CYCLIZATION OF PHENYLACETYL-L-CYSTEINYL-D-VALINE TO BENZYLPENICILLIN USING CELL-FREE EXTRACTS OF *STREPTOMYCES CLAVULIGERUS*

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Benzylpenicillin, a typical antibiotic produced by some species of fungi, was obtained by direct cyclization of the heteropeptide phenylacetyl-L-cysteinyl-D-valine using cell-free extracts of *Streptomyces clavuligerus*. This is the first description of evidence of the synthesis of benzylpenicillin from a non natural molecule using a bacterial enzyme.

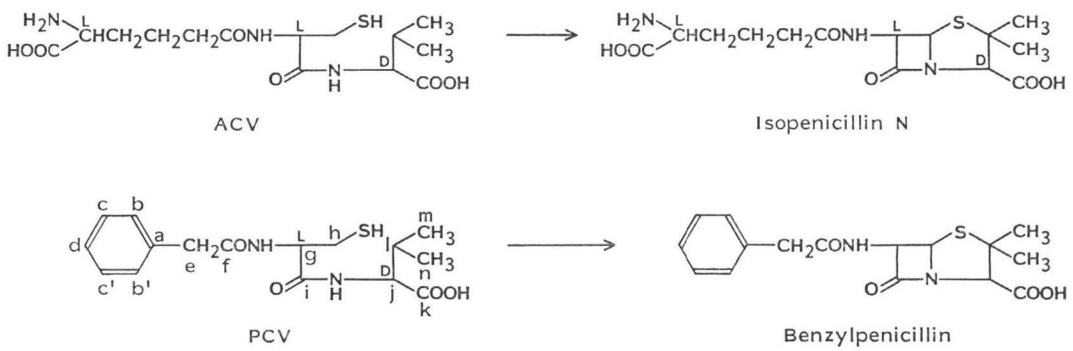
The cyclization of (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine (ACV)[1] to isopenicillin N is carried out by the enzyme isopenicillin N synthetase[2,3]. This enzyme which plays an important role in the secondary metabolism of *Acremonium chrysogenum*[4~7], *Penicillium chrysogenum*[8,9] and some species of *Streptomyces*[7~12], may accept as substrate molecules similar to ACV in which the L-valine moiety has been replaced[13,14]. Other changes in the molecular structure (the L-α-aminoadipyl moiety) are only tolerated to a certain extent[15~17] and in most cases this implies a lack of cyclization[18]. However, recent studies[19] have shown that *P. chrysogenum* and *A. chrysogenum* cyclases are able to accept as substrate peptides in which the L-α-aminoadipyl moiety has been replaced by phenylacetic acid. In the present work an attempt was made to cyclize the heteropeptide phenylacetyl-L-cysteinyl-D-valine using cell-free extracts of *Streptomyces clavuligerus*.

Materials and Methods

Materials

Phenylacetyl-L-cysteinyl-D-valine (PCV) was synthesized according to the methodology described by WOLFE and JOKINEN[20] using phenylacetic acid instead of L-α-aminoadipic acid (Fig. 1). Physical properties were as follows: MP 190°C (dec); $[\alpha]_D^{25}$ −73.05 (phosphate buffer 50 mM, pH 8.0); IR (KBr) cm^{-1} 3400 (NH), 3300 (br, acid OH), 1750 (amide, C=O), 1670 and 1550 (Ph group), 1355 and 1400

Fig. 1. Structure of ACV and PCV and their respective cyclization products isopenicillin N and benzylpenicillin.



