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BIOSYNTHESIS OF β-LACTAM ANTIBIOTICS

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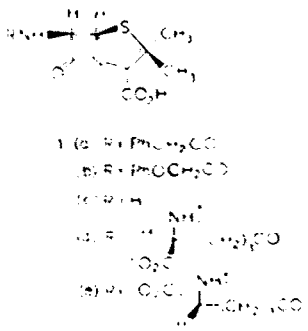
INTRODUCTION

The investigation of the biosynthesis of the β-lactam antibiotics has a history of over thirty years. Considerable progress has been made, especially in recent years, in the elucidation of the detailed pathways taken for the biosynthesis of such compounds. However, major problems in this field remain unsolved. In particular the basic mechanism for the formation of the bicyclic ring system remains unknown, although several proposed mechanisms have recently been shown to be untenable.

The objective of this review is to present a summary of current knowledge of the biosyntheses of the β-lactam antibiotics with particular emphasis on developments of the past several years. The results of chemical model studies, designed to test the chemical feasibility of certain proposed biosynthetic pathways, will be described, and some remaining problems will be delineated. The massive chemistry of the β-lactam antibiotics, still a very active field of investigation, will not be discussed, except where it has been applied in the investigation of the biosyntheses.

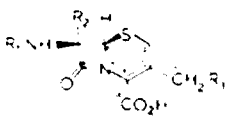
Structural types and their microbial origins

The discussion in this report will be limited to those naturally occurring β-lactam compounds which are derivatives of either the penicillin (penam) ring system **1**, or the cephalosporin (3-cephem) ring system **2**. Other



types of β-lactam-containing natural products have recently been identified,[1,2] but will be considered outside the scope of the present review.

The penicillins, produced by various species of *Penicillium*, especially *P. chrysogenum*, as well as other species of fungi,[1] all possess the same bicyclic ring



2 R₁ R₂ R₃

c) D-α-aminoadipyl H OAc
b) D-α-aminoadipyl H OH
c) H H OAc
d) D-α-aminoadipyl OCH_3 OAc
e) D-α-aminoadipyl H $OCONH_2$
f) D-α-aminoadipyl OCH_3 $OCONH_2$
g) D-α-aminoadipyl H H

system **1**, consisting of a β-lactam ring cis-fused to a thiazolidine ring. Over one hundred penicillins, differing only in the nature of an *N*-acyl side chain, can be produced by fermentations to which an appropriate side-chain precursor has been added.[3] Aliphatic or aryl-substituted aliphatic carboxylic acids, or analogues which can easily generate such acids *in vivo* constitute acceptable side-chain precursors. However, of the many penicillins producable biosynthetically, only benzylpenicillin (penicillin G) **1a** and phenoxymethylpenicillin (penicillin V) **1b** are of great clinical utility.[1] In the absence of a suitable side-chain precursor, fermentation of *P. chrysogenum* leads to the formation of 6-aminopenicillanic acid **1c**, and isopenicillin N **1d**.[5,6] The former compound (6-APA) is an important intermediate in the preparation of the so-called semi-synthetic penicillins, produced by acylation of the free amino group.

A close relative of isopenicillin N, namely penicillin N **1e**, was isolated[7-9] in 1952 from a culture of *Cephalosporium sp.* which was isolated from seawater near the outflow of a Sardinian sewer. This antibiotic has subsequently been isolated from other related species,[1] especially *C. acremonium*. No other penicillins are produced by species which synthesize penicillin N, and side-chain precursors which are easily incorporated into penicillins by *P. chrysogenum* are not utilized.[10]

Later, a new antibiotic, Cephalosporin C, was isolated[9,11,12] from *C. acremonium*, and shown to have structure **2a**.[13,14] Deacetylcephalosporin C **2b**, also is produced by *C. acremonium*.[15,16] These compounds were, until recently, the only known naturally-produced examples of the 3-cephem system. The α-aminoadipyl side chain has the D-configuration in common with penicillin N. No other side-chain analogues have been produced biosynthetically, and 7-aminocephalosporanic acid (7-ACA) **2c**, is not formed by *C. acremonium*, in contrast to the biosynthetic abilities of *P. chrysogenum*. However,

