DE-ESTERIFICATION OF CEPHALOSPORIN *PARA*-NITROBENZYL ESTERS BY MICROBIAL ENZYMES

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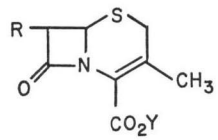
Bacteria and actinomycetes were screened for esterase enzymes capable of removing the *para*-nitrobenzyl ester from cephalosporins. An esterase preparation from *Bacillus subtilis* was used to prepare cephalexin and 7-ADCA from the corresponding *para*-nitrobenzyl esters.

The preparation of α-aminobenzyl cephalosporin antibiotics, such as cephalexin (3), requires that the C-4 carboxyl group be protected during various reactions. The protecting group must be stable to the vigorous cephalosporin modification conditions, but then must be removed easily by mild conditions to yield the C-4 carboxy cephalosporin antibiotic.

We wish to report the detection of microbial enzyme(s) capable of removing the *para*-nitrobenzyl ester protecting group of two cephalosporins.



1 R = Ph-CH(NH_3^+ tosylate$^-$)CONH- Y = $-CH_2-Ph-NO_2-p$

2 R = tosylate$^-$ NH_3^+- Y = $-CH_2-Ph-NO_2-p$

3 R = Ph-CH(NH_2)CONH- Y = -H (Cephalexin)

4 R = H_2N- Y = -H (7-ADCA)

5 R = Ph-OCH_2CONH- Y = -H

Results

Tables 1 and 2 list those cultures that were found to de-esterify compounds **1** and **2**. In general, bacteria were more proficient in removal of the *para*-nitrobenzyl moiety than were actinomycetes. No correlation was found between the ability of organisms to de-esterify compound **1** and compound **2**. *Bacillus licheniformis* de-esterified compound **2** preferentially, while *Actinomyces albocyaneous* gave a low yield of product from **1** but none at all from **2**.

Bacillus subtilis NRRL B 8079 repeatedly gave the highest yields of cephalexin from compound **1**, as well as good yields of 7-ADCA from compound **2**. The esterase from this culture was obtained in crude form. Incubation of the preparation with 500 mg of **1** or the hydrochloride salt of **2** gave, respectively, isolated quantities of 326 mg of cephalexin N-tosylate, or 76.6 mg of 7-ADCA.

The amount of product obtained from the two preparative reactions may have been limited by the insolubility of the starting compounds. Even as tosylate or hydrochloride salts, the *para*-nitrobenzyl esters of cephalexin and 7-ADCA are only partially water soluble.

The relative low yield of 7-ADCA from the hydrochloride of **2** was not due to the amount

Table 1. Formation of cephalexin and 7-ADCA from **1** and **2**, respectively, by bacterial cultures

Culture	Source	Product formed, mcg/ml	
		Cephalexin*	7-ADCA**
Bacillus subtilis	NRRL B558	40	49
Bacillus species	PCI-3 (FDA)	1.25	58
Bacillus circulans	ATCC 9966	36.0	53
Bacillus cereus	NRRL B569	29.0	39
Bacillus licheniformis	ATCC 7072	2.5	140
Bacillus cereus	ATCC 9634	15.6	19.5
Bacillus subtilis	N.I.H.	65	34
Bacillus cereus	NRRL B569	36	16
Bacillus subtilis	D-378 (Fort Detrick)	23	48
Bacillus subtilis	NRRL B1471	31.2	94
Corynebacterium equi	ATCC 6939	1.25	3.0
Staphylococcus aureus	NRRL B314	31.25	13
Micrococcus flavus	ATCC 10240	3.9	8.5
Sarcina lutea	ATCC 9341	3.9	3.0
Sarcina subflava	ATCC 7468	4.8	5.6
Salmonella enteritidis	ATCC 9221	7.3	5.6
Bacillus subtilis	NRRL B8079	92	200

* Determined by agar plate assay with *Sarcina lutea*.
** Determined by acylation of product 7-ADCA with phenoxyacetyl chloride and assay of the resulting deacetoxycephalosporin V with *Bacillus subtilis*.

