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ENZYMATIC SYNTHESIS OF THE PENICILLIN AND CEPHALOSPORIN NUCLEI FROM AN ACYCLIC PEPTIDE CONTAINING CARBOXYMETHYLCYSTEINE

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SUMMARY: The tripeptide δ-(L-carboxymethylcysteinyl)-L-cysteinyl-D-valine (L-CMC-CV) is converted sequentially into the CMC analog of isopenicillin N, the CMC analog of penicillin N, and the CMC analog of desacetoxycephalosporin C by, respectively, isopenicillin N synthetase, isopenicillin N epimerase, and desacetoxycephalosporin C synthetase, all isolated from the beta-lactam producing prokaryote *Streptomyces clavuligerus*.

The biosynthesis of desacetoxycephalosporin C (4a) from the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV)(1a) involves the action of three enzymes and their associated cofactors, and proceeds via the intermediates 2a (isopenicillin N) and 3a (penicillin N)(1). The four-equivalent oxidative cyclization of 1a to 2a requires the enzyme isopenicillin N synthetase (cyclase), and ferrous ions and ascorbate as cofactors (2); epimerization of 2a to 3a requires the enzyme isopenicillin N epimerase (3); oxidative ring expansion of 3a to 4a requires the enzyme desacetoxycephalosporin C synthetase (expandase), and ferrous ions, ascorbate and alpha-ketoglutarate (α-KG) as cofactors (4). Each of these enzymes has recently been isolated from *Streptomyces clavuligerus*, functionally purified (2-4), and immobilized successfully (5). When all three enzymes are employed in reaction mixtures, rapid and quantitative conversion of 1a into antibiotics is observed, with the nature of the product determined by the presence or absence of α-KG. The individual steps 1a → 2a, 2a → 3a and 3a → 4a have also been observed, using the appropriate functionally purified enzyme (3,4).

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lactotrophs in the anterior pituitary, and this further suggested that cyclosporine administration might alter the circulating level of prolactin. We found a 4-fold elevation of serum prolactin within 1 h of administration of 12 µg/100 g cyclosporine (66.3 vs. 16.8 ng/ml serum in the cyclosporine-treated vs. vehicle controls, $p < 0.0025$, Fig. 2). The elevation in serum prolactin could be totally blocked by bromocriptine, a dopamine agonist that inhibits release of prolactin at the level of the prolactin-secreting cells in the anterior pituitary. These data are the first report of the ability of cyclosporine to alter the serum concentration of prolactin. Further, cyclosporine does this in a dose-dependent manner.

Materials and Methods

Materials. Sprague-Dawley rats (male, 4 weeks of age) were obtained from the University of Arizona Division of Animal Resources. Cyclosporine (CsA, Sandimmune™) was obtained from Sandoz Pharmaceuticals (East Hanover, NJ). Sesame oil and 2-bromo-α-ergocryptine methane sulfonate was purchased from Sigma Chemical Co. (St. Louis, MO) and [^{125}I]iodine was obtained from ICN (Irvine, CA). Rat prolactin and rat prolactin antiserum (rabbit) were obtained from the NIADDK. Anti-rabbit gamma globulin was obtained from TLC Antibodies (Texas Tech Medical Center, Lubbock, TX). Iodo-beads were from Pierce Chemical Co. (Rockford, IL).

