Isopenicillin N Synthase: Mechanistic Studies

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I. Introduction

Although it is generally accepted that the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (LLD-ACV) (**1**) is the direct precursor to isopenicillin N (**2**),[1,2] the intimate details of the mechanism of this remarkable oxidative bicyclization have yet to be fully elucidated. A *single*, ferrous-dependent, non-heme enzyme named isopenicillin N synthase (IPNS) has been shown to catalyze this reaction.[3] This unequivocally followed from its isolation and sequence determination from *Cephalosporium acremonium* and directly led to its overexpression in *Escherichia coli*.[4] Since this initial expression, the IPNS enzyme has been isolated from at least five sources and the gene sequenced,[5–8] all showing a large degree of amino acid sequence homology and similar molecular weights (37 000–40 000), although the IPNS enzyme recently reported from *Streptomyces lactamaurans* remarkably has a molecular weight of only two-thirds that of the other IPNS enzymes.[9]

The bicyclization reaction (formally a 4-electron oxidation process) has been shown to proceed with the loss of just four hydrogen atoms, with the key abstraction of both the cysteinyl and valinyl C-3β hydrogens.[10,11] The stoichiometric consumption of molecular oxygen[12] and the observation of two distinct kinetic isotope effects for each C–H bond cleavage event (isotope effects that are not observed to be multiplicative)[13] suggest at least a two-step enzymatic process. No enzyme-free intermediates have been detected between tripeptide **1** and the bicyclic penicillin **2** despite intensive investigations using cell-free extracts of IPNS over the past decade, an observation in agreement with kinetic isotope analysis.[14] The lack of structural data relating to the IPNS enzyme has hampered a detailed mechanistic interpretation of both the binding and subsequent cyclization of the tripeptide LLHd-ACV (**1**) to isopenicillin

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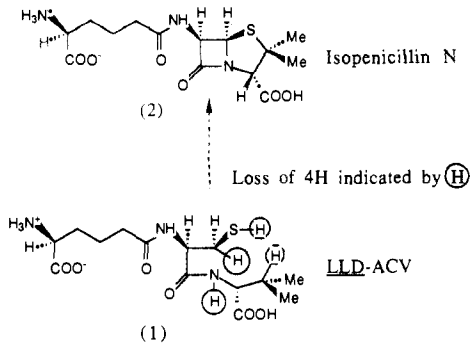
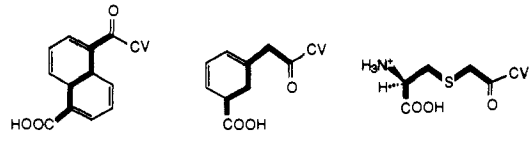


N (2) at the active site of the cyclizing enzyme. However, the binding and mechanism have been investigated, indirectly, by the systematic modification of the three amino acids of the natural substrate. Both isotopic and/or structural analogues of LLD-ACF have been used in a process that can structurally probe potential enzyme binding sites while also casting light upon the likely mechanism of cyclization. However, it should be borne in mind that only the starting materials and products are being observed and analyzed: thus, any information concerning a two-step enzymatic process and the formation of enzyme-bound intermediates can only be indirectly inferred.

This review will look at four main points of interest relating to work that has been carried out over the past decade on cell-free extracts of isopenicillin N synthase: i, aminoadipoyl variants; ii, cysteinyl variants; iii, valinyl variants; iv, evidence for initial β-lactam ring closure.

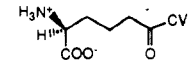
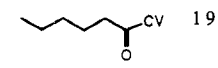
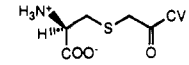
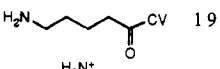
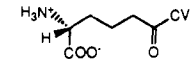
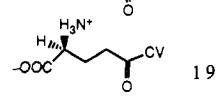
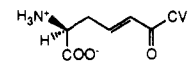
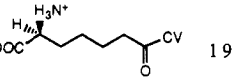
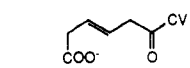
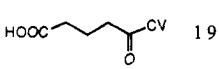
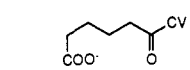
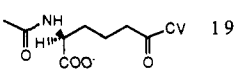
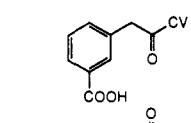
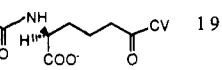
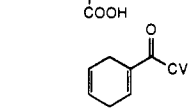
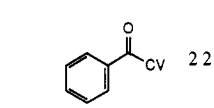
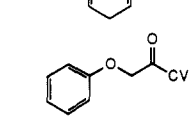
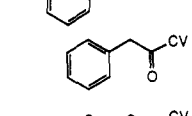
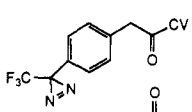
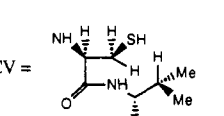
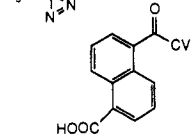
II. δ-L-α-Aminoadipoyl Variants

The various aminoadipoyl variants that have been prepared are shown in Table I, together with an indication of their acceptability to the IPNS enzyme. These suggest the possibility of the presence of a remote binding site for the aminoadipoyl side chain carboxyl group, perhaps via H-bonding interactions with lysine or arginine residues, a view supported by molecular modeling of the IPNS enzyme.[15] A requirement of a chain of six carbon atoms or equivalent is suggested (both the five- and seven-carbon chain analogues of the aminoadipoyl side chain are inactive), while the fact that substrates such as **3a–c** are converted with high efficiency again suggests that only a linear chain, terminating in a carboxyl group, is required for maximum conversion (V_{max}), even though these may contain heteroatom linkages.



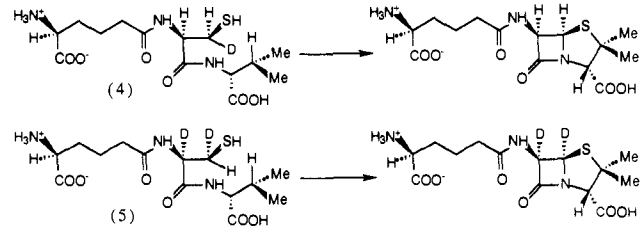
However, the fact that both the benzyl and phenoxymethyl side chains are also accepted by the IPNS enzyme (although only poorly) could also imply an active site cleft/binding region containing a large number of nonpolar alkyl/aromatic residues. Most interestingly, the acceptance of both D and L stereochemistry at the aminoadipoyl terminus, as well as the acceptance of adipic acid, suggests little binding influence for the amino terminus.