($(CH_3)_2CH$); UV λ_{max} nm 210, 250, 256, 264; ^1H NMR (80 MHz, CD_3OD) δ 0.85 and 0.90 (2×d, J= 6.8 Hz, Hm, Hn), 2.07 (m, Hl), 3.06 (m, Hg, Hh), 3.57 (s, He), 4.28 (d, J=5.4 Hz, Hj), 7.27 (s, Ph-H); ^{13}C NMR (20.1 MHz, D_2O, pH 9.0 with NaOH) δ 175.43 or 175.48 (2s, Ci, Ck), 172.9 (s, Cj), 130.01 or 130.22 (dd, s, Cc, Cc′, Ca), 128.18 or 128.40 (3×d, Cd, Cb, Cb′), 61.67 (d, Cj), 59.84 (d, Cg), 43.23 or 43.30 (2×t, Ce, Ch), 31.79 (d, Cl), 18.18 or 19.99 (2×q, Cm, Cn).

Penicillin N (95%), K-benzylpenicillin (1,580 U/mg), penicillin acylase from *Escherichia coli* and ACV were supplied by Antibióticos S. A., León, Spain. Dithiothreitol, Tris, protamine sulfate and bovine serum albumin were from Sigma Chemical Co. (St. Louis. Mo., U.S.A.). Other chemicals used were of reagent quality or HPLC grade.

Culture Conditions and Enzyme Preparation

Streptomyces clavuligerus ATCC 27064 and *Micrococcus luteus* ATCC 9341 were from the American Type Culture Collection.

S. clavuligerus ATCC 27064 was maintained and grown as described by O'SULLIVAN *et al.*[21]. Mycelium (250 ml of culture) was harvested by centrifugation at 10,000×g for 5 minutes, washed four times by resuspension in sterile saline solution and centrifuged as before. The precipitated cells, resuspended in 25 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM dithiothreitol (DTT) (TD buffer), were sonicated as previously described[12] using a Branson Sonifier Cell Disruptor (B-12). The broken cell suspension was ultracentrifuged at 100,000×g (Beckman L5-65B) for 90 minutes, treated with 0.2% (w/v) protamine sulfate and the clear extract precipitated with ammonium sulfate (30~80% saturation). The resulting precipitate was resuspended in 3 ml of TD buffer and applied to a Sephadex G-25, PD-10 column (Pharmacia). Aliquots of 1 ml were collected and assayed for cyclase activity. Fractions 4 and 5, containing maximal activity, were mixed and employed for the reaction. The extracts were stored frozen at −20°C. Protein was measured by the method of LOWRY *et al.*[22].

Cyclization activity was assayed by the method described by JENSEN *et al.*[12], slightly modified. The reaction mixture contained 30 mM Tris-HCl buffer (pH 7.5), 0.5 mM $FeSO_4$, 1 mM bis-LLD ACV or 3 mM bis PCV, 4 mM DTT, 3 mM Na-ascorbate and enzyme (10 mg of protein) in a final reaction volume of 0.5 ml. The reaction mixture was incubated at 25°C for 90 minutes and stopped by adding 0.5 ml of MeOH.

The formation of isopenicillin N and benzylpenicillin was measured by bioassay against *M. luteus* ATCC 9341 following the hole plate method[23]. Penicillin N and benzylpenicillin were used as standards. One unit of antibiotic (penicillin N or benzylpenicillin) gives a zone of inhibition equivalent to 1 μg or 1.58 μg of penicillin N or benzylpenicillin respectively with *M. luteus* ATCC 9341 as test organism.

Isopenicillin N from the reaction mixture was identified under the conditions described by JENSEN *et al.*[24]. Benzylpenicillin, obtained by cyclization of PCV, was identified using a Perkin Elmer Chromatograph, Series 3B, with a LC-75 Spectrophotometric Detector and a Sigma 10B Chromatography Data Station. A 30-cm μBondapack C-18 column (Waters Associates Inc.) and a mobile phase composed of 83% (0.1 M sodium acetate - acetic acid, pH 4.5) and 17% of acetonitrile were used. The column was eluted at 90 kg/cm using a flow rate of 2.0 ml/minute. The retention time of benzylpenicillin under these conditions was 12.3 minutes.

