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Penicillin biosynthesis: intermediates of biosynthesis of δ-L-α-aminoadipyl-L-cysteinyl-D-valine formed by ACV synthetase from *Acremonium chrysogenum*

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Abstract The tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (LLD-ACV) is synthesised by the multifunctional enzyme ACV synthetase integrating four steps of the penicillin and cephalosporin biosynthetic pathway. Peptide synthesis follows the thiotemplate mechanism from intermediates bound as thioesters to the enzyme. The formation of δ-(L-α-aminoadipyl)-L-cysteinyl-thioester in the absence of L-valine was shown by isolation of the enzyme–substrate complex and cleavage of the covalently bound intermediate with performic acid. The dipeptide was recovered as cysteic acid or cysteic acid oxime and detected by HPLC and MALDI-TOF mass spectrometry. We conclude that the first peptide bond is formed between δ-carboxyl of L-aminoadipic acid and L-cysteine, followed by addition of the dipeptidyl intermediate to L-valine.

Key words: δ-L-α-Aminoadipyl-L-cysteinyl-D-valine; ACV synthetase; Penicillin/cephalosporin biosynthetic pathway; High-pressure liquid chromatography; MALDI-TOF mass spectrometry

1. Introduction

ACV synthetase forms the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) from the respective amino acids and ATP. The relatively easy purification of higher quantities of the enzyme from penicillin- or cephalosporin-overproducing strains permits this reaction sequence to be used as a model system for mechanistic studies of peptide biosynthesis.

The classical thiotemplate mechanism [1] proposed that after activation as aminoacyl adenylates the intermediates bound as thioesters are assembled by one central swinging arm, the cofactor 4′-phosphopantethein. Molecular genetic analysis of peptide synthetase genes and affinity-labelling studies of the gramicidin S system showed the domain structure of these enzymes to contain this cofactor covalently attached to each domain [2–4]. Peptide assembly is thus accomplished by transfer of acyl intermediates between adjacent cofactors, and has been termed multiple cofactor model. This model predicts that all thioester intermediates are attached covalently to the peptide synthetases and should be detectable by specific hydrolysis.

The structural similarity of ACV to glutathion seemed to indicate a similar biosynthetic mechanism. In 1983 Adlington et al. [5] demonstrated the incorporation of radioactive-labelled amino acids into LL-AC and LLD-ACV in a crude extract. In a kinetic study Banko and Demain [6] have shown that the formation of LLD-ACV from the substrate amino acids proceeded faster than from LL-AC and valine, concluding the presence of a single multienzyme. The ACV synthetase from *Aspergillus nidulans* was then isolated by van Liempt et al., demonstrating adenylate formation of L-α-aminoadipic acid, L-cysteine and L-valine, and thioester attachment of L-cysteine and L-valine [7]. Later, the thioester formation of aminoadipic acid has been shown for the ACV synthetase from *Streptomyces clavuligerus* [8].

Recently Shiau et al. recovered *O*-methyl-seryl-D,L-valine and L-cysteinyl-D-valine in attempts to replace cysteine by *O*-methyl-L-serine and aminoadipic acid by glutamic acid, respectively [9,10]. These dipeptides were formed in small amounts, while the respective tripeptides have not been detected. From these data a new biosynthetic model has been proposed, where first LL-cysteinyl-valine is synthesised, followed by epimerization to LD-cysteinyl-valine and the formation of LLD-ACV.

We have investigated enzyme–bound intermediates to determine directly the sequence of reactions.

2. Material and methods

2.1. Strains and culture conditions

Cells of *Acremonium chrysogenum* C-10 were grown similarly as previously described for *Aspergillus nidulans* [7]. Fermentations were seeded with an overnight culture instead of spore suspension. The mycelia were harvested 40 h after inoculation before the period of transition to the stationary growth phase. The mycelia were washed with 0.8% NaCl solution, lyophilised and stored at −20°C under dry conditions.

