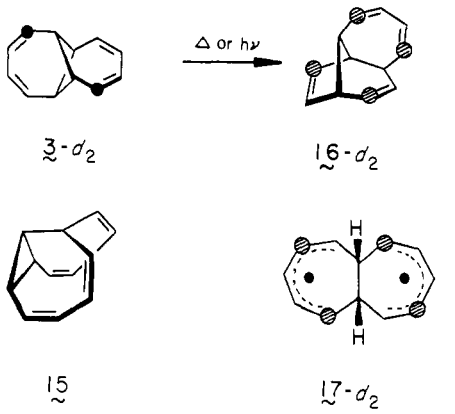
In order to assess the reactivity of **2**, solutions were prepared in $CDCl_3$ and monitored at frequent time intervals by 300-MHz ^1H NMR spectroscopy at 20 °C. Isomerization occurred quickly to give isobullvalene (**8**) and somewhat more slowly to give lumibullvalene (**9**, Scheme II).[9,10] Thus, **2** is a relatively frangible molecule.[11]

Three mechanistic options have been considered for this isomerization (Scheme III): (1) a concerted, thermally allowed [1,5]-carbon shift across the diene bridge (**2** → **10**); (2) a concerted, thermally forbidden [1,3]-carbon shift across the ethylene bridge (**2** → **11**);[12] (3) a stepwise diradical process involving homolysis of any of the four symmetry-equivalent cyclobutane σ bonds (**2** → **12**).[13] Since the pure [3,3] sigmatropy associated with the **8** → **9** process[9b] permits reliable accounting of the fate of the individual carbon atoms at this stage (e.g., **10** → **13** and **11** → **14**), suitable isotopic labeling of tricyclo[5.3.0.$0^{2,8}$]deca-3,5,9-triene can in principle distinguish between the three hypothetical pathways.

By exposure of **7** to an excess of KO-*t*-Bu in cold (−70 °C) THF containing D_2O and gradual warming of this mixture to 0 °C, it proved possible to deuteriate **2** exclusively on the olefinic bridge (0.46 D incorporation). Although the triene was likely not equivalently deuteriated at both sites, this issue is unimportant since the C_{2v} symmetry of **2** does not allow independent distinction of these positions. The species is therefore assigned as **2**-d_2 for simplicity. The smooth rearrangement to lumibullvalene-d_2 at 20 °C was monitored by ^2H NMR spectroscopy (CCl_4, 77 MHz). By means of this technique, three different types of deuterium were seen to appear (δ 6.63, 5.58, and 3.18) and in a ratio of 1:2:1.[14]

It is improbable that the 1:1 distribution of **13** and **14** arises because of entirely similar rates of [1,5] and [1,3] sigmatropy in **2**. On the other hand, formation of diradical **12** is fully compatible with our observations. Since this intermediate possesses a mirror plane, identical (save for the isotope effects) ring closure rate constants (k_{ab} and $k_{a'b'}$) explain the equal proportion of **13** and **14**. Importantly, the a priori possibility that symmetrization might originate by interconversion of **10** and **11** via a forbidden [3,5] sigmatropic rearrangement has previously been ruled out by Katz in a monodeuteriated derivative.[9b]

Force-field calculations (MMP2) give heats of formation for **2** (95.44 kcal mol^{-1}), **8** (83.70 kcal mol^{-1}), and **9** (73.63 kcal mol^{-1}) that are in good agreement with the experimental observations. In the $(CH)_{12}$ valence isomer series to which **3** ($\Delta H_f°$ = 90.50 kcal mol^{-1}) belongs, however, the semibullvalene-like structure **15** is of *higher* energy (102.35 kcal mol^{-1}) and therefore not attainable from **3** by thermal activation. The energetically feasible conversion to **16** (79.63 kcal mol^{-1}) has previously been shown to occur.[5] Following more recent preparation of the d_2 derivative,[15] the labeled tetraene has now been separately heated to 110–115 °C in CCl_4 (sealed tube) and irradiated at 366 nm in CH_2Cl_2 solution. Under both sets of circumstances, only four of the 12 possible positions in **16** showed deuterium incorporation, all with the same intensity (^2H NMR). Full symmetrization via **17**-d_2, which would have distributed the isotopic label over eight sites, clearly does not obtain. Thus, the ground- and excited-state reactivity of **3** appears limited to a formally concerted [1,3]-C migration.[12]



Finally, photoelectron spectroscopic measurements to be made on **2** are expected to reflect a very different electronic situation than that present in **3**. The possible relationship of through-bond interaction or the lack of it to the widely variant half-lives of **2** and **3** and their adoption of different mechanistic channels for rearrangement is the subject of continued study.[16]