Table 2. Formation of cephalexin and 7-ADCA from **1** and **2**, respectively, by Actinomycete cultures

Culture	Source	Product formed, mcg/ml	
		Cephalexin*	7-ADCA**
Actinomyces albocyaneous	ATCC 23872	20.0	—
Actinomyces polychromogenes	NRRL 1625	2.5	—
Streptomyces acrimycini	ATCC 19720	8.5	25.0
Streptomyces albus	ATCC 25426	3.8	—
Streptomyces argenteolus	ATCC 23822	5.0	—
Streptomyces candidus	NRRL B3218	7.0	12.5
Streptomyces longisporus	ATCC 23932	1.9	12.5
Streptomyces mutans	NCTC 10449	<2.0	—
Streptomyces olivoreticuli	ATCC 23943	<2.0	—

* Determined by agar plate assay with *Sarcina lutea*.
** Determined by acylation of product 7-ADCA with phenoxyacetyl chloride and assay of the resulting deacetoxycephalosporin V with *Bacillus subtilis*.

of product formed in the reaction. Indeed, the reaction appeared to be essentially complete within 17 hours. However, separation of crystalline 7-ADCA from inorganic salts proved difficult.

Experimental

Screening Procedures

A. Bacterial Cultures

A lyophile pellet of each of the bacterial cultures listed in Table 1 was added

to a 250-ml Erlenmeyer flask containing 50 ml of a 3% sterile Trypticase Soy Broth (Difco) medium. The cultures were incubated for 24~48 hours at 37°C on a rotary shaker revolving at 250 rpm. Five ml of each culture were transferred to 20-ml vials. To one set of vials was added an aqueous suspension of 5 mg of **1**. To the other set of vials was added an aqueous suspension of 5 mg of **2**. The vials were shaken for 24 hours at 30°C on a rotary shaker revolving at 250 rpm. The formation of product in the two sets of vials was determined by an agar plate assay of the culture broths.

To measure the formation of cephalexin (**3**) from compound **1**, paper discs saturated with the culture broths were placed on two agar plates of *Sarcina lutea*. One agar plate contained only the assay organism. The second agar assay plate contained a crude preparation of cephase. The amount of cephalexin produced by each of the bacterial cultures was determined by measuring the zone of growth inhibition obtained with the non-cephase agar assay plate. The quantity of cephalexin produced was then calculated by a dose response plot for pure cephalexin. That the zone of inhibition was indeed due to a cephalosporin was shown by the destruction of the antibiotic activity on the cephase-containing assay plate.

The amount of 7-ADCA (**4**) produced from **2** was determined by the same procedure except that the paper discs were subjected to phenoxyacetyl chloride vapors prior to plating on *Bacillus subtilis* assay plates. The phenoxyacetyl chloride converted the 7-ADCA present to deacetoxycephalosporin V (**5**).

The identity of the antibiotics produced was confirmed by paper bioautographic comparison of each culture broth reaction sample to a standard of cephalexin, or deacetoxycephalosporin V. The paper chromatography system used for cephalexin was *n*-butanol - acetic acid - water (3:1:1). The system used for deacetoxycephalosporin V was methyl ethyl ketone - water (92:8). Bioautographs of the paper chromatograms were obtained with the respective sensitive assay organism described above.

B. Actinomycete Cultures

The ability of the actinomycete cultures listed in Table 2 to conduct the desired process was tested in a manner similar to that described above for the bacterial cultures, except for the following changes. The cultures were grown at 30°C in a medium composed of the following constituents (g/liter): cerelose, 15; soybean meal, 15; corn steep solids, 8; NaCl, 5; $CaCO_3$, 2; and 1.0 liter of tap water.