2.2. Enzyme purification

All procedures were carried out at 0–4°C in succession. Dried mycelia (50 g) were ground in a mortar with sand and stirred for 40 min in 1 l of buffer A (100 mM Tris-HCl, pH 7.5, at 4°C, 10 mM DTE (Biomol), 1 mM EDTA, 50% glycerol). After 30 min of centrifugation in a Beckman centrifuge at 10 000×*g* nucleic acids were precipitated by treatment for 20 min with dialysed Polyimin solution (BASF, Ludwigshafen, Germany), final concentration 0.1%. After centrifugation as above neutralised saturated ammonium sulfate solution was gradually added to the supernatant to a final concentration of 30%

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Abbreviations: Aad, L-α-aminoadipic acid; LL-AC, δ-(L-α-aminoadipyl)-L-cysteine; LLD-ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; ATP, adenosine triphosphate; Cys, cysteine; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; D-Val, D-valine; HPLC, high-pressure liquid chromatography; MALDI, matrix-assisted laser desorption ionisation; OPA, *o*-phtalaldehyde

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saturation, stirred for 20 min and centrifuged. Subsequently ammonium sulfate solution was added to the supernatant up to a concentration of 45% and the pellet was dissolved after centrifugation in buffer B (25 mM Tris-HCl, pH 7.5, at 4°C, 0.1 EDTA, 2 mM DTE, 10% glycerol). If the suspension was turbid, it was dialysed for 1.5 h and centrifuged. The solution (7 ml) was applied to an AcA 34 (Serva) gel filtration column (3×50 cm). Fractions were analysed by SDS-polyacrylamide gel electrophoresis for protein composition and frozen. The final enzyme concentrations were defined by Bradford [11] and the extinction coefficient method [12]. From the absorption at 280 nm a final enzyme concentration of 360 pmol/ml calculated, using the extinction coefficient for ACV synthetase of 369 280 $M^{-1}\cdot cm^{-1}$. The Bradford procedure gave 365 pmol/ml with BSA as a standard. With an estimated purity of 85% from SDS-PAGE, the concentration of ACV synthetase was estimated to 300 pmol/ml.

2.3. Thioester formation

ACV synthetase (300 pmol) was incubated with 1 mM Aad, 1 mM Cys, 6 mM DTE, 40 mM Mg acetate, 8 mM ATP (final volume 1.2 ml) for 30 min at 25°C. The enzyme was precipitated with TCA (final concentration 10%), centrifuged after 15 min and the pellet was washed twice with 10% TCA and once with 100% ethanol. The pellet was dissolved in performic acid (300 μl of 95–98% formic acid, Merck and 60 μl of 30% H_2O_2, Fluka; [13]) and dried in a vacuum centrifuge. The AC standard was prepared analogue to the enzyme sample. A defined amount of the dipeptide was dissolved in 300 μl of formic acid and 60 μl of H_2O_2 and dried in a vacuum centrifuge. This method was developed in analogy to the sample preparation for the classical TLC analysis of radioactively labelled intermediates as thioesters [14].

2.4. HPLC analysis

Analysis of free amino groups was performed by precolumn derivatization with OPA (chiral amino acid analysis kit, GROM, Germany) dissolving the dry pellet from the precipitation in 60 μl of water and loading 7.5 μl to the column.

2.5. MALDI-TOF mass spectrometry

All samples were analysed in 150 mg/ml dihydroxybenzoic acid in acetone and 0.1% trifluoroacetic acid. A mixture of 1 μl of matrix and 1 μl of sample dissolved in 10 μl of water/ethanol (1:1) was prepared directly on the plate. The samples were analysed using a MALDI VOYAGER ELITE time of flight mass spectrometer from PerSeptive Biosystems (Framingham, MA) containing a nitrogen laser giving a 337 nm output. The ions were accelerated with a voltage of 20 kV. Measurements were performed in the delayed extraction mode, allowing the determination of monoisotopic mass values. The mass spectrometer was used in the positive ion detection and reflector mode.