TABLE I

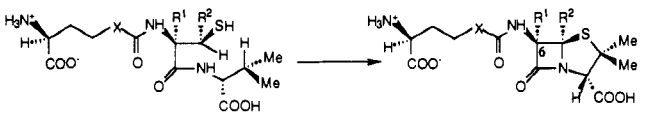
ACCEPTABLE SIDE CHAINS	Ref	UNACCEPTABLE SIDE CHAINS	Ref
			19
	16,17,19		19
	18,19		19
	20		19
	20		19
	19		19
	21		19
	22		22
	22		
	22,23		
	24,25		
	26		

III. L-Cysteinyl Variants

In 1986, cell-free extracts of IPNS were shown to catalyze the completely stereospecific cyclization of specifically deuterated cysteinyl-labeled tripeptides **4** and **5** (against a primary kinetic isotope effect in the case of tripeptide **4**), with retention of configuration at the cysteinyl C-3 position.[10]



Other variants have shown the L-cysteinyl moiety is essentially inviolate, very few structural modifications having been found to be acceptable. This may be understandable if one considers a binding site that must distort the cysteinyl region of the tripeptide into the strained "transition structure" necessary for β-lactam ring formation. Hence, the cysteinyl residue probably has a large number of potential binding/contact sites with the enzyme. Three structural analogues **6–8** have been made that have been accepted by the IPNS enzyme, perhaps indicative of the crucial role played in binding of this residue.



(6) R^1=Me, R^2=H, X=CH_2

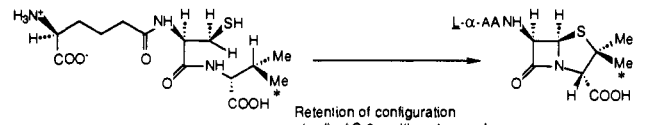
(7) R^1=H, R^2=Me, X=CH_2

(8) R^1=OMe, R^2=H, X=O

Some structural information has thus been obtained from these analogues, such as the acceptance of the α-methyl- and α-methoxycysteinyl-containing tripeptides 6–8.[28] Of special interest is the 6-methoxypenicillin tripeptides isolated from incubation of tripeptide 8, due to its similarity to the cephamycins.[29,30]

IV. D-*Valinyl Variants*

In 1986 it was shown, with use of cell-free extracts, by ^{13}C NMR spectroscopy that the natural substrate LLD-AC[3*S*,4-^{13}C]V was converted to isopenicillin N with complete retention of the valinyl C-3 stereochemistry.[31] However, numerous other structural valinyl

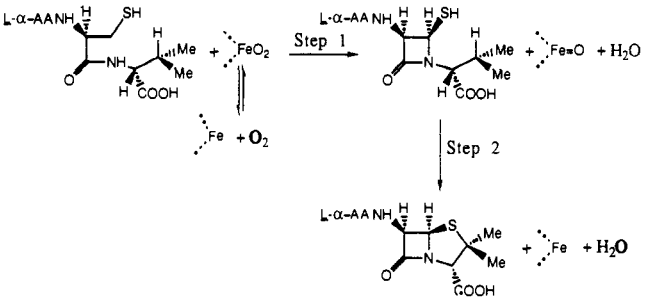


analogues have been accepted. The D configuration of the terminal amino acid in the tripeptide seems at first glance to be the only major constraint of the IPNS enzyme with regard to variation of this valinyl moiety, a remarkably large degree of substrate variability being observed. It is probably worth emphasizing that the β-lactam ring is common to all the new products isolated from these substrates[32] in spite of, as will be seen later, the variation in second ring size (5–8). This is perhaps evidence in itself of initial formation of the β-lactam ring, a hypothesis maintained throughout this section and enlarged upon and substantiated in section D. A large amount of data are available with respect to the acceptabilities/nonacceptabilities of substrate variants in the valinyl position. These results have allowed a structural hypothesis (mapping) for the substrate/enzyme complexes, as well as mechanistic investigations utilizing both isotopomers and chemical probes. These results can be conveniently divided into five sections: i, chemical probes; ii, sensitivity to bond strengths; iii, sensitivity to polarity of analogues; iv, steric effects; v, conformational effects.

A. Mechanistic Investigations Utilizing Chemical Probes

Since the overall redox change catalyzed by IPNS is a 4-electron change, resulting in the case of the natural substrate in the complete reduction of oxygen to two molecules of water, and since kinetic isotope effects observed in IPNS are indicative of a two-step mechanism (vide infra), then the simplest working hypothesis is that the iron atom mediates two separate, 2-electron reductions of iron-bound dioxygen by the substrate. In the case of substrate 1, this sequence is shown in Scheme I, in which the following step 1 the enzyme contains a ferryl species (oxoiron (IV)) that mediates the second ring closure, step 2. This scheme is further simplified by postulating that the cysteinyl sulfur atom

