Cell-Free Conversion of δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine into an Antibiotic with the Properties of Isopenicillin N in *Cephalosporium acremonium*

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Cell-free extracts of antibiotic-negative mutants of *Cephalosporium acremonium* converted δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-tripeptide) into an antibiotic that was destroyed by penicillinase. The enzymic activity of the extracts was destroyed by boiling, but was not inhibited by cycloheximide. LLL-Tripeptide was totally inactive as substrate. The product resembled isopenicillin N, but not penicillin N, in its antibacterial spectrum. We propose that isopenicillin N is the first product of cyclization of LLD-tripeptide.

The use of protoplasts and cell-free extracts for studying β-lactam antibiotic biosynthesis was introduced by the Oxford group (Duncan & Newton, 1970; Fawcett *et al.*, 1973). Duncan & Newton (1970), by using sonicated protoplasts of *Cephalosporium acremonium*, observed incorporation of DL-[^{14}C]valine into a compound that, on oxidation, behaved like penicillaminic acid. They concluded that valine had been converted into penicillin N. Fawcett *et al.* (1976*a*) reported briefly that ^{3}H from labelled δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-tripeptide) was incorporated by lysed protoplasts into a compound that could be oxidized to penicillaminic acid; they assumed that this compound was penicillin N. In a detailed description of these experiments Fawcett *et al.* (1976*b*) also reported the LLL- and DLD-tripeptides to be inactive as precursors. They pointed out that the compound produced in the cell-free system could have been isopenicillin N, a compound known to be produced by *Penicillium chrysogenum*.

Kohsaka & Demain (1976) reported the conversion of penicillin N, by lysed protoplasts, into a cephalosporin-like compound that was later identified as deacetoxycephalosporin C (Yoshida *et al.*, 1978). However, Bost & Demain (1977) did not find incorporation of L-[^{14}C]valine into a cephalosporin, even though it was incorporated into an antibiotic with the chromatographic and electrophoretic properties of penicillin N. It therefore seemed possible

that the product observed in the cell-free systems was isopenicillin N, rather than penicillin N. The present paper offers evidence to support this possibility.

Materials and Methods

Unless otherwise stated, media and conditions for growing *C. acremonium* and its mutants were those given by Drew & Demain (1975) and all experimental methods were as described by Yoshida *et al.* (1978).

The cultures used in the present work were the penicillin N- and cephalosporin C-producing *C. acremonium* CW-19 (*Acremonium strictum* A.T.C.C. 36255) and its mutants. These mutants included M-0198 and M-0199, which produce neither antibiotic in fermentations, and M-1836, which produces only penicillin N. Mutants were isolated as described by Yoshida *et al.* (1978).

The cell-free extract was prepared as described by Yoshida *et al.* (1978), except that at the end of the procedure the lysed protoplast pellet was gently homogenized in a Teflon homogenizer instead of being placed in the freezer for 30–60 min. After homogenization, the suspension was centrifuged at 3000 *g* for 5 min and the supernatant (the cell-free extract) was placed in the freezer until used. Extracts were stable at −20°C for at least 15 days.

The cell-free reaction was carried out in the manner described by Yoshida *et al.* (1978), except that LLD-tripeptide was used as substrate in place of penicillin N. The total volume was also reduced to 1 ml, which included 0.8 ml of cell-free extract. Product formation was determined by pipetting 25 μl samples of the reaction mixture at various times on the filter-paper assay discs (Schleicher & Schuell, Keene, NH,

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U.S.A.; diameter 6.35mm); the discs were placed in the freezer until the end of the incubation. Then all discs, including the cephalosporin C standard discs, were placed on seeded antibiotic medium no. 5 plates (Difco Laboratories, Detroit, MI, U.S.A.). The assay organism used was the supersensitive strain *Escherichia coli* Ess or *Pseudomonas aeruginosa* Pss (Yoshida *et al.*, 1978); both strains are about equally sensitive to cephalosporin C and penicillin N. One unit of antibiotic activity is defined as the amount of antibiotic that produced the same sized clear zone as 1 μg of cephalosporin C.