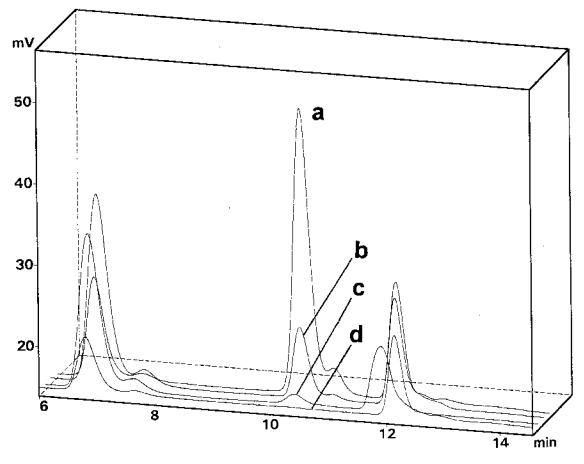
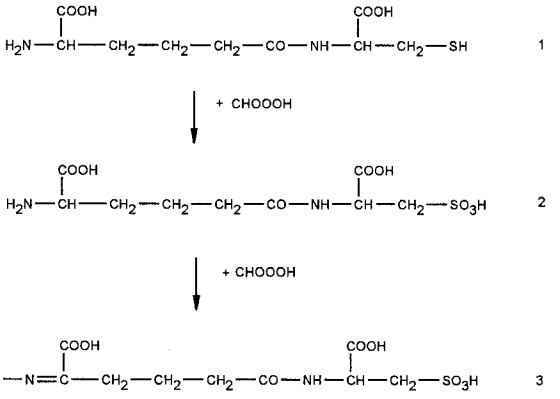


Fig. 1. Chiral HPLC analysis with OPA derivatisation: (a) standard AC treated with perfomic acid; (b) AC product of ACV synthetase treated with performic acid after 30 min incubation; (c) ACV synthetase treated with performic acid at $t=0$; (d) water. The peaks at retention times around 7 and 12 min have not been identified but are also contained in control samples containing water instead of the intermediates.

Scheme 1: oxidation products of AC treated with performic acid



1 δ-(L α-aminoadipyl)-L-cysteine (264,07 m/z)
2 δ-(L α-aminoadipyl)-L-cysteic acid (312,06 m/z)
3 δ-(L α-aminoadipyl)-L-cysteic acid oxime (326,05 m/z)

Scheme 1.

3. Results

To determine the order of peptide bond formation catalysed by ACV synthetase we have analysed covalently bound intermediates interrupting the catalytic cycle by substrate depletion. Conditions selected would permit enzymatic formation of ACV for at least 3 h at a constant rate [15]. Omission of the last amino acid valine was expected to lead to the accumulation of either cysteinyl- or aminoadipyl-L-cysteinyl intermediates. The covalent intermediates were obtained by TCA precipitation, thioester bonds were cleaved by performic acid treatment, and analysis was carried out by HPLC with OPA detection. No variation in the product patterns was found varying the incubation times between 5 min and 1 h. Controls of the isolated ACV synthetase without incubation did not show significant peaks of intermediates in the case of the *Aspergillus nidulans* enzyme. In the elution pattern obtained a compound barely detectable at a retention time of 10.3 min without incubation increases upon charging with aminoadipate and cysteine (Fig. 1). The same compound is obtained if a sample of δ-(L-α-aminoadipyl)-L-cysteine is treated with performic acid. As shown in Scheme 1 oxidation of the dipeptide intermediate to the cysteic acid derivative (Scheme 1, 1) and further oxidation to the oxime (Scheme 1, 2) are expected to occur in performic acid [13,16]. For further identification the intermediates were subjected to MALDI-TOF mass spectrometry (Fig. 2). In both the treated control sample and the intermediate preparation peaks corresponding to δ-(L-α-aminoadipyl)-L-cysteic acid oxime (326.05 Da) were observed. The method, however, did not permit detection of the cysteic acid derivative due to matrix interference. Even if dihydroxybenzoic acid was replaced by an α-cyano-4-hydroxycinnamic acid matrix peaks occurred in the 312 Da region. The oxidized sample is concluded to contain both δ-(L-α-aminoadipyl)-L-cysteic acid and the respective *N*-oxime, the latter not being detectable as fluorescent derivative in HPLC. The molar ratio