Methods. CsA was dissolved in EtOH (120 mg/ml) to make a stock solution which was stored under refrigeration. The stock stolution was mixed with sesame oil for injection; bromocriptine was suspended in sesame oil for injection. Rats were fed laboratory rodent chow and water ad libitum and were on a 0600-1900 h photoperiod. All rats were used at approximately 4½ weeks of age. For the dose response study, vehicle control rats received sesame oil (100 µl, i.p.) and other rats received 1200, 120, 12, 1.2, or 0.12 µg per 100 g body weight CsA in 100 µl sesame oil, i.p. For the CsA/bromocriptine study, rats received sesame oil (200 µl, i.p.) as the vehicle control or 12 µg CsA/100 g body weight and/or 75 µg bromocriptine/100 g body weight in a total sesame oil volume of 200 µl, i.p. Bromocriptine was given 10 min prior to CsA when both were given. One h after injection, rats were rapidly killed by decapitation and bled. Blood was allowed to clot at room temperature and after centrifugation, serum was removed, frozen and stored until assay. Iodination of prolactin for radioimmunoassay was done with Pierce Iodo-beads using a procedure modified from Markwell (9). The serum prolactin levels were determined by radioimmunoassay using the method of the NIADDK.

Results

Effect of various doses of cyclosporine on serum prolactin levels in the rat

Serum prolactin levels exhibited a bell-shaped dose response curve to CsA. A dose of 0.12 µg/100 g body weight CsA elevated the serum prolactin significantly to a level 2.9-fold above vehicle controls ($p < 0.025$, Fig. 1).

3b → 4b have also been characterized, and it has been shown for the first time that the action of the epimerase leads to a 1:1 equilibrium mixture of 2 and 3.

MATERIALS AND METHODS

Synthesis of Substrates: The peptide 1b was synthesized from L-CMC and S-trityl-L-cysteinyl-D-valine benzhydryl ester (8), following the general procedure developed (10) for the synthesis of ACV. This leads to the disulfide, tlc (butanone-water-acetic acid, 4:1:1) R_f = 0.1; nmr (D_2O, δ): 0.95 (6H, br t), 1.24 (1H, br t), 2.99-3.28 (4H, m), 3.44 (2H, s), 3.98 (1H, q, 6, 8 Hz), 4.35 (1H, d, 9 Hz), 4.82 (1H, m). The penicillins 2b and 3b were synthesized from the ammonium salt of bromomethylpenicillin (11) by a modification of the procedure of Troonen et al (12): the ammonium salt (1.0 mmol) in deoxygenated water (2.0 mL) was treated dropwise at 2°C with an ice-cold solution of cysteine (1.0 mmol) and ammonium carbonate (1.0 mmol) in deoxygenated water (2.0 mL). After 10 min, the pH was adjusted to 7.4 with 7% ammonium hydroxide, and the solution was lyophilized. The penicillin crystallized out following addition of absolute ethanol to a solution of the residue in water (2.0 mL). 2b: tlc (butanone-water-acetic acid, 4:1:1) R_f = 0.45; nmr (D_2O, δ): 1.52 (3H, s), 1.63 (3H, s), 3.10 (1H, q, 15, 7.5 Hz), 3.23 (1H, d, 15, 5 Hz), 3.43 (2H, s), 4.00 (1H, q, 7.5, 5 Hz), 4.31 (1H, s), 5.50 (1H, d, 4 Hz), 5.58 (1H, d, 4 Hz). 3b: nmr (D_2O, δ): 1.52 (3H, s), 1.64 (3H, s), 3.10 (1H, q, 15, 8 Hz), 3.24 (1H, q, 15, 4.7 Hz), 3.44 (2H, s), 3.98 (1H, q, 8, 4.7 Hz), 4.31 (1H, s), 5.49 (1H, d, 4 Hz), 5.59 (1H, d, 4 Hz). The cephalosporin 4b was synthesized analogously, from 7-aminodesacetoxycephalosporanic acid, bromoacetyl chloride and D-cysteine; tlc (butanone-water-acetic acid, 4:1:1) R_f = 0.3; nmr (D_2O, δ): 1.83 (3H, s), 2.99 (1H, q, 14.8, 8 Hz), 3.11 (1H, q, 14.8, 4 Hz), 3.14 (1H, 3, 18 Hz), 3.32 (2H, s), 3.47 (1H, d, 18 Hz), 3.85 (1H, q, 8, 4 Hz), 4.99 (1H, d, 4 Hz), 5.46 (1H, d, 4 Hz).