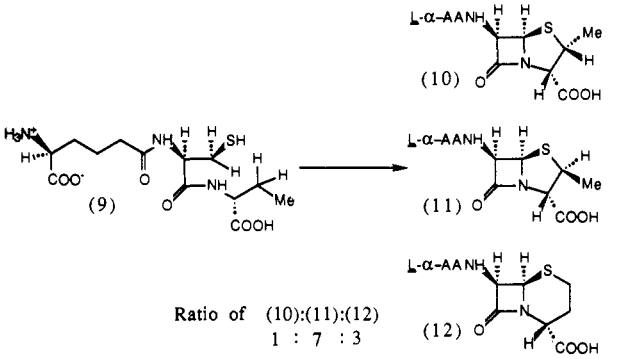
SCHEME I



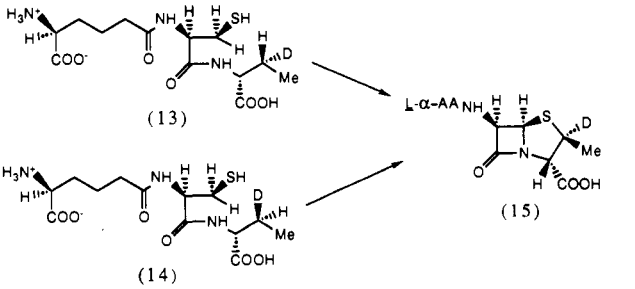
is actually coordinatively bound to the iron atom, since in this way the first electron-transfer reaction, step 1, is directly mediated through the iron–sulfur conductor. The four modes of second ring closure, step 2, may now be rationalized on the basis of four discrete mechanistic types characteristic of the ferryl species: i, radical abstraction/recombination; ii, 1,3-shifts/oxo–ene reaction; iii, [2π + 2π] cycloaddition/reductive elimination; iv, epoxide formation/inverting displacement. Recognition of these four pathways has emerged from the study of a wide range of valine analogues incorporated into the tripeptides and challanged with IPNS. Some of the more significant results are described as follows.

i. Radical Abstraction/Recombination

The aminobutyrate-containing tripeptide **9** upon incubation with IPNS[33,34] gave rise to the three β-lactam-containing metabolites: these were the two penams

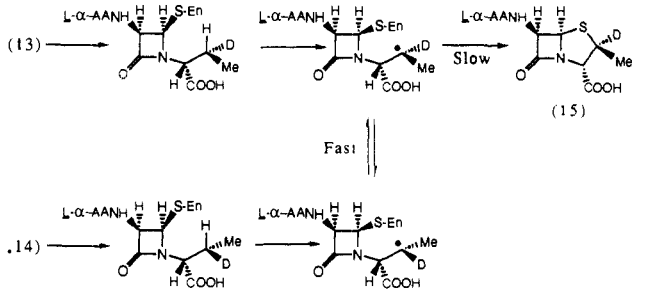


10 and **11** and the cepham **12**. When the specifically deuterated aminobutyrates **13** and **14** were prepared and incubated,[35] they both gave rise to the same β-methyl-α-deuteriopenam (**15**), obviously proceeding with inversion of stereochemistry in the case of tripeptide **14** and retention in **13**.



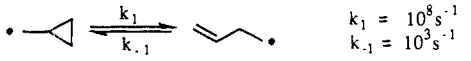
This suggests a common intermediate from both of the starting tripeptides. A hydrogen atom in both cases was removed in preference to that of a deuterium atom, and these results led to the proposal of a radical-type intermediate (see Scheme II). The deuterated aminobutyrate results have implications with respect to

SCHEME II

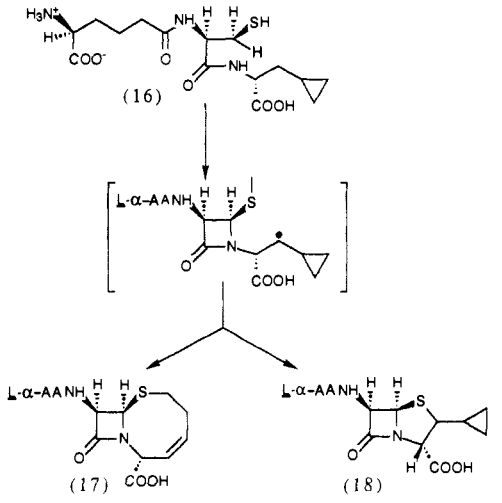


both the preferred β-group orientation of the methyl group and the sensitivity of IPNS toward C–H bond strengths. To test this radical hypothesis, chemical probes such as cyclopropyl[36,37] and cyclobutyl[38,39] groups have been utilized to try to detect free-radical behavior in these systems.[40]

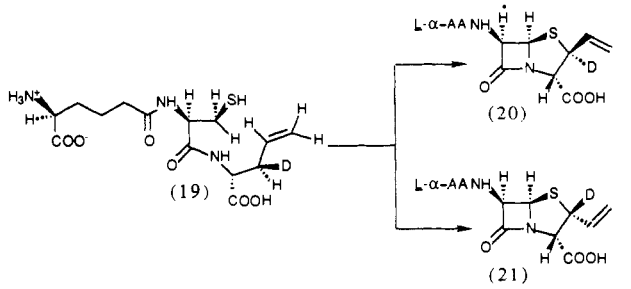
Cyclopropylcarbinyl radicals are well-known to rearrange to the allylcarbinyl system, in a reversible manner. The time scale of this rearrangement is such



that it was hoped that formation of a cyclopropylcarbinyl radical in a substrate such as tripeptide **16** would lead to ring opening relatively quickly with respect to radical trapping. Indeed, when tripeptide[41] **16** was prepared and incubated with IPNS, two products **17** and **18** were isolated, suggestive of a free-radical mechanism:

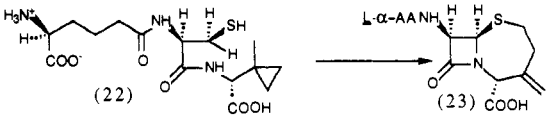


Further evidence for the presence of a radical intermediate comes from the incubation of the specifically deuterated tripeptide **19** with IPNS and the isolation of penams **20** and **21**.[42,43]

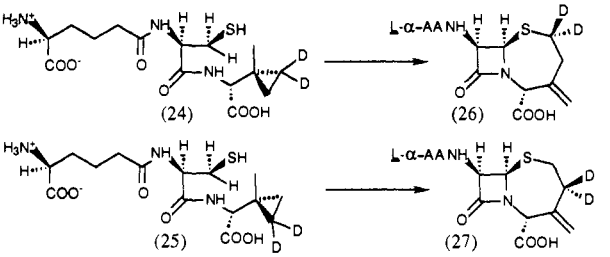


ii. 1,3-Shifts/Oxo–Ene Reaction

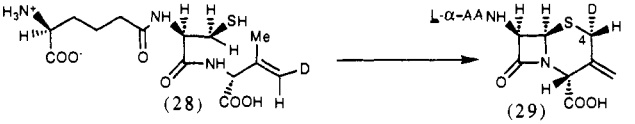
The (methylcyclopropyl)glycine analogue **22** was found to be a reasonably good substrate with IPNS,[41] showing complete rearrangement to give *exo*-methylenehomocepham (**23**). Initially this was again