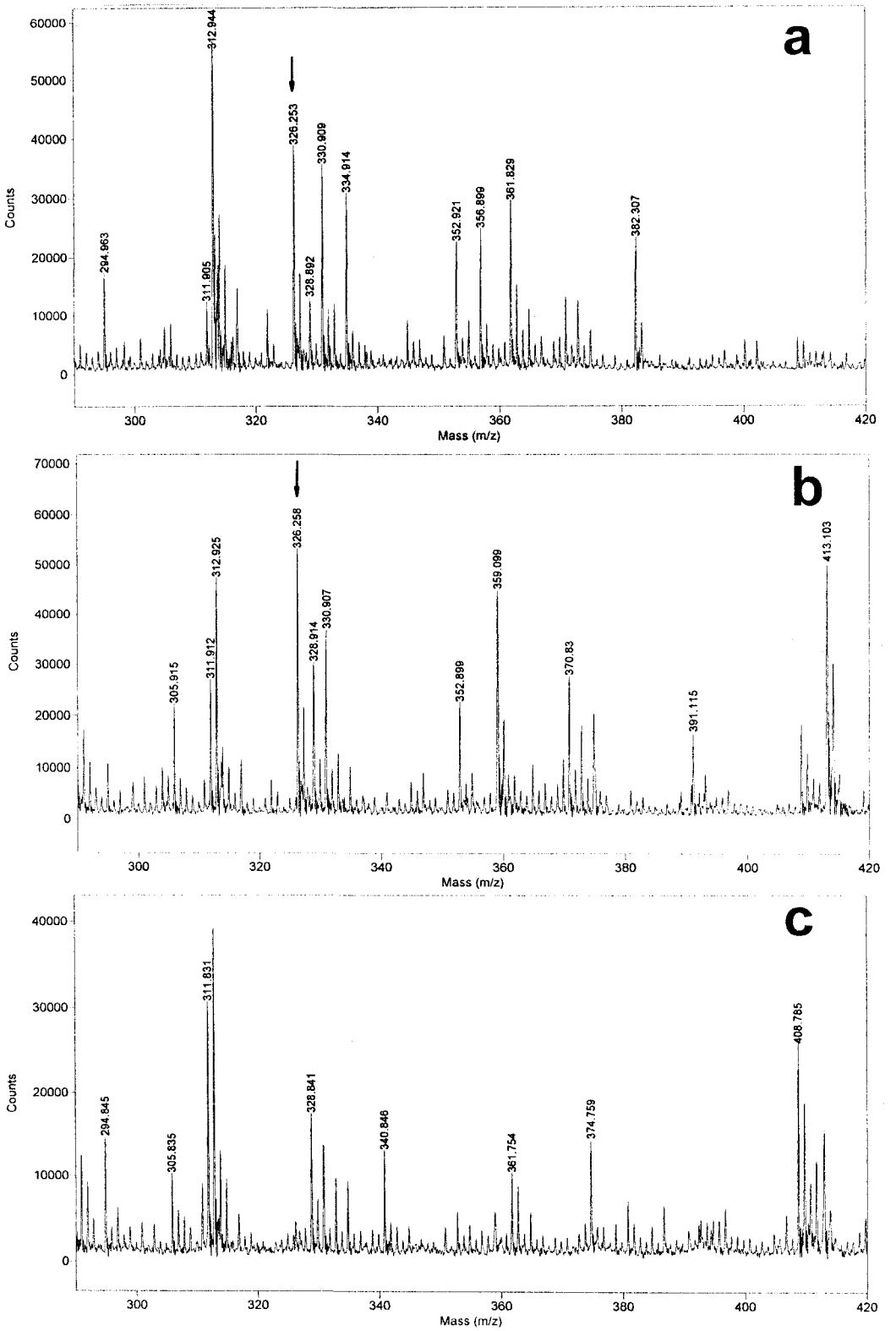


Fig. 2. MALDI analysis with dihydroxy benzoic acid matrix: (a) standard AC oxime (M. 326,25); (b) AC product (M. 326,25) of ACV synthetase treated with performic acid after 30 min incubation; (c) matrix dihydroxybenzoic acid.

Scheme 2: Reactions catalysed by ACV synthetase.

$$E1 + A + ATP \rightleftarrows E1(A\text{-}AMP) + PPi \rightleftarrows E1\text{-}S^{p1}\text{-}A + AMP \quad (1)$$

$$E2 + C + ATP \rightleftarrows E2(C\text{-}AMP) + PPi \rightleftarrows E2\text{-}S^{p2}\text{-}C + AMP \quad (2)$$

$$E1\text{-}S^{p1}\text{-}A \rightarrow E2\text{-}S^{p2}\text{-}C \xrightarrow{C1} E2\text{-}S^{p2}\text{-}C\text{-}A + E1\text{-}S^{p1}H \quad (3)$$

$$E3 + V + ATP \rightleftarrows E3(V\text{-}AMP) + PPi \rightleftarrows E3\text{-}S^{p3}\text{-}V + AMP \quad (4)$$

$$E2\text{-}S^{p2}\text{-}C\text{-}A + E3\text{-}S^{p3}\text{-}V \xrightarrow{C2} E3\text{-}S^{p3}\text{-}V\text{-}C\text{-}A + E2\text{-}S^{p}H \quad (5)$$

$$E2\text{-}S^{p2}\text{-}C^{*}\text{-}A + E3\text{-}S^{p3}\text{-}V \xrightarrow{C2} E3\text{-}S^{p3}\text{-}V\text{-}C^{*} + E2\text{-}S^{p}H \quad (6)$$

$$E3\text{-}S^{p3}\text{-}V\text{-}C\text{-}A \overset{Ep3}{\rightleftarrows} E3\text{-}S^{p3}\text{-}DV\text{-}C\text{-}A \xrightarrow{TE} A\text{-}C\text{-}DV \quad (7)$$

$$E3\text{-}S^{p3}\text{-}V\text{-}C \overset{Ep3}{\rightleftarrows} E3\text{-}S^{p3}\text{-}DV\text{-}C \xrightarrow{TE} C\text{-}DV \quad (8)$$

Scheme 2. The synthetase consists of the three modules: E1, E2 and E3. Each module is composed of an activation site forming the (amino) acyl adenylate, a carrier domain which is post-translationally modified with 4′-phosphopantetheine (S^p), and a condensation domain (C1, C2) or alternatively a structurally similar epimerization domain (Ep). Activation of aminoadipate (A) leads to an acylated enzyme