ENZYMATIC CONVERSION OF THE UNNATURAL TRIPEPTIDE δ-(D-α-AMINOADIPYL)-L-CYSTEINYL-D-VALINE TO β-LACTAM ANTIBIOTICS

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Incubation of the unnatural tripeptide δ-(D-α-aminoadipyl)-L-cysteinyl-D-valine (DLD-ACV) with a partially purified extract of *Cephalosporium acremonium* resulted in the production of deacetoxycephalosporin C. The extract contained isopenicillin N synthetase (cyclase) and deacetoxycephalosporin C synthetase (expandase) but no penicillin epimerase activity, and was incubated aerobically in the presence of the components of the cyclase and expandase reaction mixtures (Fe^{++}, ascorbate, dithiothreitol, α-ketoglutarate and ATP). The reaction was sensitive to penicillinase, indicating penicillin N to be an intermediate. However, when ring expansion was prevented by omission of α-ketoglutarate and ATP, no penicillin N was detected.

The normal biosynthetic pathway to cephalosporins in *Cephalosporium acremonium* is shown in Fig. 1. It involves the cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) to isopenicillin N by isopenicillin N synthetase (cyclase), the epimerization of isopenicillin N to penicillin N by penicillin epimerase (epimerase), and the ring expansion of penicillin N to deacetoxycephalosporin C by deacetoxycephalosporin C synthetase (expandase)[1]. Each of these enzyme activities has been detected in cell-free extracts of *C. acremonium*[2,3] and *Streptomyces clavuligerus*[4]. Since the cyclase accepts adipyl-L-cysteinyl-D-valine[3] as a substrate, suggesting that the amino group of the aminoadipyl moiety is not essential, we wondered whether a tripeptide containing the D-aminoadipyl moiety, *i.e.*, δ-(D-α-aminoadipyl)-L-cysteinyl-D-valine (DLD-ACV) would undergo cyclization. Frozen extracts of *C. acremonium* normally lack epimerase activity because of the lability of this enzyme; these extracts therefore convert tripeptides only to the penicillin oxidation level. However, were DLD-ACV to behave as a substrate, the need for an epimerase would be obviated, since penicillin N would be produced directly. This compound should undergo ring expansion to deacetoxycephalosporin C. The present paper examines this point and reveals an unexpected finding.

Materials and Methods

Organism

C. acremonium CW-19 (*Acremonium chrysogenum* ATCC 36225) was used throughout the study.

Medium and Culture Conditions

C. acremonium was maintained on agar slants and inoculated into seed and fermentation media as

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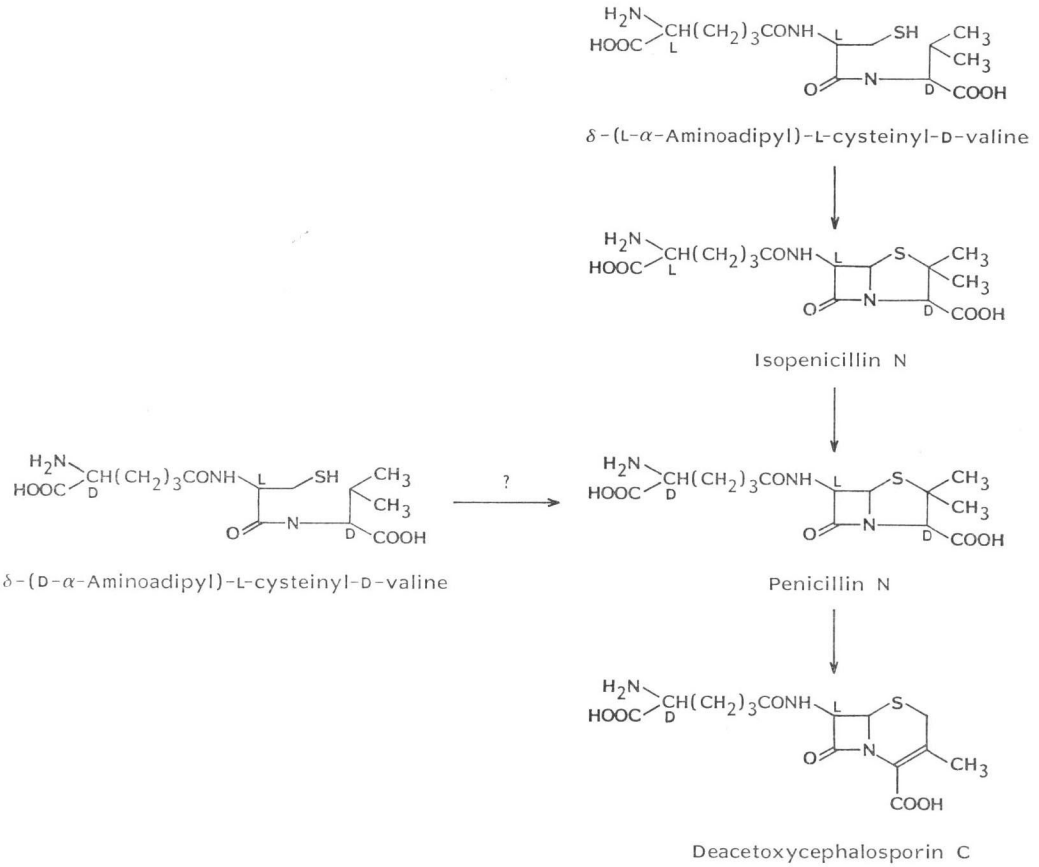
Fig. 1. Diagram depicting the conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to deacetoxycephalosporin C.

