AM, equipped with a flame ionization detector was used. The glass tube column (3 mm inner diameter and 1 m long) was packed with chromosorb W-AW/SE-30. After 4 mg of C-II was hydrolyzed with 0.5 ml of 6 N HCl for 15 hours at 105°C in a sealed tube, the hydrolyzate was extracted twice with each 10 ml of ethyl acetate. The solvent layer was washed with water, dehydrated with Na_2SO_4 and concentrated to dryness *in vacuo*. The sample was dissolved in 0.1 ml of pyridine followed by trimethylsilylation with 0.1 ml of a mixture of N,O-bis (trimethylsilyl)-acetamido and trimethylchlorosilane (4: 1) and was subjected to gas liquid chromatography (GLC). Trimethylsilylated glutaric acid was used as the standard sample.

Antimicrobial activity

The minimal inhibitory concentrations (MIC) of cephamycins against various bacteria were determined by conventional two-fold agar-dilution method.

Results

Substrate Specificity of D-Amino Acid Oxidase

Substrate specificity of D-amino acid oxidase was examined using the enzyme preparation of the cell-free extracts of *T. variabilis* and hog kidney. As reported by MAZZEO *et al.*[7,8], cephalosporin C was catalyzed by D-amino acid oxidase either from *T. variabilis* or hog kidney (Table 1). Cephamycin C, however, was not catalyzed by hog kidney enzyme as shown in Table 1.

Effect of Substrate Concentration on D-Amino Acid Oxidase from *T. variabilis*

At higher concentrations of cephamycin C, deviations from linearity typical for substrate inhibition were observed (Fig. 1). The apparent MICHAELIS constant calculated from LINEWEAVER-BURK plot was 2.5×10^{-3} M.

Table 1. Substrate specificity of D-amino acid oxidase.

Substrate*	Relative activity (%)	
	T. variabilis	Hog kidney
Cephamycin C	1.2	N.D.**
Cephalosporin C	1.0	0.9
DL-α-Aminoadipic acid	34.4	9.1
D-Alanine	100	100

* Substrate conc: 20 mM
** N.D.: not detected

Table 2. Reaction mixture of D-amino acid oxidase.

D-Amino acid oxidase activated cells of *T. variabilis* (catalase involved)	10 g	10 g
Cephamycin C	8 mM	8 mM
Sodium azide (catalase inhibitor)	—	10 mM
Pyrophosphate buffer, pH 8.1	0.1 M	0.1 M
final volume	100 ml	100 ml
Product	C-I	C-II

Chart 1. Isolation of C-I and C-II.

Reaction mixture
|—centrifugation at 5,000×*g*, 10 minutes
Supernatant
|—pH 2.5 with 2 N HCl
|—extracted with EtOAc
Solvent layer
|—conc. *in vacuo*
Syrup
|
Sephadex G-15 column (H_2O)
↓
C-I or C-II
|—2,4-DNP
2,4-DNP derivative of C-I

Fig. 1. LINEWEAVER-BURK plot for the D-amino acid oxidase reaction with cephamycin C.

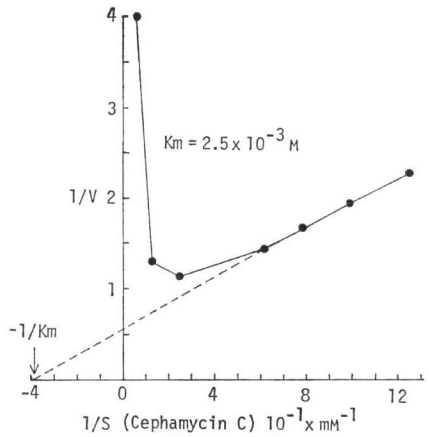
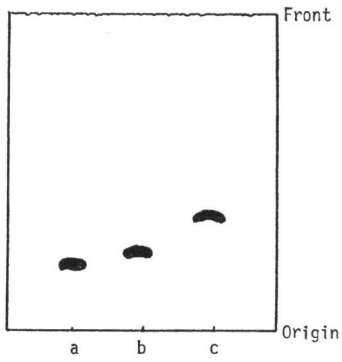


Fig. 2. TLC patterns of the cephamycins.
The TLC developed with *n*-butanol - acetic acid - water (4: 1: 2, v/v).
(a): cephamycin C; UV and ninhydrin positive, 2,4-DNP negative, (b): C-I; UV and 2,4-DNP positive, ninhydrin negative, (c): C-II; UV positive, ninhydrin and 2,4-DNP negative.



Enzymatic Conversion of Cephamycin C

The reaction products of enzymatic conversion of cephamycin C was determined as shown in Table 2.

After incubation under aeration by gentle shaking for 4 hours at 28°C, each product (C-I and C-II) of the reaction mixture was monitored by TLC, respectively. The results are given Fig. 2. C-I was produced in the absence of sodium azide in the reaction mixture, and it was positive for UV and 2,4-DNP and negative for ninhydrin. C-II was produced in the presence of sodium azide as catalase inhibitor, and it was positive for UV, and negative for 2,4-DNP and ninhydrin.

Isolation of the Product Formed by Enzymatic Reaction

The reaction products were isolated from the reaction mixtures by the procedures summarized in Chart 1.

Table 3. Physico-chemical properties of C-I.