Organism and Culture Conditions: *S. clavuligerus* NRRL 3585 was maintained and cultivated for the preparation of enzyme extracts as described previously (2).

Enzyme Preparations: Salt-precipitated enzyme concentrate, which contains the cyclase, epimerase and expandase, was prepared as described previously (4). The functional separation of enzyme activities was carried out by ion exchange chromatography on DEAE-trisacryl, as described in Ref 3.

Cyclase Assay: This was carried out using partially purified cyclase, as described previously for 1a (2). The disulfide of 1b (100 μg) was prereduced with dithiothreitol (10 mg/mL, 25 μL) at 21°C for 15 min prior to its addition to the reaction mixture.

Epimerase Assay: This was carried out using partially purified epimerase, as described previously (3).

Expandase Assay: This was carried out using partially purified expandase, as described previously (4).

Multistep Enzyme Assay: This employed salt-precipitated enzyme concentrate and the conditions of the cyclase assay or, alternatively, the conditions of the cyclase assay supplemented with α-KG, KCl and $MgSO_4$ as in the expandase assay.

Biological Assays: Antibiotic in reaction mixtures was estimated by the agar diffusion method as described previously (2). *Micrococcus luteus* ATCC 9341 was used as the indicator organism for the bioassay of cyclase reaction mixtures. *Escherichia coli* Ess was used for the bioassay of epimerase, expandase and multistep reaction mixtures. Where indicated, the agar bioassay medium was supplemented with sterile penicillinase to distinguish penicillin from cephalosporin antibiotics.

High Performance Liquid Chromatography (HPLC): Cyclase and epimerase reaction mixtures were analyzed as described previously (2). Expandase and multistep

reaction mixtures were analyzed by ion pair reversed phase HPLC as described previously (4).

All experiments involving comparisons between the α-aminoadipyl and CMC side chains differed only in the nature of the substrate.

RESULTS AND DISCUSSION

The bioassay data summarized in Table I indicate that each of the steps 1b → 2b, 2b → 3b, and 3b → 4b can be achieved using a salt-precipitated enzyme concentrate (M) which contains the cyclase, epimerase and expandase, or, alternatively, a functionally purified enzyme. In the former case, conversion of the peptide to the penicillin or cephalosporin oxidation level is predetermined by the choice of cofactors. Representative data for the natural sequence 1a → 2a → 3a → 4a are included (in parentheses) for comparison, and demonstrate that the CMC and α-aminoadipyl side chains provide compounds having comparable properties as substrates towards these enzymes.

Antibacterial activity versus *E. coli* Ess following cyclization of ACV in crude enzyme extracts suggested that the product contains both penicillin N and isopenicillin N, since this indicator organism is twenty times more

Table I. Bioassay of Cyclization, Epimerization and Ring Expansion Reaction Mixtures

Substrate	Enzyme	Assay	Reaction Time (min)	Zone of Inhibition (mm) *M. luteus*	*E. coli* Ess[a]	*E. coli* Ess[b]
1b	M[c]	cyclase	15	16(23)[d]	19(25)[d]	0(0)[d]
	M	cyclase	60	24(28)[d]	25(26)[d]	0(0)[d]
	M	expandase	60		22(24)[d]	16(24)[d]
	M	expandase	120		24(26)[d]	23(26)[d]
1b	cyclase		15	27(30)[d]		
	cyclase		30	31(31)[d]		
	cyclase		60	34(32)[d]		
2b	epimerase		0		0	
	epimerase		15		25	
	epimerase		30		26	
3b	epimerase		0		29	
	epimerase		15		27	
	epimerase		30		27	
3b	expandase		15			20(17)[e]
	expandase		30			22(18)[e]
	expandase		60			24(19)[e]

[a]Penicillinase not present in the assay. [b]Penicillinase included in the assay. [c]Refers to the salt-precipitated mixture of enzymes. [d]Data in parentheses refer to 1a as substrate. [e]Data in parentheses refer to penicillin N (3a) as substrate.