Synthetic LLD- and LLL-tripeptides, penicillin N and isopenicillin N were prepared as described by Herchen (1977).

Results

Production of antibiotic from LLD-*tripeptide*

When LLD-tripeptide was incubated in the cell-free system, we observed generation of antibiotic activity. Assay discs containing samples of the reaction mixture taken at various times produced zones of increasing size on both supersensitive assay organisms (*E. coli* Ess and *P. aeruginosa* Pss). However, the zones did not appear when penicillinase was added to the agar assay medium, indicating that the product was a penicillin and not a cephalosporin.

Production of the antibiotic from LLD-tripeptide was observed with extracts from the parent culture CW-19, its antibiotic-negative mutants M-0198 and M-0199, and the penicillin N-positive cephalosporin C-negative mutant M-1836. Most of the experiments were done with extracts from mutant M-0198 (Fig. 1a).

Effect of incubation conditions on the conversion

Decreasing the speed of the rotary shaker from 250rev./min to 120rev./min had no effect on antibiotic production and the production was not greatly inhibited by elimination of agitation (Fig. 1a). Elevation of the temperature from 25 to 30°C had no effect. A stepwise increase in the concentration of the extract from 0.2 to 0.8ml/ml total volume increased the antibiotic production from <0.6 to 1.7units/ml. Boiling the cell-free extract completely eliminated antibiotic formation. Addition of cycloheximide (100μg/ml) had no effect on the reaction.

Increasing the substrate concentration up to 400μg/ml resulted in increasing amounts of product synthesis (Fig.1b).

Comparison of extracts from M-0198 mycelia harvested at 44, 56 and 68h showed highest activity in the extracts from the youngest culture.
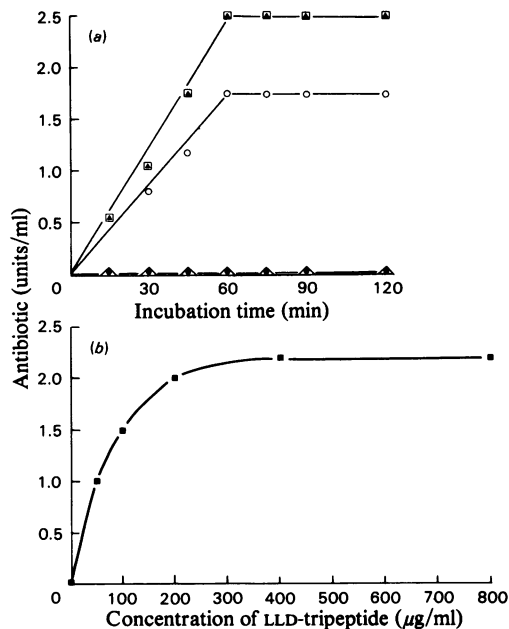

Fig. 1. *Production of penicillinase-labile antibiotic from* LLD-*tripeptide by cell-free extracts of C. acremonium M-0198*

In (*a*) the concentration of each LLD-tripeptide was 200μg/ml. The LLL-tripeptide was inactive as a substrate at various concentrations from 100 to 800μg/ml. (*a*) shows the effect of incubation conditions. Symbols: ▲, LLD-tripeptide, shaking at 120rev./min; □, LLD-tripeptide, shaking at 250rev./min; ○, LLD-tripeptide, no shaking; △, LLL-tripeptide, shaking at 250rev./min; ●, no LLD-tripeptide, shaking at 250rev./min. (*b*) shows the effect of tripeptide concentration. Symbol: ■, LLD-tripeptide, incubation time 60min.

Specificity of the reaction

LLL-Tripeptide was compared with LLD-tripeptide as substrate of the reaction; LLL-tripeptide was totally inactive, as shown in Fig. 1(*a*).