Free form	2,4-DNP derivative of C-I
Elementary analysis (%) Found: C 42.99, H 4.61, N 9.12, S 7.02 Calcd.: C 43.18, H 4.30, N 9.43, S 7.20 for $C_{16}H_{19}O_{10}N_3S$ MW=445	Elementary analysis (%) Found: C 41.87, H 4.05, N 14.56, S 5.23 Calcd.: C 42.24, H 3.71, N 15.01, S 5.13 for $C_{22}H_{23}O_{13}N_7S$ MW=625
UV ($\lambda_{max}^{H_2O}$, nm); 263	UV (λ_{max}^{MeOH}, nm); 263, 370
IR (KBr, cm^{-1}); 1780 (β-lactam)	IR (Liquid, cm^{-1}): 1780 (β-lactam)
NMR (δ ppm DMSO-d_6) 1.5~2.6: 6H, m, $-CH_2-CH_2-CH_2-$ 3.3: 2H, ABq, J=18 Hz, $2-CH_2-$ 3.5: 3H, s, $7-OCH_3$ 4.9: 2H, ABq, J=12 Hz, $3-CH_2OCO-$ 5.1: 1H, s, 6-CH-	NMR (δ ppm acetone-d_6) 1.5~2.6: 6H, m, $-CH_2-CH_2-CH_2-$ 3.3: 2H, ABq, J=18 Hz, $2-CH_2-$ 3.5: 3H, s, $-OCH_3$ 4.9: 2H, ABq, J=12 Hz, $3-CH_2OCO-$ 5.1: 1H, s, 6-CH- 8.5~9.05: 3H, m.

Table 4. Physico-chemical properties of C-II.

Elementary analysis (%)	Found: C 43.01, H 4.88, N 9.57, S 7.30 Calcd.: C 43.16, H 4.58, N 10.07, S 7.67 for $C_{15}H_{19}O_9N_3S$ MW=417
Hydrolysis (GLC)	glutaric acid
UV ($\lambda_{max}^{H_2O}$, nm)	263
IR (KBr, cm^{-1})	1780 (β-lactam)
NMR (δ ppm DMSO-d_6)	1.7~2.7: 6H, m, $-CH_2-CH_2-CH_2-$ 3.3: 2H, ABq, J=18 Hz, $2-CH_2-$ 3.5: 3H, s, $7-OCH_3$ 4.65: 2H, ABq, J=12Hz, $3-CH_2OCO-$ 5.05: 1H, s, 6-CH-

Scheme 1. Enzymatic conversion of cephamycin C by D-amino acid oxidase from *T. variabilis*.

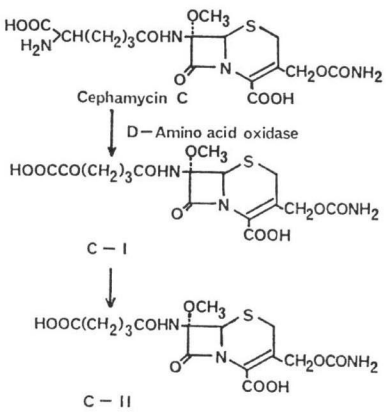


Table 5. Antimicrobial spectra of glutaryl cephamycin C and cephamycin C

Test organism	MIC (mcg/ml)	
	Glutaryl cephamycin C	Cephamycin C
Staphylococcus aureus FDA 209P JC-1	100	>200
S. aureus 56	100	200
Micrococcus luteus PCI 1001	25	25
Bacillus subtilis PCI 219	50	12.5
Mycobacterium smegmatis ATCC 607	200	>200
Escherichia coli NIHJ JC-2	12.5	12.5
E. coli SANK 72375	12.5	12.5
Klebsiella pneumoniae 835	25	12.5
Proteus vulgaris OX19	25	3.12
P. rettgeri SANK 73675	25	50
P. mirabilis SANK 73975	50	3.12
Pseudomonas aeruginosa SANK 73860	25	12.5
P. sp. SC-8756	>200	>200
P. sp. SC-1046	>200	>200

Medium: Heart infusion agar

Characterization and Identification of C-I and C-II

Physico-chemical properties of C-I and C-II are shown in Tables 3 and 4, respectively. The structures of C-I and C-II were assigned to 7β-(5-carboxy-5-oxovalerylamido)-7α-methoxy-3-carbamoyl-3-cephem-4-carboxylic acid, and 7β-(4-carboxybutylylamido)-7α-methoxy-3-carbamoyl-3-cephem-4-carboxylic acid (glutaryl cephamycin C), respectively. As indicated in Scheme 1, C-I is formed from cephamycin C by the action of D-amino acid oxidase from *T. variabilis*, and then C-II is formed from C-I by the action of hydrogen peroxide.

Biological Properties

The antimicrobial spectrum of glutaryl cephamycin C was compared with that of cephamycin C. As shown in Table 5, the antimicrobial activity of glutaryl cephamycin C was weaker than that of cephamycin C against most of the microorganisms tested, but stronger against *Staphylococcus aureus* and *Proteus rettgeri*.

Discussion

Cephamycin C differs from cephalosporin C in its chemical structure by possessing a methoxy group at the 7α-position of the cephem nucleus, but also differs from the view point of the substrate character for enzymatic reaction. As already well known, cephamycins, in contrast to 7-H cephalosporins, are resistant to β-lactamase[6].

The present studies further revealed that glutaryl cephamycin C is also resistant to mammalian D-amino acid oxidase which easily catalyzes its 7-H analogs (Table 1). On the other hand, if acylase attacks the acyl side chain of cephamycin C to form 7-aminocephamycinoic acid (7-ACMA) in the culture broth, glutaryl cephamycin C also might be the key intermediate. Although cephamycin C is difficult to extract from fermentation broth due to its amphoteric and hydrophilic nature, glutaryl cephamycin C can easily be extracted with organic solvent owing to its more hydrophobic nature than cephamycin C, giving some advantage for purification.

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