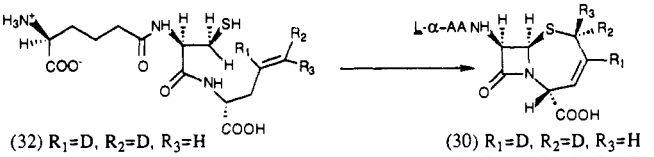
thought to be perhaps indicative of a free-radical process. However, the specifically deuterated methylcyclopropyl systems **24** and **25**[44] each gave only one specific deuterated product (**26** and **27**, respectively), a result suggestive of either a nonradical mechanism (such as a concerted 1,3-cleavage) or an enzymatically constrained system leading to cleavage of only the α-cyclopropyl bond (perhaps due to stereoelectronic effects). The results from incubation of the specifically



deuterated dehydrovaline-containing tripeptide **28**, in which the label in the unsaturated portion of the molecule ends up stereospecifically at position C-4 of *exo*-methylenecepham (**29**), are also indicative of a highly controlled concerted type reaction.[45] The for-



mation of the homoceph-3-ems **30** and **31** from the deuterated allylglycine-containing tripeptides **32** and **33**, respectively, also provides evidence for the possibility of a 1,3-shift, (although a conjugatively rigid allylic radical is also a possibility).[42,43]

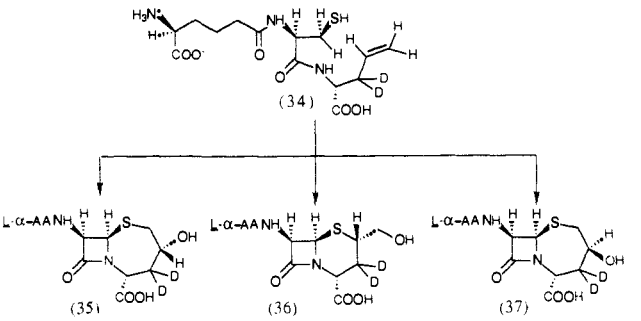


(32) R_1=D, R_2=D, R_3=H
(33) R_1=H, R_2=H, R_3=D

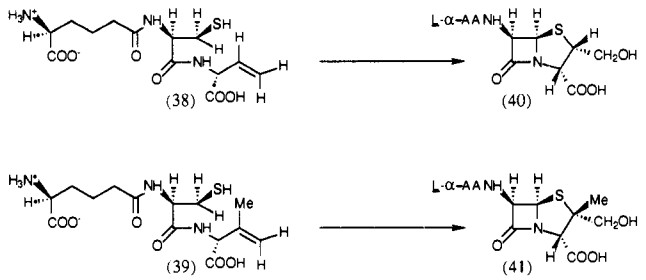
(30) R_1=D, R_2=D, R_3=H
(31) R_1=H, R_2=H, R_3=D

iii. [2π + 2π] Cycloaddition/Reductive Elimination

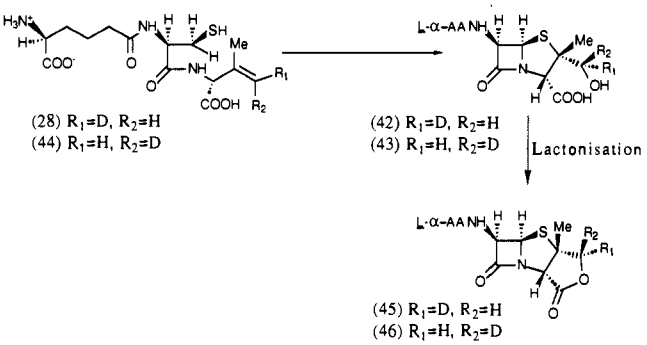
Incubation of the allylglycine-containing tripeptide **34** with IPNS led to the isolation of three oxygenated products **35–37**, the oxygen being shown to be derived



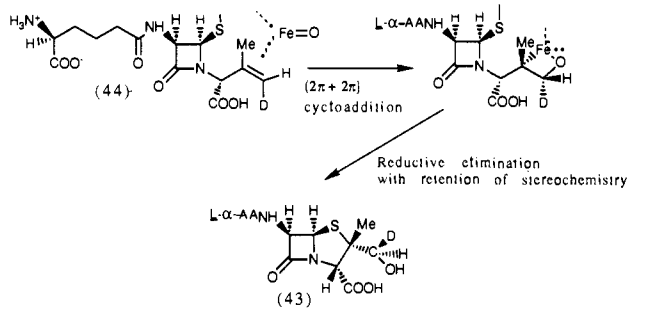
from O_2 (using $^{18}O_2$) and showing the first evidence of the monooxygenase (−2H + 1O) type activity of IPNS.[27,46] Such a result is not in isolation. Tripeptides **38** and **39**[47] have given the same type of oxygenated products **40** and **41**. The stereochemical course of these



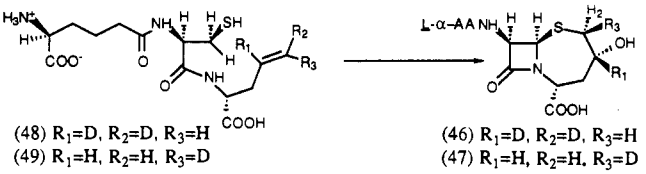
oxidation reactions was followed by labeling studies and produced some quite remarkable results, giving evidence of two further types of pathway from IPNS. One isomer of (hydroxymethyl)penams **42** and **43** is produced from tripeptides **28** and **44**, respectively. Lactones **45** and **46** are produced with the stereochemistry shown.[45] Mechanistically both the oxygen and the



sulfur have added from the same face of the olefin. Epoxide intermediates are obviously ruled out, and a $[2\pi + 2\pi]$ addition of a reactive iron–oxene across the olefin has been suggested, followed by reductive insertion of the sulfur atom. Such a result is also the