Antibacterial spectra of penicillin N, isopenicillin N and the product

To distinguish penicillin N from isopenicillin N by the disc-plate assay, a series of bacteria were examined as test organisms. The most suitable were two Gram-negative species (*Salmonella typhimurium* A.T.C.C. 13311 and *P. aeruginosa* Pss) and two Gram-positive bacteria (*Staphylococcus aureus* A.T.C.C. 25923 and *Sarcina lutea* A.T.C.C. 9341). Penicillin N was more active against the Gram-

Table 1. *Activity of reaction product on selected bacteria in vitro*

Solutions of penicillin N and isopenicillin N were made up to approx. 5 μg/ml according to the hydroxylamine assay. Discs were dipped into these solutions and applied to the surface to the seeded agar. An inhibitory zone around the 6.35 mm diameter disc is indicated by + and no inhibitory zone by −.

| | | | Preparation tested | | |
| | | | Reaction product | | |
Bacterium	Penicillin N	Isopenicillin N	Preparation 1	Preparation 2	Preparation 3
Staph. aureus A.T.C.C. 25923	−	+	+	+	+
Sarcina lutea A.T.C.C. 9341	−	+	+	+	+
Salm. typhimurium A.T.C.C. 13311	+	−	−	−	−
Pseudomonas aeruginosa Pss	+	−*	−	−	−

* An inhibitory zone was detected when the concentration of isopenicillin N was 10 μg/ml.

negative than against the Gram-positive species, whereas the reverse was true for isopenicillin N. Table 1 shows that the activity of the reaction product resembled that of isopenicillin N and not that of penicillin N.

Discussion

Identification of the product made in the cell-free system from LLD-tripeptide was not a simple task. Since the structures of penicillin N and isopenicillin N are identical except for the configuration of the α-aminoadipyl side chain, the compounds are indistinguishable by chromatography, electrophoresis, ninhydrin staining or by the rate of acid or penicillinase destruction (Flynn *et al.*, 1962; Cole & Batchelor, 1963). With the small amounts of material produced in our cell-free system, we therefore considered differentiation of the two antibiotics by antibacterial spectra. Flynn *et al.* (1962) reported that the activity of isopenicillin N against *Staph. aureus* paralleled that of penicillin N. Cole & Batchelor (1963) found isopenicillin N 'significantly less' active against *Salm. typhimurium* than penicillin N, though their activities against *Bacillus subtilis* were similar. The results of Vanderhaeghe *et al.* (1974) showed a slightly greater activity of penicillin N than isopenicillin N on the Gram-negative *E. coli* and *Proteus mirabilis*. Usher *et al.* (1975) reported that the quotient (activity against *Salm. typhimurium* activity against *Staph. aureus*) was much lower with isopenicillin N than with penicillin N. In the present work we found isopenicillin N to be more active on two Gram-positive than on two Gram-negative species and the opposite to be true for penicillin N. Since our reaction product was more active against the Gram-positive than against the Gram-negative species, we concluded that it was isopenicillin N. In the preceding paper O'Sullivan *et al.* (1979) described completely different experiments that have led them to the conclusion that isopenicillin N is the primary product in a cell-free system.

The use of blocked *C. acremonium* mutants for these studies was very helpful, since the cell-free extract produced only about 1–2 units of antibiotic/ml. Because extracts of the parent culture carry over antibiotic activity from the fermentation, it is difficult to detect product formation by using the parent. The observation that cultures M-0198 and M-0199 can convert LLD-tripeptide into isopenicillin N indicates that their genetic block is at some stage of peptide formation.

We suggest that a racemase that converts isopenicillin N into penicillin N is a labile enzyme. This would account for the lack of incorporation of radioactivity from labelled valine into a cephalosporin (via penicillin N) in the experiments of Bost & Demain (1977). Furthermore we have observed production of traces of a cephalosporin (i.e. a penicillinase-resistant antibiotic) from LLD-tripeptide in experiments in which fresh extracts were used. When frozen for a day or more, these extracts no longer produced the cephalosporin.

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