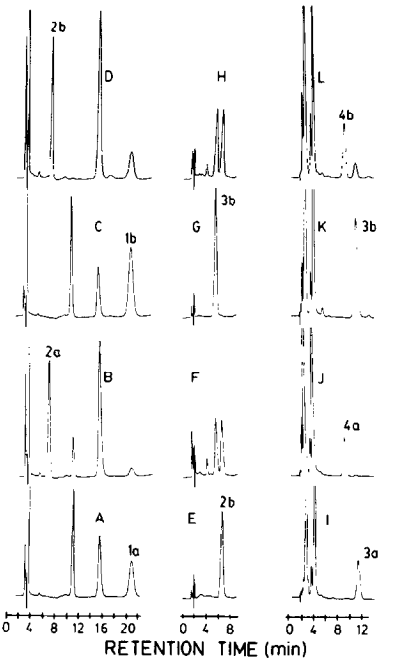

Figure 1. HPLC data for the cyclization of 1a (A, t=0; B, t=15 min) and 1b (C, t=0; D, t=60 min), for the epimerization of 2b (E, t=0; F, t=30 min) and 3b (G, t=0; H, t=30 min), and for the ring expansion of 3a (I, t=0; J, t=15 min) and 3b (K, t=0; L, t=60 min). For A - H, twenty microliter aliquots of reaction mixtures were analyzed with a mobile phase consisting of 5% methanol and 95% KH_2PO_4 (0.05M, adjusted to pH 4.0 with concentrated H_3PO_4). For I - L, the mobile phase was supplemented with 0.002% tetramethylammonium bromide. Retention times are (min): A-H: 2a, 5.9; 3b, 6.1; 2b, 7.2; reduced dithiothreitol, 10.3; oxidized dithiothreitol, 15.2; 1a, 21.1; 1b, 21.2. For I-L: 4b, 9.5; 4a, 9.7; 3b, 11.5; 3a, 12.0.

sensitive towards penicillin N than towards isopenicillin N. This finding led to the discovery of the epimerase (3). Analogous behavior towards *E. coli* Ess is seen in Table I for 2b and 3b, the CMC analogs of isopenicillin N and penicillin N.

It is clear from the bioassay data that each of the enzymatic transformations is rapid under the conditions of these experiments, but bioassay data alone do not allow a quantitative description of the compositions of reaction mixtures. Therefore, all reaction mixtures were monitored concurrently by HPLC; these results are presented in Figure 1 (A-L).

In the case of the natural substrates, the normal HPLC operating conditions (2) do not separate 2a, 3a and 4a; ion pair HPLC (4) separates 2a and 3a from 4a, but not from each other. A quantitative study of the action of the epimerase has thus not previously been possible.

On the other hand, as seen in Figures E-H, the analogs 2b and 3b are well separated under the normal HPLC conditions. These data demonstrate that the epimerase acts on both epimers, and leads to a 1:1 equilibrium mixture of 2b and 3b.

Figures A and B indicate that the cyclization of 1a is complete within 15 min; from C and D it is seen that the cyclization of 1b is somewhat slower. A corresponding difference in the specificity of the expandase is found upon comparison of I and J with K and L. Nevertheless, it is evident that the CMC side chain allows all of these transformations to proceed efficiently.

Penicillins and cephalosporins having the carboxymethylcysteinyl side chain have been found to have interesting antibacterial activity, with the D-epimer much more active than the L- (12,13). Unfortunately, chemical syntheses of the D-epimers of these compounds require D-cysteine ($58/g). Because the epimerase leads to a mixture of 2b and 3b, an enzymatic synthesis of 3b from 2b would not be practical, at least under the conditions reported here. However, an enzymatic synthesis of the cephalosporin 4b from 2b is now feasible, using the combined action of the epimerase and expandase.

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