intermediate, where A is attached to the terminal cysteamine of the cofactor ($E1\text{-}S^{p1}\text{-}A$) (1). Likewise activation of cysteine (C) leads to cysteinylated module 2 (2). For the condensation reaction to occur between aminoadipate as donor and cysteine as acceptor, both intermediates are thought to react at the condensation site of module 1 (C1). Each condensation site is composed in analogy to the ribosomal peptide formation of an aminoacyl and a peptidyl site, and in this case of initiation the thioester of A enters the P-site, while the thioester of C enters the A site. Condensation occurs and leaves the dipeptidyl intermediate AC at the carrier protein of the second module (3). The third amino acid valine is activated on module 3, and V is attached to the carrier protein 3 (4). Formation of the tripeptide occurs at the second condensation site C2, with the dipeptidyl-intermediate entering the P site, and the valinyl-intermediate the A site (5). The synthesis of dipeptides is seen as a side reaction, where the cysteinyl-thioester enters the P site of C2, and this minor reaction seems to be favoured in the case of *O*-methyl-serine replacing C (C*), or if an unproductive glutamyl adenylate occupies the first module (6). A similar reaction occurs in the synthesis of poly-phenylalanine in the ribosomal system, where the phenylalanyl ester of tRNA enters the P site to initiate peptide synthesis. Finally epimerization of the tripeptide (or dipeptide) intermediate occurs at the epimerization site of module 3 (Ep3), and the stereospecific peptide release is controlled by the thioesterase (TE) (7). This release occurs likewise with the dipeptide Cys–D–Val. Since the dipeptide *O*-methyl-serinyl-D,L-valine is released as a racemate, it is apparently not a substrate of the thioesterase, but is hydrolysed non-enzymatically.