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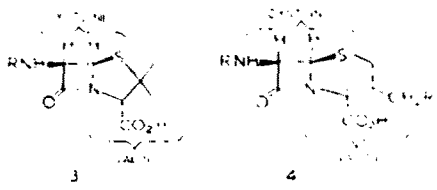
7-ACA can be prepared chemically or biologically from cephalosporin C, and it serves as the precursor for the preparation of some clinically valuable antibiotics.[18] One of the problems in this approach has been the relatively low yield of cephalosporin C produced in cultures, as compared with the yields of penicillins by *P. chrysogenum*. This problem has therefore stimulated extensive research on the development of synthetic routes for converting the readily available penicillins into analogues having the 3-cephem ring system. This goal has now been accomplished.[19]

Recently several analogues of cephalosporin C **2d**, **2e**, **2f** were isolated[20] from various species of *Streptomyces*. They differ from cephalosporin C in having a 7α-methoxy group and/or a 17-carbamoyl group in place of the acetoxy group.

BIOSYNTHETIC PRECURSORS OF THE PENICILLINS AND CEPHALOSPORINS

The results of studies, up to the early 1970s, on the establishment of biosynthetic precursors of the penicillins and cephalosporins have been the subject of a number of reviews.[1,21-24] Consequently, the earlier results will be only briefly described for completeness. Biosynthetic studies on penicillin were started in the 1940s as a part of the massive international collaborative efforts on the development of penicillin as an antibiotic.[1] The main objectives of the biosynthetic work were the improvement of the yield of penicillin by fermentation methods, and the possible production of new penicillins by adding appropriate precursors to the culture. Both objectives of this work were reached, to some extent, and a large number of new penicillins was produced, having modified *N*-acyl side chains. However, penicillins structurally modified in the penam ring system were not obtained. The studies were conducted by observing the effect on penicillin yield of adding to the cultures various putative biosynthetic precursors.[25-28] Unfortunately the results are rather misleading from the point of view of elucidating the nature of the direct, *in vivo* precursors of penicillin. As pointed out by Behrens,[26] stimulation of penicillin production may result, not from direct incorporation of the added nutrient, but through its interaction in some indirect manner with the microbial metabolism. For example, the addition of L-cysteine, now accepted as a direct precursor of penicillin, reduced the yield of penicillin in these studies. Similarly DL-valine did not stimulate penicillin production and higher concentrations of DL-valine were inhibitory, although valine is now accepted as a direct penicillin precursor. Thus the approach of observing the increase or decrease in penicillin yields in cultures of *P. chrysogenum* is not a valid method for evaluating biosynthetic precursors or intermediates, although it has been used in a distressing number of investigations in this field. Real progress in the study of biosynthetic precursors to penicillin commenced with the application of tracer techniques to the problem.

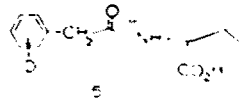
Examination of the structures of both the penicillins and the cephalosporins suggests as a working hypothesis the assumption that their ring systems could be formed from the carbon skeletons of valine and cysteine as in **3** and **4**. In both types of β-lactam antibiotic, the cysteine part of the molecules has a configuration in common with that of L-cysteine. In penicillin, the valine part of the molecule has a configuration like that of D-valine, whereas in the cephalosporins, the configurational identity of the valine part is lost. The following discussion will describe the results of investigations up to the present time, establishing the involvement of valine and cyst(e)ine as penicillin and cephalosporin C precursors. Also included will be the results of experiments designed to determine which atoms of these amino acids are incorporated into the final metabolites, and the results of certain stereochemical investigations. The discussion will be subdivided into the work on valine and the work on cysteine, although in some cases both precursors were studied in the same work.



3 4

Incorporation of valine into penicillins and cephalosporins

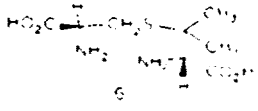
In an attempt to demonstrate the direct incorporation of valine as well as phenylacetyl derivatives into benzylpenicillin, Behrens *et al.*[26] prepared deuterophenylacetyl - ^{15}N - DL - valine **5**, having 41% of the phenyl



5

group hydrogens replaced by deuterium and containing 32 atom per cent ^{15}N. Upon incubation of **5** with *P. chrysogenum*, benzylpenicillin **1a** was isolated. The labelling results indicated that 92% of the benzyl group in **1a** had been formed from the added precursor, but that very little of the ^{15}N was incoporated. The use of **5** as precursor is rather curious, since it is clear from the structure of **1** that **5** would have to undergo some kind of fragmentation, probably to phenylacetate and valine before either could be incorporated into benzylpenicillin. Thus the incorporation experiment could have been done simply by using a mixture of deuterated phenylacetic acid and ^{15}N-valine. The poor incorporation of the ^{15}N of valine suggested that valine was not incorporated intact. Fortunately this incorrect conclusion has been amended in an impressively large number of subsequent ^{15}N valine experiments ranging over the past twenty years.