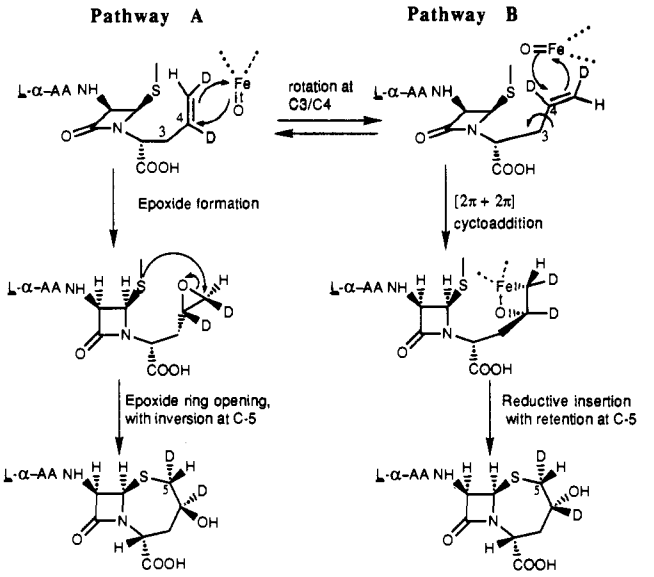


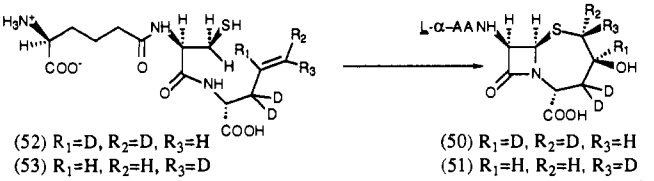
conclusion drawn for the formation of the major isomer of the isolated 4α-hydroxyhomocephams **46** and **47** prepared from the deuterated tripeptides **48** and **49**.[42,43]



However, epoxide formation must be postulated for 4β-hydroxyhomocephams **50** and **51** following their isolation from incubations of deuterated tripeptides **52** and **53**, respectively, with oxygen and sulfur appearing to have added from opposite faces of the olefin.[46]

SCHEME III





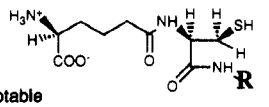
iv. Epoxide Formation/Inverting Displacement

These results with the formation of 4β-hydroxyhomocephams **50** and **51** are best explained as shown in Scheme III. The reactive iron–oxygen species attacks either the *re* or *si* face of the olefin, whichever is presented to it, leading to the two intermediates shown (either by two direct $[2\pi + 2\pi]$ cycloadditions followed by collapse to the epoxide or by direct epoxidation). Second ring formation then takes place by either reductive insertion (with retention of stereochemistry at C-5) or epoxide ring opening (with inversion of stereochemistry at C-5). These results have led to the postulation of an iron–oxene species (Fe^{IV}=O) as a reactive intermediate in penicillin biosynthesis, similar to those implicated in P450 cytochromes,[48,49] phenylalanine hydroxylase,[50] peroxidases[51] and bleomycin.[52] These species are known to be able to activate primary C–H bonds to generate radicals and to undergo hydroxylation reactions, as well as to add across double bonds to form epoxides (in the case of some model studies these epoxidation reactions have been suggested to proceed via a four membered iron–oxo intermediate).[53] Thus, we consider an iron–oxene species to be a viable intermediate, able to undergo the remarkable range of chemistry illustrated above, and the basic four mechanisms described can account for the products and the labeling patterns shown.

B. Sensitivity of Product Distribution to Bond Strengths

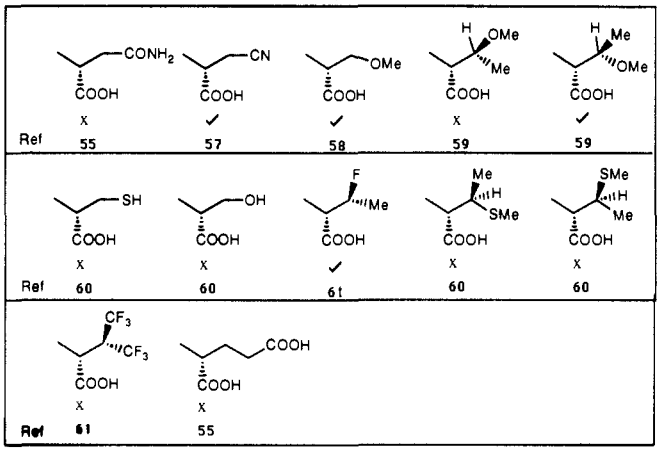
As was shown in the aminobutyrate cases, preferential removal of the hydrogen atom over that of deuterium took place for isotopomers **13** and **14**, suggesting a

TABLE II

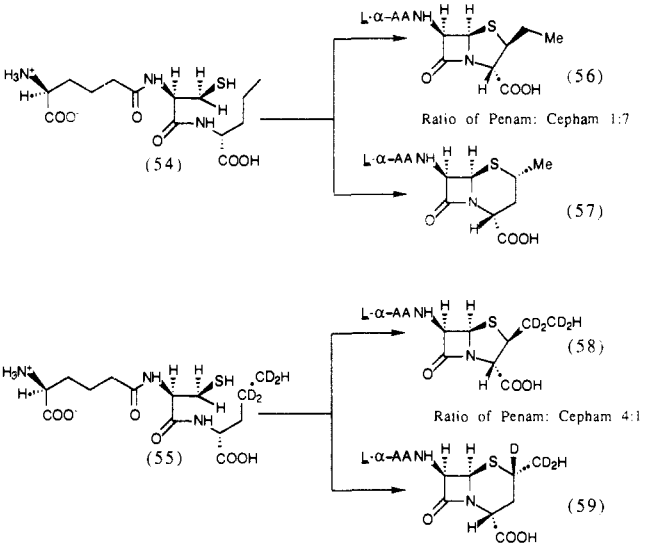


✓ = acceptable
x = not accepted

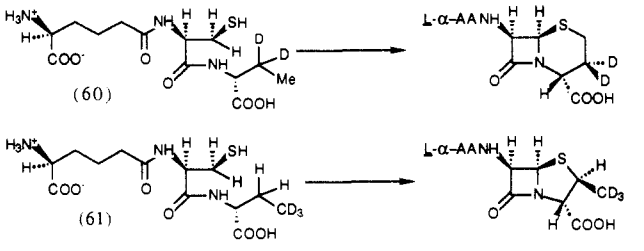
Polar side chains = R



sensitivity of the enzyme's catalytic center to C–H bond strengths. This effect can be viewed with many substrates, for example the norvalinyl analogues **54** and **55**, which give the ratios of penams **56** and **58** and cephams **57** and **59** shown below:[54,55]