of bound dipeptide as cysteic acid to ACV synthetase has been estimated to 0.27, but no final conclusion can be drawn, since the ratio of cysteic acid derivative to oxime is not known. A sample of *Acremonium chrysogenum* ACV synthetase yielded a more significant peak of the oxidized dipeptide even without incubation with substrates (results not shown).

4. Discussion

The investigation of reaction intermediates in biosynthetic sequences operating with covalent intermediates poses detection and identification problems due to the availability of the respective multienzymes in the low picomolar range. Thus aminoacyl- and peptidyl-intermediates in non-ribosomal peptide synthesis so far have been studied using radiolabeled substrates and cochromatography by TLC [14,17–19]. We have attempted here to apply HPLC and mass spectrometry to (a) study non-labeled substrates for reasons of availability and (b) to obtain more detailed structural informations.

Thioester intermediates may be distinguished by performic acid oxidation from oxygen esters [20]. The only modification described so far has been the formylation of amino groups [19]. The analysis of intermediates of ACV biosynthesis involves di- and tripeptides containing both amino and thiol groups presumably susceptible to oxidation. It is known that the oxidation of amines to oximes by peroxide leads to the formation of barely soluble equimolar complexes of the oximes formed with the respective amines, and thus no complete oxidation can be achieved [16]. The oxidation of cysteine and cysteine peptides generally proceeds in yields of 80–90%, and so far products besides sulfonic acids have not been identified [21]. We have shown here the presence of the δ-(L-α-aminoadipyl)-L-cysteic acid oxime, and indirect evidence for the cysteic acid derivative in HPLC. No evidence for the unmodified peptide has been obtained. The level of charging has been estimated to at least 27%, but the ratio of oxime to cysteic acid derivative of the dipeptide, the hydrolytic stability of the dipeptide, and the possible occupation by cysteine have not been considered. In conclusion evidence is presented that ACV synthetase contains detectable amounts of δ-(L-α-aminoadipyl)-L-cysteine as a thioester, the amount of which is increased upon incubation with the respective substrate amino acids and ATP. The rapid formation of this covalent intermediate indicates this peptide to be formed first in the ACV biosynthetic cycle.

The formation of *O*-methyl-seryl-D,L-valine or cysteinyl-D-valine in the presence of glutamic acid [9] indicates either a side reaction initiating peptide synthesis in position 2, or an N-terminal cleavage of either N-terminal aminoadipoyl or glutaminyl side chain of the respective tripeptide formed. Although prolonged incubation times were used (16 h), we would favour the first interpretation since intermediate starts leading to dipeptide formation have been observed recently in actiniomycin and cyclosporin forming multienzymes [22,23]. In addition, L-cysteinyl-D-valine is not a substrate for ACV biosynthesis [9], while δ-(L-α-aminoadipyl)-L-cysteine was found to be accepted as a substrate for adenylation and biosynthesis [6,24,25].