Following a suggestion by Hockenhull,[29] Süs[30] and later Stevens *et al.*[31] investigated the possibility that the thioether **6** might be a penicillin precursor. In the experiments by Stevens,[31] the pure isomer **6** formed from

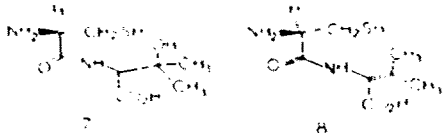


6

L-cysteine and D-penicillamine was synthesized. Its ability to serve as a precursor was tested by observing the resultant depression in the utilization of L-cystine-^{35}S added to the medium at the same time. In fact, no depression of the utilization of ^{35}S-L-cystine was observed. The results are inconclusive, since it was not shown that the compound was taken up by the mycelium. Also it is not clear that **6** would have been incorporated intact had it been taken up.

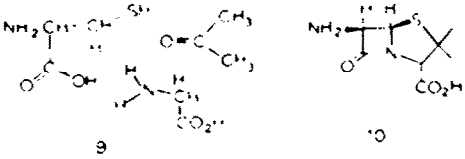
At this time, Arnstein and Grant[32,33] showed that 4-^{14}C-DL-valine could be incorporated into benzylpenicillin. They showed that 95% of the label was located in the penicillamine (valine) part of the metabolite, although the exact labelled position was not established. Similarly Stevens *et al.*[34] incorporated carboxyl-labelled ^{14}C-DL-valine into benzylpenicillin, and demonstrated that the label was located exclusively in the free carboxyl group through degradation of the penicillin to penicillamine and decarboxylation of the latter with ninhydrin. From these experiments, the reasonable extrapolation can be made that the entire carbon skeleton of valine is incorporated into penicillin, although it does not follow that the skeleton is incorporated intact. Stevens and coworkers then examined the relative rates of incorporation of D and L-valine-1-^{14}C into benzylpenicillin. It was observed that the conversion of L-valine into penicillin was considerably more rapid that that of D-valine, the latter exhibiting a lag time of *ca.* 6 hr before rapid incorporation. The conclusion from these observations that D-valine is not a normal penicillin precursor is, however, not necessarily valid, since it is possible that D-valine might be slowly absorbed from the medium by the mycelium and then be rapidly converted to penicillin. In contrast, L-valine might be rapidly taken up by the mycelium and then used for penicillin biosynthesis. In fact, in a later study by Stevens and DeLong,[36] it was shown that L-valine was taken up by the mycelium much more rapidly than D-valine. An analysis of the valine recovered from hydrolysis of the mycelial protein showed that it was almost entirely L-valine, whether the added precursor was D- or L-valine. The result can be explained by assuming that, in the mycelium, D-valine is incorporated into penicillin whereas only L-valine (from the isomerization of D-valine) is used for protein biosynthesis. In the same work,[36] the authors showed that several valine analogues all bearing the valine carbon skeleton, namely 3-hydroxy-DL-valine, 2-ketoisovaleric acid, DL-2,3-dihydroxyisovaleric acid, and 3,3-dimethylacrylic acid, did not compete significantly with 1-^{14}C-L-valine for penicillin biosynthesis. It was not demonstrated that these compounds were taken up by the mycelium. Stevens and DeLong also showed that in short-time incubations, ^{15}N from ^{15}N-L-valine was incorporated into the valine-part of the penicillin molecule with approximately the same efficiency as it was converted into mycelial protein. In both cases, the ^{15}N isotope dilution was rather extensive (*ca.* 1:22 up to 1:30). However, the result does suggest that both penicillin and protein are biosynthesized from the same precursor, L-valine. Also the result shows that in the overall penicillin biosynthesis in which the L-configuration of valine is inverted to the D-configuration of penicillin, at least some of the nitrogen must be retained.