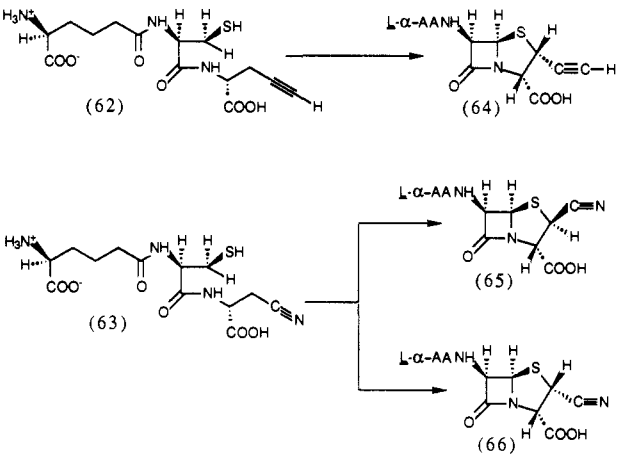
A product biasing of over 28-fold was observed. Similar effects of deuteration have also been observed with the deuterated aminobutyrate-containing tripeptides **60** and **61**.[56] A distinction is thus drawn by the enzyme on the



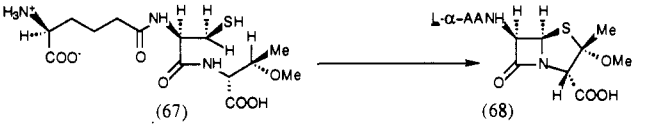
basis of bond strengths. This would also be expected to influence product ratios (in terms of penam/cepham ratios) for processes involving cleavage of primary vs secondary vs tertiary bonds, examples of which have already been shown, but these may also be influenced by product ring strain as shown for the norvaline-containing tripeptides.

C. Sensitivity of Isopenicillin N Synthease to Polar Valinyl Analogues

Few polar analogues of valine have been accepted as substrates (see Table II). Overall, heteroatoms appear to reduce acceptability to the enzyme. This is perhaps most vividly demonstrated with the two tripeptides **62** and **63**. In the case of the acetylene-containing tripeptide, conversion to the α-acetylenic penicillin **64** is virtually quantitative, while nitrile analogue **63** gives penicillins **65** and **66** in low overall conversion.[57] This



is interesting as the nitrile and acetylene groups are structurally very similar. Thus, the bias may result from electronic grounds (polarity) alone, a factor that may have influenced the relatively poor conversion of **63** when compared with the acetylene-containing tripeptide. This low level of conversion is also found for the *O*-methylserine valinyl analogue, while only the (*R*)-methyl-*O*-methylthreonine isomer (**67**) was accepted as a substrate by IPNS[59] giving penicillin **68**.

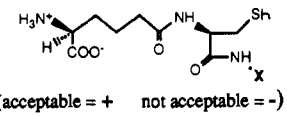


D. Steric Effects—Active Site Mapping

From the structures shown in Table III it is observed that tripeptides that are alkyl branched at or beyond C-4 of the D-amino acid side chain are not acceptable as substrates for the IPNS enzyme. Branching at C-3 is accepted, while alkyl chains up to C-5 are accepted, but not beyond this unless they are unsaturated. These ideas can perhaps be rationalized in terms of the "binding pockets" surrounding the valinyl region of the tripeptide probably being relatively nonpolar, a fact that can perhaps be rationalized with respect to (1) natural substrate binding for which one would expect relatively nonpolar residues and (2) the possibility of a highly reactive iron–oxene type intermediate and the region of the protein in proximity to this species being relatively nonreactive.

In Table IV a series of monosubstituted penicillins that have been isolated following incubations of tripeptide analogues of LLD-ACV are shown. It is of interest to see how we can relate the observed stereo-

TABLE III



(acceptable = + not acceptable = -)

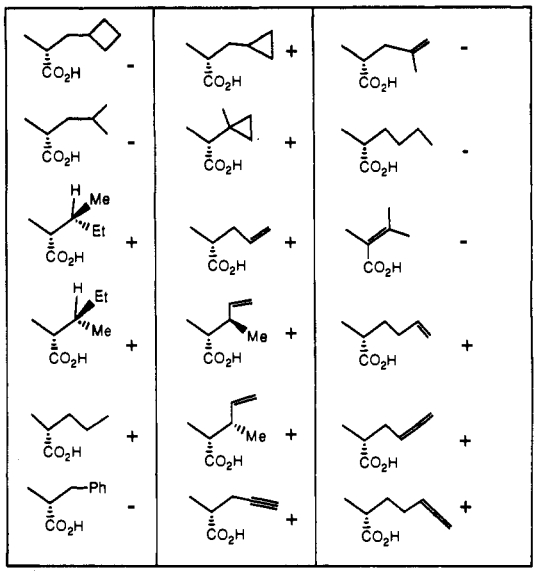
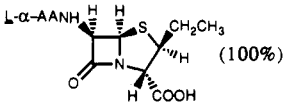
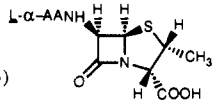
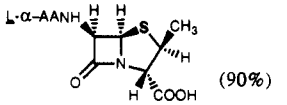
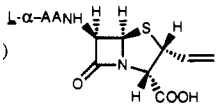
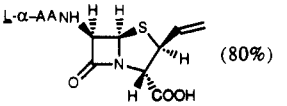
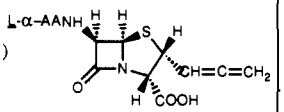
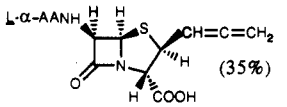
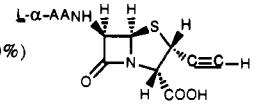
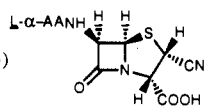
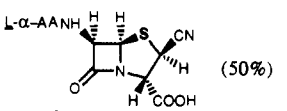