Results and Discussion

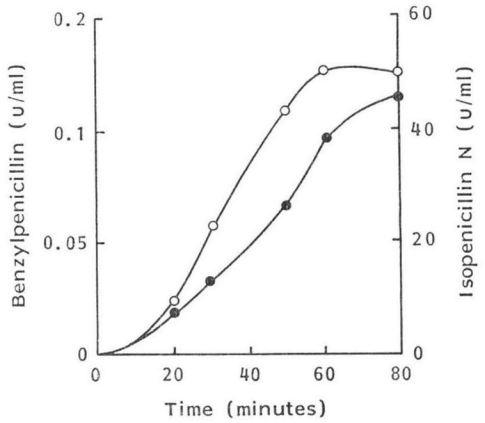
The cyclization of PCV using a preparation of isopenicillin N synthetase from *S. clavuligerus* leads to the formation of a molecule active against *M. luteus* ATCC 9341 and sensitive to β-lactamase I from *Bacillus cereus* (Difco) (Table 1). This product, in contrast to the isopenicillin N obtained from ACV, loses over 90 percent of its antibacterial activity when incubated with 2 I.U. of penicillin acylase (10 minutes, 30°C) (Table 1). Furthermore, the antibiotic once extracted with amylacetate at pH 2.5, and after transferring to 50 mM phosphate buffer (pH 7.0), was identified by HPLC[19] as benzylpenicillin. Fig. 2 shows that in the above cyclization conditions only 0.15 U/ml of benzyl-

Table 1. Conversion of tripeptides to penicillin by *Streptomyces clavuligerus* cyclase.

Substrate	Reaction mixture	Isopenicillin N[a] or benzylpenicillin[b] (u/ml)
ACV	Control	40[a]
	+Penicillin acylase (2 I.U., 10 minutes)	38[a]
	+β-Lactamase	0[a]
PCV	Control	0.15[b]
	+Penicillin acylase (2 I.U., 10 minutes)	0.01[b]
	+β-Lactamase	0.00[b]

[a] Isopenicillin N was measured using penicillin N as standard.
[b] Benzylpenicillin was measured using benzylpenicillin as standard.

Fig. 2. Time course of formation of benzylpenicillin (○) and isopenicillin N (calculated as penicillin N) (●) from PCV and ACV respectively using cell-free extracts of *Streptomyces clavuligerus* (Bioassay against *Micrococcus luteus*).



penicillin are produced, while in similar conditions, 40 u/ml of isopenicillin N (evaluated as penicillin N) were obtained (see Materials and Methods). This result, which implies a very low cyclization efficiency (not more than 0.01% of the PCV is cyclized) supports the hypothesis of BALDWIN *et al.*[15] according to which the L-α-aminoadipic acid moiety plays an important role in the formation of the enzyme substrate complex. However, the ability of this enzyme to accept PCV as substrate appears conflict with other previous observations[16] to the effect that isopenicillin N synthetase does not tolerate much of a change in the L-α-aminoadipyl moiety of the normal substrate (ACV) and although to some degree it accepts the removal of the terminal amino group or substitution of a CH_2 by a sulfur atom[17], it does not tolerate any shortening of the side-chain even by one carbon[18]. This apparent discrepancy is accounted for by low efficiency of PCV cyclization. For the same reason, the hypothesis of BALDWIN *et al.*[15] concerning the minimal structural requirements of the *N*-acyl group may be accepted as valid although the side chain of PCV (phenylacetic acid) has more than six carbons and does not terminate in a carboxylgroup. Similar results to those described here have been reported by us[19] and the Oxford group who demonstrated, by bioassay an mass spectral analysis, that isopenicillin N synthetase from *A. chrysogenum* is able to cyclize to the corresponding antibiotic, peptides in which the L-α-aminoadipyl moiety has been changed by phenylacetyl, phenoxyacetyl and other related molecules[25]. The isolation of mutants in which the cyclase has a wider substrate specificity could be important for the fermentation industry in the future.

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