In a similar experiment, Arnstein and Clubb[37,38] incorporated ^{15}N-DL-valine, mixed with 2-^{14}C-DL-valine into benzylpenicillin. Labelled nitrogen was retained in the biosynthetic product, although the dilution of ^{15}N was considerably larger than the dilution of ^{14}C (possibly as a result of the use of DL- rather than L-valine). These workers also showed that (U-^{14}C)-L-valine was incorporated nearly exclusively (95%) into the valine-part of the penicillin molecule, thus providing further evidence that the valine skeleton is incorporated intact. 3-Hydroxy-DL-valine was very poorly incorporated and was presumed not to be a direct precursor. However, it was suggested that a 3-hydroxyvaline peptide such as **7** could not be ruled out as a penicillin precursor if the hydroxylation occurred after synthesis of the deoxy peptide. In addition, the cysteinyldehydrovaline **8** was suggested as a reasonable intermediate in penicillin biosynthesis.



7 **8**

Some time later, Shimi and Iman[39,40] proposed an alternative pathway for the biosynthesis of the thiazolidine ring of penicillin. In a study of the degradation of penicillin by washed mycelial mats of *P. chrysogenum* or a penicillin-producing strain of *Aspergillus flavus*, it was observed that benzylpenicillin was degraded to phenylacetic acid, cysteine, and valine, the latter apparently being further metabolized to glycine and acetone or isopropanol. It was then observed[40] that the addition of glycine and acetone or isopropanol to washed pre-formed mats of *P. chrysogenum* resulted in the formation of significant quantities of valine. Furthermore, the production of benzylpenicillin by these mycelial mats was markedly stimulated by the addition of acetone, isopropanol or glycine, even more that by added cysteine or valine. The authors therefore suggested that incorporation of exogenous valine into penicillin might occur by cleavage of the added valine into glycine and acetone followed by reassembly of these components as the D-valine part of the thiazolidine ring, **9**→**10**. Unfortunately the above



9 **10**

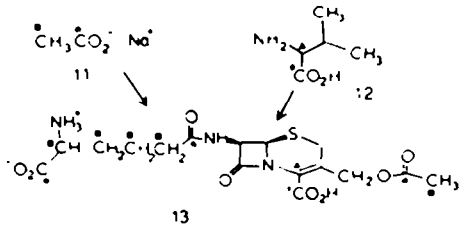
experiments were not performed with labelled additives and therefore it cannot be concluded that the acetone and glycine were directly incorporated into valine or into the "valine-derived" part of the penicillin molecule without prior metabolic transformation. In addition, certain stereochemical results, to be discussed later, obtained with chirally labelled valine indicate that Shimi and Iman's biosynthetic route cannot be a major one in systems supplied with adequate quantities of valine.

Abraham and coworkers[41] showed that 1-^{14}C-DL-valine served as a precursor of penicillin N **1d** and cephalosporin C **2a** in *Cephalosporium* sp. The label in cephalosporin C was mainly (90%) located in the expected part of the molecule, as in **4**. The remaining 10% of the label presumably arose from labelled cysteine and α-aminoadipic acid,[42] newly synthesized *in vivo* from $^{14}CO_2$ released in the catabolism of valine. The C-17 acetoxy group of cephalosporin C was formed from labelled acetate.[43] Demain[44] independently observed that L-valine efficiently labelled cephalosporin C.

Abraham *et al.*[45] later found that cephalosporin C biosynthesis was not depressed by addition to the cultures of D-valine, but that the synthesis of penicillin N was depressed by D-valine. As earlier observed in *P. chrysogenum* by Stevens,[36] the uptake of L-valine by *Cephalosporium* sp. was rapid but uptake of D-valine or

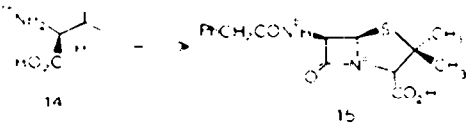
α-ketoisovaleric acid was slow. The observations supported the view that the biosynthesis of both antibiotics in *Cephalosporium* sp. occured directly from L-valine rather than from D-valine.

In the first of several applications of ^{13}C NMR spectroscopy to studies on β-lactam antibiotic biosynthesis, Neuss *et al.*[46-48] investigated the incorporation of several specifically labelled precursors into cephalosporin C. The labelled positions could be directly determined from the ^{13}C NMR spectrum, as all ^{13}C signals of cephalosporin C could be unambiguously assigned. In confirmation of earlier work,[43] ^{13}C-acetate caused significant labelling of C-18 and C-19 (acetoxy group) and of the aminoadipyl



side-chain carbons **11** → **13**. The labelling pattern in the side-chain was consistent with its biosynthesis from acetyl coenzyme A and α-ketoglutarate, the latter in turn also being biosynthesized from acetyl CoA via the Krebs cycle. No appreciable labelling appeared in the 3-cephem ring system. Incorporation of 1-^{13}C-DL-valine resulted in specific labelling of the C-16 carboxyl, while 2-^{13}C-DL-valine specifically labelled C-4, **12** → **13**.