The arrow with the question mark shows the possible entry of the unnatural tripeptide δ-(D-α-aminoadipyl)-L-cysteinyl-D-valine into the pathway.



previously described[5]. The chemically defined fermentation medium was prepared, adjusted to pH 7.3 with NaOH, and autoclaved without the sugar components. These were sterilized separately and added aseptically before inoculation. Ten percent inoculum was used. Fermentations were carried out in 250-ml flasks containing 40 ml of medium at 25°C on a rotary shaker at 250 rev/minute.

Preparation of Cell-free Extract

At 90 hours, 80 ml of fermentation broth was withdrawn and the mycelia were recovered by filtration. After thorough washing with distilled H_2O, 6 g of cells (wet weight) were suspended in 15 ml of Tris-salts (0.05 M Tris-HCl (pH 8), 0.01 M $MgSO_4$) and the suspended cells were ruptured by sonication as previously described[3]. The extract was partially purified by protamine sulfate treatment (0.8%), $(NH_4)_2SO_4$ precipitation (50~80% saturation) and Sephadex G-25 gel filtration. The partially purified preparation was kept at −65°C until use. The preparation as used contained cyclase and expandase but no epimerase activity.

Cyclase Assay[3]

The 1-ml final reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM KCl, 10 mM $MgSO_4$, 0.2 mM $FeSO_4$, 0.67 mM ascorbic acid, 0.75 mM dithiothreitol (DTT), 0.28 mM LLD-ACV (or DLD-ACV), and 100~200 μl enzyme solution (containing 0.17~0.55 mg protein). Before the assay, the bis-LLD-ACV (or bis-DLD-ACV) was reduced to its monomeric form by preincubation with 3.75 mM DTT in 0.2 ml total volume for 15 minutes at room temperature. The cyclization reaction was started by add-

ing the ACV-DTT solution to the mixture containing enzyme and cofactors. Incubation lasted up to 60 minutes at 25°C and 250 rev/minute. The reaction was stopped by the addition of 1 ml MeOH. After removal of the precipitated proteins by centrifugation, the supernatant fluid was analyzed for isopenicillin N (or penicillin N) formation by bioassay against *Micrococcus luteus* ATCC 381, with penicillin N serving as standard.

Expandase Assay[6]

The 1-ml final reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM KCl, 10 mM $MgSO_4$, 0.04 mM $FeSO_4$, 0.67 mM ascorbic acid, 0.8 mM ATP, 0.6 mM α-ketoglutarate, 0.28 mM penicillin N [or DLD-ACV (bis-DLD-ACV pretreated with DTT as described above)] and 400~800 μl of enzyme solution (0.68~2.2 mg protein). The reaction was started by the addition of penicillin N (or DLD-ACV) and the mixture was incubated up to 60 minutes at 25°C and 250 rev/minute. The reaction was stopped by the addition of 1 ml methanol. After removal of the precipitated proteins, the supernatant fluid was analyzed for cephalosporins by bioassay against *Pseudomonas aeruginosa* Pss, seeded in agar containing 500 units/ml of penicillinase (Difco Laboratories, Detroit, MI).