TABLE IV. **Monosubstituted Penicillins Isolated from Substrate Analogues of LLD-ACV after Incubation with IPNS (Relative Ratios, %)**

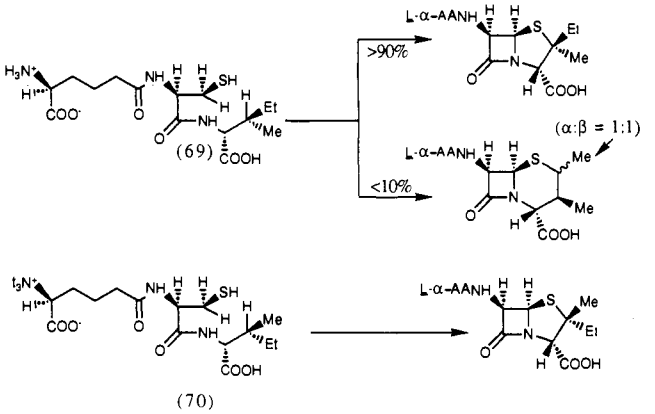
α-Penicillins Isolated	β-Penicillins Isolated
	 (100%) β-ethyl penicillin
 (10%) α-methyl penicillin	 (90%) β-methyl penicillin
 (20%) α-vinyl penicillin	 (80%) β-vinyl penicillin
 (65%) α-allenyl penicillin	 (35%) β-allenyl penicillin
 (100%) α-acetylenic penicillin	
 (50%) α-cyano penicillin	 (50%) β-cyano penicillin

chemistries of the isolated penicillins to both enzymic and chemical considerations and consider the possible reasons for the bias found.

It becomes apparent that alkylpenicillins tend to have predominantly β-stereochemistry, while with the unsaturated systems α-orientation begins to become favored. It is possible to view this effect as arising from active site constraints, which play a role in maintaining and stabilizing the stereochemical orientation of the substituent group, after radical-type hydrogen abstraction from the C-3 position of the valinyl moiety of the tripeptide analogue. [Radical formation is considered to be the most likely possibility considering the results of the two specifically deuterated aminobutyrate-containing tripeptides δ-(L-α-aminoadipoyl)-L-cysteinyl-D-(3*R*)-2-amino-3-deuteriobutyrate and δ-(L-α-aminoadipoyl)-L-cysteinyl-D-(3*S*)-2-amino-3-deuteriobutyrate, which both gave the same β-methyl-α-deuteriopenam[35] and results with the cyclopropylalanine-containing tripeptide.[41]]

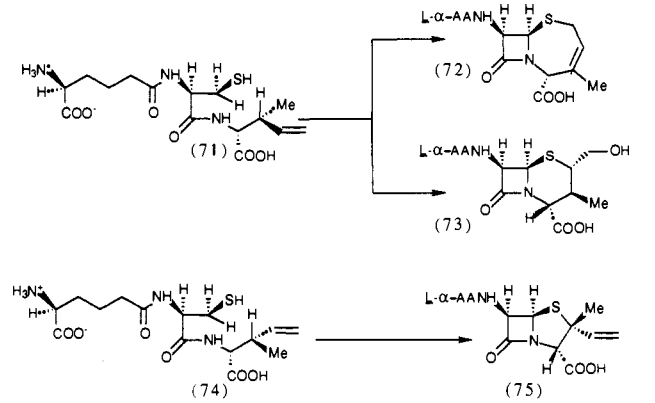
E. Conformational Restrictions

A table of substrate acceptabilities/nonacceptabilities is shown (Table III). In terms of the products formed, the most vivid structural constraints are shown to arise with the two isoleucine/alloisoleucine tripeptides[63] **69** and **70**, which can be contrasted with the two deuter-



(70)

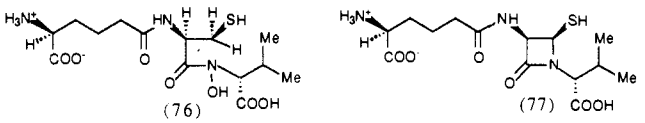
ated aminobutyrate tripeptides **13** and **14** giving rise to the same β-methyl-α-deuteriopenam (**15**). These conformational effects can be even more vividly demonstrated with tripeptide **71**, giving products **72** and **73**, and tripeptide **74**, giving penicillin **75**.[64] The contrast between these products can be rationalized if one considers the conformational restriction around the C_α and C_β bond of the valinyl analogue and the geometric relationship between the iron–oxene and the two differing substrate environments. Thus, in the case of tripeptide **71** the enzyme undergoes a [2π + 2π] cycloaddition and an "ene"-type reaction to give 3-methylhomoceph-3-em (**72**) and 3-methyl(hydroxymethyl)cepham (**73**). In contrast tripeptide **74** gave only penam **75** with retention of configuration.



V. Evidence for First Ring Closure in Penicillin Biosynthesis

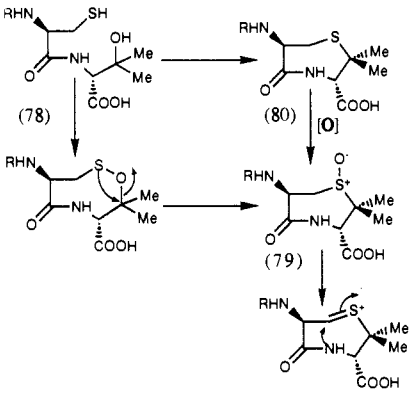
As has been shown there is much circumstantial evidence that β-lactam ring formation is the first ring formed in penicillin biosynthesis. This follows not only from the isolation of numerous β-lactam-containing metabolites from valinyl analogues but also from the different labeling patterns and stereochemical outcomes of ring closures upon the valinyl analogues; a common enzyme-bound intermediate leading to these various products is a much more realistic rationale than the alternative scenario.