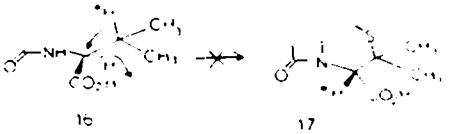
Recently the question of the retention or loss of the nitrogen of L-valine in the biosynthesis of penicillin was reinvestigated by Bycroft and collaborators,[49] using ^{15}N labelling with the application of ^{15}N NMR spectroscopy. The ^{15}N signals of benzylpenicillin were assigned from the spectrum of an ^{15}N-enriched sample produced biosynthetically from $Na^{15}NO_3$. Benzylpenicillin **15** produced biosynthetically from ^{15}N-L-valine **14**, showed



enhancement only in N^a. The incorporation of ^{15}N was estimated to be 84% ± 10% of the incorporation of the carbon skeleton of valine (estimated by simultaneous incorporation of (U-^{14}C)-L-valine). The results thus confirm the earlier conclusion of Arnstein[18] and Stevens[36] that at least some of the L-valine nitrogen is retained in the D-valine part of the penicillin molecule, although more exchange of nitrogen had been observed in the earlier work.

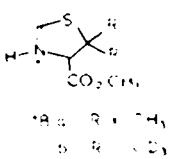
In some other recent studies, the questions of the retention or loss of the C-2, C-3 and methyl group hydrogens of valine in biosynthesis of penicillin and cephalosporin C have been examined. Bycroft *et al.*[50] showed, in a double label experiment with 2-^3H-D- or L-valine, mixed with (U-^{14}C)-L-valine or 1-^{14}C-D-valine, respectively, that the C-2 hydrogen of both L and D-valine is eliminated. The total incorporation of ^{14}C into benzylpenicillin was remarkably high (37%) and moreover was essentially identical whether D or L-^{14}C-valine was used as precursor. However, the rate of absorption of these precursors differed in that L-valine was rapidly absorbed, whereas D-valine was rather slowly absorbed. The authors concluded that D-valine was not directly incorporated into penicillin but may first be converted to L-valine, either via α-ketoisovaleric acid or via an alternative pathway not involving C–N cleavage. The interpretation of the results is complicated by the problem which has plagued investigators in this field from the outset, namely that absorption of the precursor by the cells and biosynthesis of antibiotic are two separate, (probably) independent processes. It is conceivable, even if unlikely, that D-valine could be used in the rapid biosynthesis of penicillin in the cells once the D-valine has been absorbed. It would appear to be worthwhile to examine the retention or loss of ^{15}N from ^{15}N-D-valine in penicillin (and cephalosporin C) biosynthesis, since all published ^{15}N experiments have been done with either DL or L-valine only.

In a similar experiment, Vanderhaege *et al.*[51] showed that 2-^3H-DL-valine did not produce labelled phenoxymethylpenicillin under conditions where 1-^{14}C-DL-valine was efficiently taken up by the medium. Since the incorporation of the tritiated precursor was not performed with a ^{14}C co-tracer, it is difficult to evaluate the extent of penicillin biosynthesis in this experiment. However, the conclusions match those of Bycroft with regard to the loss of the valine C-2 hydrogen. As part of their work, Vanderhaege *et al.*[51] prepared 3-^3H-DL-valine by α-hydrogen exchange of 2,3-$^3H_2$-L-valine.[52] A mixture of 3-^3H-DL-valine plus 1-^{14}C-DL-valine was incorporated into phenoxymethylpenicillin with retention of only 2% of the ^3H as compared with ^{14}C incorporation. This amount of retained tritium is slightly larger than the amount estimated to be present in the methyl groups of the tritiated precursor. However, the results still appear to rule out an intramolecular hydrogen shift from C-3 to C-2 in the overall transformation of valine into penicillin, **16** → **17**.



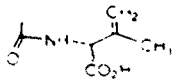
Such a shift would otherwise have offered a possible mechanism for the inversion of the C-2 carbon of L-valine to the D-configuration in the biosynthesis of penicillin.