Analysis of Assay Mixture by HPLC

Separation of reaction products was achieved using an M 6000 A pump, U6K septumless injector, model 440 absorbance detector (fixed wavelength, 254 nm), M 730 data module, and System M 720 controller, all from Waters Associates (Milford, MA). Cephalosporins were eluted from a DuPont (Zorbax NH_2) column (3.6×150 mm), using AcOH - MeOH - CH_3CN - H_2O, 2: 4: 7.5: 86.5, mobile phase[7]. Reaction mixtures (100 μl) were injected and eluted at about 210 kg/cm^2 (3,000 psi, 1 psi= 6.895 KPa) at a flow rate of 4 ml/minute.

Protein Determination

Protein was measured by the method of BRADFORD[5]. Bovine serum albumin was used as standard.

Results

Activity of DLD-ACV as Substrate in the Cyclase and Expandase Assays

The replacement of LLD-ACV by DLD-ACV in the cyclase reaction did not generate antibiotic activity against *M. luteus*, which was not surprising given the known inactivity of other unnatural tripeptides[3]. Nevertheless antibiotic activity was generated against *P. aeruginosa* Pss in the expandase reaction assay, when DLD-ACV was substituted for penicillin N (Table 1). As expected, LLD-ACV showed no activity in the expandase assay. The product(s) formed from DLD-ACV was presumed to be a cephalosporin(s), since the penicillinase in the expandase assay (*P. aeruginosa* Pss) plates would destroy penicillin products. This was confirmed by HPLC analysis, which showed deacetoxycephalosporin C as a major product and deacetylcephalosporin C as a minor product.

Effect of Penicillinase on the Reaction

One possible explanation of the generation of antibiotic activity from DLD-ACV is that DLD-ACV

Table 1. Comparison of LLD-ACV and DLD-ACV as substrates in the cyclase and expandase assays.

Substrate	Antibiotic zone diameter (mm)								
	Cyclase assay (minutes)					Expandase assay (minutes)			
	0	10	20	30	60	0	30	60	90
LLD-ACV	—*	25.5	25.5	27.5	28.0	—	—	—	—
DLD-ACV	—	—	—	—	—	—	trace	13.5	14.0

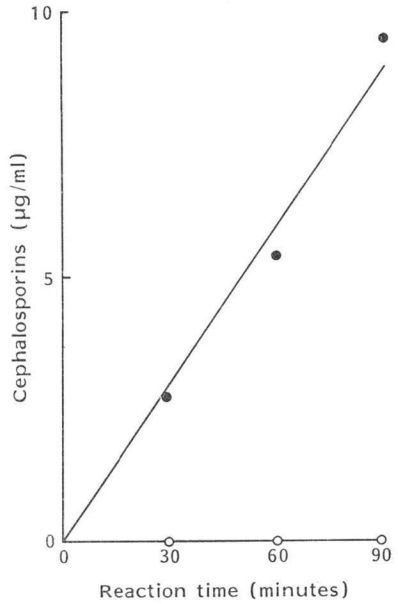
* — No zone around paper disk of 6.35 mm diameter.

is cyclized to penicillin N and is rapidly expanded to deacetoxycephalosporin C in the expandase assay because the assay mixture contains the components of the cyclase reaction mixture. If this explanation were correct, the process would be sensitive to penicillinase because penicillin N produced transiently would be destroyed. As seen in Fig. 2, addition of penicillinase to the reaction mixture completely inhibits the formation of cephalosporins. Penicillin N is thus implicated as an intermediate in the conversion of DLD-ACV to deacetoxycephalosporin C. If this is the case, it is necessary to explain why penicillin N does not accumulate in the cyclase assay. To check whether penicillin N inhibits the cyclase*, this penicillin was added to a normal cyclase reaction mixture using LLD-ACV as substrate. No inhibition of cyclization of LLD-ACV to isopenicillin N was observed even in the presence of a 3-fold excess of penicillin N.

Fig. 2. Effect of penicillinase on conversion of DLD-ACV to cephalosporins.

Penicillinase was used at 500 units/ml and DLD-ACV was used at 0.56 mM.

● DLD-ACV, ○ DLD-ACV+penicillinase.



Discussion

It is clear that DLD-ACV can be cyclized to penicillin N and the latter compound expanded to deacetoxycephalosporin C when the peptide is incubated with the cofactors necessary for both the cyclase and expandase reactions. However, penicillin N does not accumulate when only the cyclase assay components (Fe^{++}, ascorbate, DTT and air) are present. This suggested that penicillin N inhibits the cyclization of DLD-ACV to penicillin N. We attempted to study this possibility indirectly, *i.e.*, by determining its effect on cyclization of LLD-ACV to isopenicillin N, but no inhibition was observed; however, penicillin N may specifically inhibit its own formation.

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