STEREO-SPECIFIC SYNTHESIS OF 3-TRIFLUOROMETHYLCEPHALOSPORIN DERIVATIVE BY MICROBIAL ACYLASE

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(Received for publication July 7, 1979)

Cephalosporin acylase (EC 3.5.1.11) obtained from *Kluyvera citrophila* ATCC 21285 was found to catalyze synthesis of 7-[2-(2-thienyl)acetamido]-3-trifluoromethyl-3-cephem-4-carboxylic acid from methyl thienylacetate and *dl*-7-amino-3-trifluoromethyl-3-cephem-4-carboxylic acid.

The enzymatically-synthesized compound showed $[\alpha]_D^{25}$ +42.7° (*c* 0.058, MeOH) and its biological activity was about twice as much as that of racemic 7-[2-(2-thienyl)acetamido]-3-trifluoromethyl-3-cephem-4-carboxylic acid chemically synthesized.

As a result, N-acylation by this enzyme was demonstrated to be asymmetric synthesis.

The synthetic chemistry of cephalosporin compounds based on structure-activity relationships has been extensively explored through total synthesis and molecular modifications of naturally occurring cephalosporins and penicillins. An electron-withdrawing substituent, such as CF_3 group, introduced at the 3-position of cephalosporin derivatives facilitates the nucleophilic cleavage of β-lactam amido bond to result in enhancement their antimicrobial activity.

As reported in a recent paper, WATANABE *et al.*[1] succeeded in the total synthesis of (6R, 7R) and (6S, 7S) mixture of 3-trifluoromethylcephalosporin derivatives.

On the other hand, the acylase which hydrolyzes β-lactam antibiotics, such as penicillins and cephalosporins has widely been demonstrated in bacteria, yeast and fungi[2,3]. This enzyme also catalyzes N-acylation of 7-amino-3-cephem compounds or 6-aminopenicillanic acid (6-APA) with organic acid esters to produce their corresponding β-lactam antibiotics.

However, stereo-specificity of these enzymes has not been clarified because of using naturally occurring stereo-specific (6R, 7R) 7-amino-3-cephem compounds and (5R, 6R) 6-APA as substrates.

It was therefore assumed that stereo-specificity of acylase will be demonstrated, if we use a cephalosporin derivatives chemically synthesized as substrates for the enzymatic reaction.

This report describes the enzymatic synthesis of an optically active 3-trifluoromethylcephalosporin derivative by the acylase from *Kluyvera citrophila* ATCC 21285.

Materials and Methods

Chemicals

A *dl*-7-[2-(2-thienyl)acetamido]-3-trifluoromethyl-3-cephem-4-carboxylic acid was prepared as described by WATANABE *et al.*[1] Thienylacetic acid was purchased from Tokyo Kasei Kogyo Ltd.

Microorganism

A strain of *Kluyvera citrophila* ATCC 21285, isolated by NARA *et al.*[4], was used in this study.

Cell preparation

Bacteria was aerobically cultured in a medium: Polypepton (Daigo Nutritive Chemicals 1.0%, w/v), NaCl (0.25%, w/v) and phenylacetic acid (0.15%, w/v), pH 7.0 before sterilization, at 28°C, for 48 hours.

The cells of 100 ml culture broth were collected by centrifugation (10,000 × *g*, 10 minutes) and suspended in 0.1 M phosphate buffer, pH 6.0, to give a final volume of 4 ml.

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on a silica gel plate Kieselgel 60 F_{254} (E. Merck) using the solvent system of chloroform - methanol (2: 1, v/v).

Bioautography

TLC plate was overlaid on the agar plate seeded with *Staphylococcus aureus* FDA 209P JC-1 for 10 minutes. After removal of the TLC plate, the agar plate was incubated at 37°C overnight.

Results and Discussion

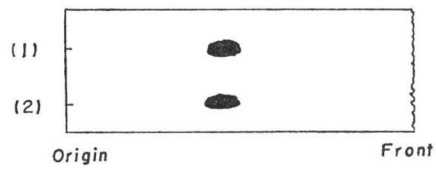
Enzymatic Synthesis of 3-Trifluoromethylcephalosporin Derivative

The transfer reaction of acyl group from acyl donor to *dl*-7-amino-3-trifluoromethyl-3-cephem compound was carried out at 37°C for 4 hours in a 30-ml reaction mixture containing 10 mM *dl*-7-amino-3-trifluoromethyl-3-cephem-4-carboxylic acid, 20 mM methyl thienylacetate and 20 ml of the cell suspension.

After incubation, the supernatant of the reaction mixture was monitored by TLC. As shown in Fig. 1, the enzymatically-synthesized compound was detected at the corresponding position to the chemically-synthesized one.