The current view of the reaction sequence of ACV synthetase has been summarised in Scheme 2. Experimental demonstration of adenylate formation as well as thioester binding of α-aminoadipic acid, cysteine and valine has been carried out. We here have shown that the dipeptidyl-thioester δ-(L-α-aminoadipyl)-L-cysteine is an actual intermediate of ACV biosynthesis, so that reactions 1–4 have been verified. The verification of the intermediates of reactions 5–8 is currently investigated.

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References

[1] Lipmann, F. (1973) Sciences 173, 875–884.
[2] Schlumbohm, W., Stein, T., Ullrich, C., Vater, J., Krause, M., Marahiel, M., Kruft, V. and Wittmann-Liebold, B. (1991) J. Biol. Chem. 266, 23135–23141.

[3] Stein, T., Vater, J., Kruft, V., Wittmann-Liebold, B., Franke, P., Panico, M., Mc Dowell, R. and Morris, H.R. (1994) FEBS Lett. 340, 39–44.
[4] Stein, T., Kluge, B. and Vater, J. (1995) Biochemistry 34, 4633–4642.
[5] Adlington, R.F., Baldwin, J.E., Nieto, M.L., Murphy, J.A. and Patel, N. (1983) Biochem. J. 213, 573–576.
[6] Banko, G. and Demain, A.L. (1987) J. Am. Chem. Soc. 109, 2858–2860.
[7] Van Liempt, H., von Döhren, H. and Kleinkauf, H. (1989) J. Biol. Chem. 264, 3680–3684.
[8] Schwecke, T., Aharonowitz, Y., Palissa, H., von Döhren, H., Kleinkauf, H. and van Liempt, H. (1992) Eur. J. Biochem. 205, 687–694.
[9] Shiau, C.Y., Baldwin, J.E., Byford, M.F., Sobey, W.J. and Schofield, C.J. (1995) FEBS Lett. 358, 97–100.
[10] Shiau, C.Y., Baldwin, J.E., Byford, M.F. and Schofield, C.J. (1995) FEBS Lett. 373, 303–306.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Gill, S.C. and von Hippel, P.H. (1989) Anal. Biochem. 182, 319–326.
[13] Sanger, F. (1949) Biochem. J. 44, 126–128.
[14] Keller, U. (1987) J. Biol. Chem. 262, 5852–5856.
[15] W. Kallow, Thesis, Technical University Berlin, 1996.
[16] H. Metzger, in: E. Müller (Ed.), Methoden der Organischen Chemie (Houben and Weyl), Vol. 10/3, 4th edn., Georg Thieme, Stuttgart, 1968, pp. 7–308. .
[17] Zimmer, T.-L. and Laland, S.G. (1975) Meth. Enzymol. 43, 567–579.
[18] Dittmann, J., Wenger, R.M., Kleinkauf, H. and Lawen, A. (1994) J. Biol. Chem. 269, 2841–2846.
[19] Gocht, M. and Marahiel, M.A. (1994) J. Bacteriol. 176, 2654–2662.
[20] Lynen, F. (1959) J. Cellular Comp. Physiol. 1, 33.
[21] Hirs, C.H.W. (1967) Meth. Enzymol. 11, 197–199.
[22] M. Glinski, M. Walther, and R. Zocher, Abst. Symp. Enzymology Biosynthesis of Natural Products, Technical University Berlin, 1996.
[23] Stindl, A. and Keller, U. (1994) Biochemistry 33, 9358–9364.
[24] Aharonowitz, Y., Bergmeyer, J., Cantoral, J.M., Cohen, G., Demain, A.L., Fink, U., Kinghorn, J., Kleinkauf, H., MacCabe, A., Palissa, H., Pfeifer, E., Schwecke, T., van Liempt, H., von Döhren, H., Wolfe, S. and Zhang, J. (1993) Bio/Technology 11, 807–810.
[25] Baldwin, J.A., Bird, J.W., Field, R.A., O'Callaghan, N.M. and Schofield, C.J. (1994) J. Antibiot. 43, 1055–1056.