Preparation of the Cephalosporin Esterase from *Bacillus subtilis*

A lyophile pellet of *Bacillus subtilis*, NRRL B8079, was used to inoculate 50 ml of 3% sterile Trypticase Soy Broth (Difco) medium in a 250-ml Erlenmeyer flask. After shaking for 8 hours at 37°C and 250 rpm on a rotary shaker, the culture was used to provide a 5% (volume/volume) vegetative inoculum for more flasks of sterile broth. After incubating for 18 hours under the above described conditions, the culture was used to provide a 0.6% inoculum for 500-ml Erlenmeyer flasks containing 150 ml of Trypticase Soy Broth medium. After shaking for 24 hours at 30°C, the flasks were pooled and the resulting 40 liters of culture were centrifuged with a Sharples centrifuge and 308.7 g (wet weight) of cells were collected. The cells were suspended in 1 liter of 0.05 M phosphate buffer, pH 6.8, and centrifuged at 5,000 rpm. The collected cells were suspended in 475 ml of the same buffer and sonicated for 15 minutes with a Raytheon Model DF101 Sonic Oscillator, 250 watt, 10 KC. The sonicated cell suspension was centrifuged for 25 minutes at 15,000 rpm. To the resulting supernatant was added ammonium sulfate to 0.8 saturation. The resulting precipitate was collected by centrifugation of the mixture for 30 minutes at 16,250 rpm. The precipitate was resuspended in 300 ml of 0.05 M phosphate buffer and dialyzed against the same buffer. The resulting dialyzate

was lyophilized to give 9.6 g of powdery esterase preparation. LOWERY protein analysis of the preparation gave 0.46 mg of protein per mg of preparation.

Preparation of Cephalexin from **1** with the *B. subtilis* Esterase Preparation

To 1 liter of 0.05 M phosphate buffer, pH 6.8, was added 2.5 g of *B. subtilis*, NRRL B8079, esterase preparation and 500 mg of **1**. After shaking for 18 hours at 30°C, the pH of the reaction mixture was adjusted with 0.1N HCl to 2.5 and the mixture was passed over a 2.5×70 cm column of XAD-4 resin. The column was washed with 2 liters of deionized water and then eluted with methanol. Concentration of the antibiotic-containing methanolic eluate fractions gave 326 mg of cephalexin, N-tosylate. The nmr, uv, infrared spectra, and antimicrobial activity of the material was identical to that of authentic material. Paper chromatographic bioautography of the sample [solvent system, *n*-butanol-acetic acid-water (3:1:1); assay organism, *Sarcina lutea*] gave one zone of inhibition whose Rf value was identical to that of authentic cephalexin.

Preparation of 7-ADCA from 7-ADCA *para*-Nitrobenzyl Ester Hydrochloride with the *B. subtilis* Esterase Preparation

To 1 liter of 0.05 M phosphate buffer, pH 6.8, was added 2.5 g of *B. subtilis*, NRRL B8079, esterase preparation and 500 mg of 7-ADCA *para*-nitrobenzyl ester hydrochloride. After shaking for 18 hours at 37°C, the pH of the reaction mixture was adjusted with 0.1 N HCl to 2.5 and was passed over a 3.5×60 cm column of XAD-2 resin. Elution of the column with water gave 800 mg of a mixture of 7-ADCA and inorganic salts. This material was dissolved in 220 ml of water, the pH of the solution was adjusted to 6.5 with 0.2 N NaOH, and the solution was passed over a 2.0×60 cm column of XAD-2 resin. Elution of the column with 1 liter of water gave 7-ADCA, 76.6 mg free of salts. The nmr of this material was identical to that of authentic 7-ADCA. The paper chromatographic bioautography of the sample after the chromatography paper tapes were treated with phenoxyacetyl chloride [solvent system, *n*-butanol-acetic acid-water (3:1:1); assay organism *Bacillus subtilis*] gave one zone of inhibition with an Rf value identical to that of deacetoxycephalosporin.

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