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δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine Synthetase (ACV Synthetase): A Multifunctional Enzyme with Broad Substrate Specificity for the Synthesis of Penicillin and Cephalosporin Precursors

Gerald Banko† and Arnold L. Demain*

Fermentation Microbiology Laboratory
Department of Applied Biological Sciences
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Saul Wolfe*

Department of Chemistry, Queen's University
Kingston, Ontario, Canada K7L 3N6
Received January 27, 1987

The biosynthesis of penicillins and cephalosporins is a linear process in both eukaryotic and prokaryotic organisms.[1,2] The process begins, at the amino acid oxidation level, with the coupling of L-α-aminoadipic acid, L-cysteine, and L-valine to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV, **1**).[3] This peptide is then converted sequentially into isopenicillin N (**2**),[4] penicillin N (**3**),[5] desacetoxycephalosporin C (**4**),[6] desacetylcephalosporin C (**5**),[7] and cephalosporin C (**6**)[8] or carba-

† Present Address: Chemie Linz AG, A-4020, Linz, Austria.

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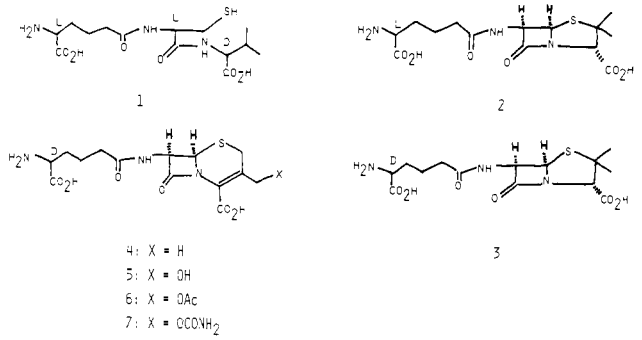
moyloxycephalosporin C (**7**),[9] the nature and oxidation level of the ultimate product depending upon the organism.



Each of the steps from **1** to **7** has been observed under cell-free conditions, using homogeneous enzymes,[10] functionally purified enzymes,[11] or mixtures of enzymes,[12] and the sequence **1** → **2** → **3** → **4** → **5** has also been accomplished in quantitative yield on a single immobilized enzyme reactor.[13] A variety of analogues of ACV, in which the L-α-aminoadipyl and/or D-valinyl moieties have been altered by chemical synthesis,[14] are accepted by one or more or the antibiotic-forming enzymes and converted into nuclear- and/or side-chain-modified penicillins/cephalosporins. Several of these nuclear-modified cephalosporins have also been prepared by multistep chemical syntheses from penicillin precursors.[1,15] It has thus been demonstrated, on a laboratory scale, that novel ring systems, in sufficient quantities for biological evaluation, are more readily accessible from peptide precursors and the appropriate combinations of enzymes and cofactors than by the more traditional methods of organic chemistry.

Nonetheless, the strategy just described still requires a peptide precursor. Although the requisite chemical syntheses have been solved,[16] and L-carboxymethylcysteine (**8**) has been found[11,17] to be an effective alternative to L-α-aminoadipic acid, a replacement of the valinyl moiety of the peptide requires the synthesis, resolution, and incorporation of a D-amino acid. In addition, an average of 10–13 protection, coupling, and deprotection steps are necessary, from the amino acids, for each new peptide. An enzymatic synthesis of tripeptide analogues of LLD-ACV would, therefore, be of value.

The formation of LLD-ACV from its amino acid precursors has received only sporadic biosynthetic attention. In an early study,[18] a tripeptide was reported to be formed by a cell-free preparation of *Penicillium chrysogenum*, but the configurations of the amino acids in this peptide were not determined. Experiments[19] with particulate fractions of *Cephalosporium* sp. suggested that L-α-aminoadipyl-L-cysteine (LL-AC) is an intermediate in the formation of LLD-ACV and that L to D epimerization of valine occurs during its attachment to LL-AC. More recently,[20] AC synthetase activity in cell-free extracts of *P. chrysogenum* has been confirmed, and soluble extracts of a nonantibiotic producing mutant of *Cephalosporium acremonium*[21] were found[22] to give 0.1–0.4% incorporation of labeled amino acids into LLD-ACV and 0.03–0.15% incorporation into LL-AC. It was thought[23] that LLD-ACV biosynthesis parallels glutathione biosynthesis[24] and involves the action of two separate enzymes.