The stepwise nature of this reaction (which is also perhaps indicated by the stoichiometric consumption of molecular oxygen,[12] since it is considered extremely unlikely that the conversion $O_2 \rightarrow 2H_2O$ could occur in just one step) suggested that it may be possible to observe or incorporate a synthesized intermediate into the penicillin nucleus. Among those hypothesized intermediates exposed to cell-free extracts of IPNS include the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-(N-OH)valine (**76**)[67] and 1-(1-D-carboxy-2-methylpropyl)-3(*S*)-(5-L-aminoadipamido)-4(*R*)-mercaptoazetidin-2-one (**77**), a compound with a turbulent history.[68-70] Other proposed and tested intermediates in



(76) (77)

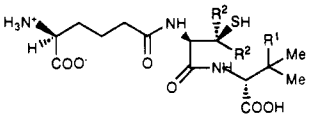
penicillin biosynthesis include the β-hydroxyvalinyl tripeptide **78** as well as the thiazepines **79** and **80**.[71,72]



(78) (80) (79)

However, none of these proposed intermediates have even been observed to be incorporated into isopenicillin N, while all attempts to detect enzyme-free intermediates from cell-free systems of IPNS by various techniques including ^{13}C NMR and the substrate LLD-A[3-^{13}C]C[^{15}N]V have also met with failure.[73] Such results however are always negative and have restricted utility in that problems with the correct redox potential of the enzyme could lead to failure of incorporation. However, these results along with the kinetic isotope data shown below have led to the tentative conclusion that *no* enzyme-free intermediates can exist in the cyclization of the peptide precursor to isopenicillin N. However, there is much additional evidence to suggest that β-lactam ring closure is the initial oxidative step in penicillin biosynthesis.

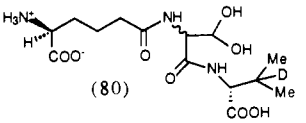
In 1984, isotopic competition experiments[74] were published by Baldwin. The basis for these experiments is that IPNS is exposed to an approximately 1:1 ratio of two tripeptides, either (LLD-ACV and LLD-A[3,3-$^2H_2$]CV) or (LLD-ACV and LLD-AC[3-^{2}H]V) and the remaining *substrate* ratio is determined with respect to time by mass spectrometry techniques. It was observed in the former case that the remaining substrate pool was becoming enriched in the dideuterated cysteinyl tripeptide, suggesting that there were only equilibrium steps before the irreversible step of cysteinyl C-3–H bond cleavage. In the latter experiment no discrimination was observed between the two substrates. The rationale behind this result is either that valinyl C-3–H bond cleavage is a reversible event, before or after the cysteinyl event, or that if irreversible it occurs after cysteinyl C-3–H bond cleavage. The kinetic isotope effects (under saturating substrate conditions) for each of the two C–H bond cleavages necessary for bicyclization were reported in 1988 and are shown below:[13]



R^1	R^2	D_V	$D_{V/K}$
H	H	1	-
H	D	5.6 ± 0.3	1.4
D	H	13 ± 2	1.0
D	D	18 ± 2	-

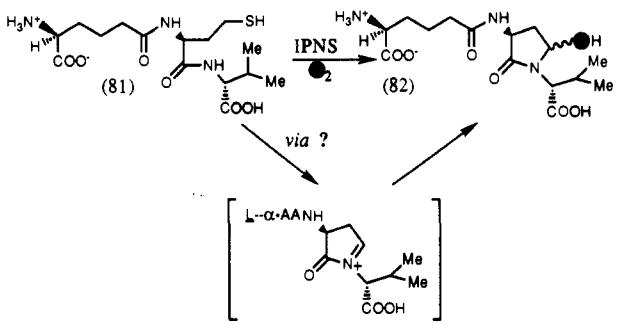
Since there is a large kinetic isotope effect for both cysteinyl C-3–H and valinyl C-3–H bond cleavages (i.e., both steps are partially rate limiting), then it would be difficult to envisage how discrimination could *not* occur for valinyl C-3–H bond cleavage unless it followed an irreversible event, the cysteinyl C-3–H bond cleavage event. It is also perhaps worth making the point that the cosubstrate is dioxygen for which an equilibrium process involved in the cleavage of the valinyl C-3–H bond would be an unlikely reaction. (The isotopic discrimination experiments also suggest that there is no enzyme-free intermediate after initial β-lactam ring formation as this would have led to a discrimination between the deuterated and nondeuterated substrates in both mixed substrate experiments.)

The kinetic isotope effects mentioned above suggest that oxidation at the cysteinyl C-3 position is at least partially rate limiting, perhaps leading to an enzyme-bound thioaldehyde; however, it does not provide direct evidence either kinetically or chemically for initial β-lactam ring formation. However, a solution to this problem was sought by three approaches, one of which led to isolation and characterization of the "shunt metabolite" (**80**),[75] arising from a stereospecific oxida-

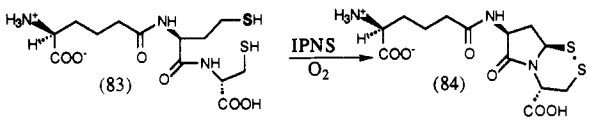


(80)

tion at the cysteinyl C-3 position and loss of the cysteinyl sulfur atom. This has been postulated to arise via an enzyme-bound intact β-lactam intermediate that collapses before thiazolidine ring closure. The tripeptide δ-(L-α-aminoadipoyl)-L-homocysteinyl-D-valine (**81**) has been incubated with IPNS and led to γ-lactams **82** as the only isolable products.[76] These can be thought



to be derived by a mechanism similar to that of the shunt metabolite (80) above. The hydroxy group in these products was found to be at least in part partially derived from molecular oxygen, an idea supported by the incubation of tripeptide 83 and isolation of disulfide 84.[77] Thus, in conclusion, all evidence points to the formation of a β-lactam ring as the first ring formed in penicillin biosynthesis.



Mechanistically, isopenicillin N synthease remains a remarkably intriguing and unique enzyme from both a chemical and biological viewpoint, with much evidence pointing toward the involvement of a reactive, high-valency iron–oxygen species mediating second ring closure in the formation of the plethora of products shown above.

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Registry No. IPNS, 78642-31-6.

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