Fig. 1. Bioautogram of the enzymatically-synthesized compound.

The TLC developed with chloroform-methanol (2: 1, v/v). (1): reaction mixture, (2): authentic chemically-synthesized compound.



Isolation of the Product Formed by Enzymatic Reaction

The reaction mixture was centrifuged at 10,000 × *g* for 10 minutes. The supernatant fluid was concentrated and adjusted to pH 2.5 with 1 N HCl. Extraction with ethyl acetate was performed three times. The combined extract was concentrated to 2 ml *in vacuo*, and the residue purified by silica gel column chromatography using methanol - chloroform (1: 3) as an eluent. Biologically active fractions were combined and concentrated to give 21 mg of a colorless powder.

Characterization and Identification of Enzymatically-synthesized Compound

The NMR and IR spectra of both enzymatically- and chemically-synthesized compounds were compared in Fig. 2.

From the data described above, the enzymatically-synthesized compound was assigned to 7-[2-(2-thienyl)acetamido]-3-trifluoromethyl-3-cephem-4-carboxylic acid. Although the enzymatically-synthesized compound showed $[\alpha]_D^{25}$ +42.7° (*c* 0.058, MeOH), the chemically-synthesized one showed no specific rotation.

On the other hand, the same enzymatic N-acylation was performed by using chemically-synthesized (6R, 7R) and (6S, 7S) mixture of 7-aminodeacetoxycephalosporanic acid and D(−)-α-phenylglycine methylester hydrochloride. Specific rotation of the compound thus obtained was identical with that of cephalexin (6R, 7R).

Fig. 2. The IR (KBr) and NMR spectra of both enzymatically (1) and chemically (2) synthesized compounds.

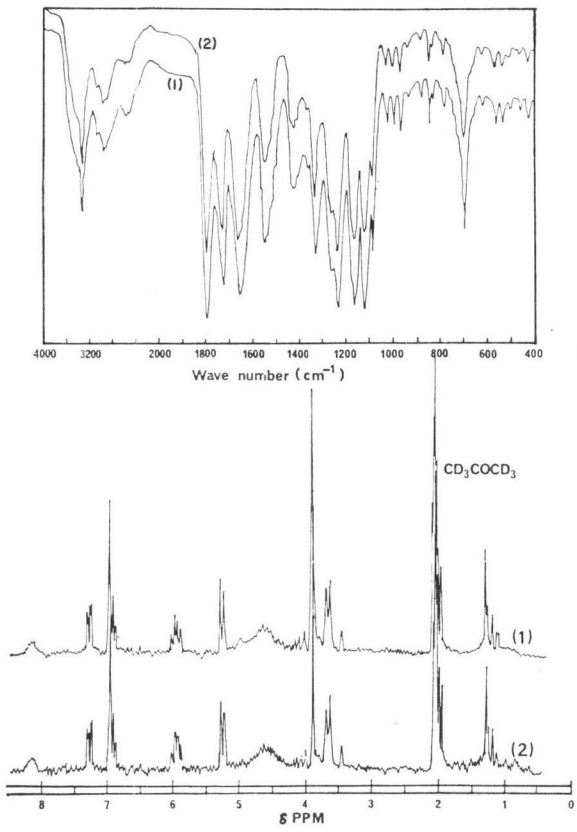


Table 1. Comparison antimicrobial activity of both enzymatically- and chemically-synthesized compounds.

	Antimicrobial activity			
	S. aureus 209P		*E. coli* SANK 70164	
	Conc. (μg/ml)	Activity*	Conc. (μg/ml)	Activity*
Enzymatically-synthesized compound	0.5	29.2	10	21.2
	1.0	31.7	20	26.7
Chemically-synthesized compound	1.0	28.8	20	22.0
	2.0	31.3	40	26.2

* Diameter of inhibitory zone (mm).

Therefore, the enzymatically-synthesized 3-trifluoromethylcephalosporin derivative was confirmed to possess (6R, 7R) configuration.

The Antimicrobial Activity

The antimicrobial activity of both enzymatically- and chemically-synthesized compounds against *Staphylococcus aureus* FDA 209P JC-1 and *Escherichia coli* SANK 70164 was determined by the disc plate method. As shown in Table 1, the activity of the enzymatically-synthesized compound against various microorganisms was about twice as much as that of the chemically-synthesized one. This result clearly indicates that the (6S, 7S) compound is totally inactive biologically.

It is therefore concluded that the stereo-specific synthesis of an optically and biologically active 3-trifluoromethylcephalosporin derivative from *dl*-7-amino-3-trifluoromethyl-3-cephem-4-carboxylic acid is proceeded by microbial acylase which distinguishes the enantiomer from racemic substrates.

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