Evidence is now presented that the ACV synthetase of *C. acremonium* C-10 is a single multifunctional enzyme, with broad substrate specificity, whose behavior is more properly compared to that of the multifunctional enzymes associated with the biosyntheses of the peptide antibiotics gramicidin S,[25] tyrocidine,[26] bacitracin,[27] polymyxin,[28] and enniatin.[29]

We believed initially that the ATP- and Mn^{2+}- or Mg^{2+}-dependent activity that is stabilized by addition of glycerol during the preparation of cell-free extracts[30] was associated with the presence of a barely detectable AC synthetase. If two enzymes were required for LLD-ACV synthesis and the first exhibited inhibition by product, LLD-ACV formation via the reaction LL-AC + L-valine would be faster than LL-AC formation from L-α-aminoadipate + L-cysteine. That this is the case is seen in Table

Table I. Peptide Formation by ACV Synthetase[a]

reactants	product	conversion, μg/mL 15 min	30 min	60 min
LD-AV + C	none			
A + LD-CV	none			
A + C	LL-AC			0.2
LL-AC + V	LLD-ACV	0.9	1.1	
A + C + V	LLD-ACV	5.2	10.2	

[a] Typical reaction mixtures contained desalted extract (about 0.5 mg of protein), 20 μg of cycloheximide, 10 mM ATP, 10 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM L-α-aminoadipic acid, 1 mM L-cysteine, and 5 mM L-valine, all components dissolved in 100 μL of 100 mM MOPS/KOH at pH 7.5. Reactions were performed at 25 °C in a water bath shaker and terminated by addition of 25 μL of 20% trichloroacetic acid. Precipitated protein was removed by centrifugation and the supernatant stored at −20 °C prior to analysis.

Table II. Formation of ACV Analogues by Single Amino Acid Replacements

amino acid replacement (5 mM)	tripeptide formed, pmol/mg min
none	130
For L-α-Aminoadipic Acid	
L-carboxymethylcysteine	63.5
L-glutamic acid	1.9
L-aspartic acid	<1.0
adipic acid	<1.0
phenylacetic acid	<1.0
For L-Valine	
L-*allo*-isoleucine	24.9
L-α-aminobutyric acid	8.4
L-isoleucine	<1.0
glycine	<1.0
D-valine	<1.0

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I, which shows data for the two reactions.[31] However, Table I also shows the unexpected finding that the conversion of L-α-aminoadipic acid + L-cysteine + L-valine to LLD-ACV is far more rapid. These observations are not compatible with the hypothesis that two enzymes are required for the synthesis of LLD-ACV from its components. Rather, a single enzyme, here termed ACV synthetase, must carry out the synthesis of LLD-ACV, and this enzyme requires binding of all three amino acids for maximum activity.

The stereochemistry of the tripeptide produced in these experiments was established by HPLC comparison with authentic LLD-ACV and also by its isolation from reaction mixtures and conversion to isopenicillin N using isopenicillin N synthetase. The L → D epimerization of valine must occur during tripeptide formation, since D-valine is not accepted as a substrate (Table II), and incubation of ACV-synthetase with LLL-ACV in the presence of isopenicillin N synthetase, oxygen, and the cofactors Fe^{2+} and ascorbate does not lead to isopenicillin N.

The optimal concentrations of ATP and Mg^{2+} were found to be 10 mM each, and the specific activity of ACV synthetase was about 20% higher in the presence of 10 mM Mg^{2+} than in the presence of 10 mM Mn^{2+}. The apparent K_m values of the synthetase for the amino acids are, A, 0.17 mM; C, 0.026 mM, and, V, 0.34 mM. No other cofactors are necessary.

The formation of ACV analogues was examined under the same conditions as for LLD-ACV (100 mM MOPS/KOH buffer at pH 7.5, 25 °C and shaking at 250 rpm). Table II summarizes the activities observed upon various single amino acid replacements. These reaction mixtures were analyzed by HPLC, and the retention time for each analogue was established by using the corresponding authentic tripeptide prepared by chemical synthesis.[16] Addition of the authentic tripeptide to a reaction mixture resulted in a single peak in each case. As seen in Table II, peptide analogues of LLD-ACV were obtained by replacement of A with L-carboxymethylcysteine or L-glutamic acid and by replacement of V with L-*allo*-isoleucine or L-α-aminobutyric acid. Tripeptide formation was not observed when L-valine was replaced by glycine. When glycine was added as a fourth amino acid, no tetrapeptide[32,33] was formed and LLD-ACV synthesis was unaffected.

Fructose 1,6-diphosphate,[34] glucose 1-phosphate,[34] orthophosphate,[34,35] and L-glutamate[36,37] were found to inhibit ACV synthetase. L-Methionine, glutathione, L-leucine, L-isoleucine, and D-valine (all added at a concentration of 5 mM) had no effect. No component having a positive effect upon the synthetase could be found.

Purification and further characterization of ACV synthetase are in progress.

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Supplementary Material Available: Experimental procedure for synthetase formation (1 page). Ordering information is given on any current masthead page.

(31) General experimental and analytical procedures are described in ref 30.

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