In independent investigations by Sih *et al.*,[53] and in our laboratory in collaboration with a group at the Lilly laboratories,[54] it was established that penicillin biosynthesis proceeds with retention of all six of the methyl group hydrogens of the valine precursor. In our work, DL-valine-*methyl*-d_6 was converted by *P. chrysogenum* into phenoxymethylpenicillin **1b**, which was then converted into the methyl ester. This compound produces in its mass spectrum[53,55] a very intense peak, *m/e* 174, **18a**,



which in the biosynthetically labelled sample was accompanied by a new peak (*ca.* 20% as intense as *m/e* 174) at *m/e* 180, **18b**. No significant peaks appeared

between *m/e* 174 and *m/e* 180. The results therefore eliminate the possible involvement of an isodehydrovaline-type intermediate **19** in penicillin biosynthesis.



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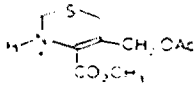
Similar results were obtained by Sih *et al.*[53] upon incorporation of DL-valine-d_8 into penicillin N by washed cells of *Cephalosporium acremonium*. Complete retention of all six deuterons was observed. In addition, the formation of cephalosporin C from valine-d_8 was studied in this organism. In the mass spectrum of cephalosporin C methyl ester,[57] appears a prominent fragment **20** *m/e*



20

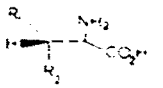
230. In the labelled product from valine-d_8, this fragment was accompanied by fragments corresponding to d_4 species (1%; d_0 = 72%), d_1 (10%), d_2 (7%) and d_3 (10%). Thus the results were rather ambiguous and tended to suggest that some exchange of the valine methyl hydrogens may occur during the conversion of valine into cephalosporin C. Alternatively the observed isotopic ratios could have resulted from some intermolecular deuterium scrambling in the mass spectrometer between labelled (d_4?) and unlabelled cephalosporin C molecules. The ^{1}H NMR spectrum of the cephalosporin C (N-benzoate) biosynthesized from valine-d_8 showed an equal signal intensity for the C-2 and C-17 hydrogens. However, this does not prove that the two hydrogens at each of these positions are derived from valine-d_8 without exchange. An equal loss of deuterium at each position would also lead to a Cephalosporin C sample which would give an equal signal intensity for these hydrogens. This possibility was rendered less likely by the observation that cephalosporin C biosynthesized in D_2O did not possess deuterium at C-2 or C-7, although several other positions were labelled.

It should also be mentioned that Katz *et al.*[58] had shown earlier that deuterium from D_2O was not incorporated into the methyls of penicillin.

Stereochemistry of valine incorporation into penicillin and cephalosporin C

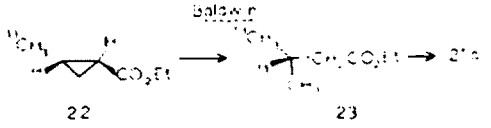
A question of considerable recent interest has concerned the fates of the diastereotopic methyl groups of valine in its conversion into the β-lactam antibiotics. Assuming that the biosyntheses proceed in a stereospecific manner in common with practically all biochemical reactions,[59,60] it was of interest to determine whether the ring closure process, leading to the formation of the thiazolidine ring of penicillin, proceeds with an overall inversion or retention of configuration at C-3 of the valine precursor. In addition, it was of interest to establish which of the valine methyls served as the precursor of C-2 (–CH_2S–) and which the precursor of C-17 (–CH_2O–) of cephalosporin C.

For the purposes of these and certain other studies involving valine metabolism, four different syntheses of asymmetrically labelled valine ("chiral valine") were independently developed. In the work of Baldwin *et al.*[61]

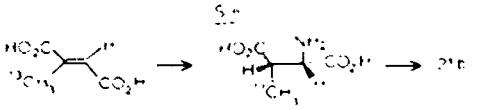


21

	R_1	R_2	
a	$^{13}CH_3$	CH_3	(2RS,3R)
b	CH_3	$^{13}CH_3$	(2S,3S)
c	CD_3	CH_3	(2S,3R)
d	CH_3	CD_3	(2S,3S)
e	CH_2D	CH_3	(2S,3R)
f	CD_3	CH_3	(2RS,3R)
g	CH_3	CD_3	(2RS,3S)
h	CH_3	$^{13}CH_3$	(2RS,3R)
i	$^{13}CH_3$	CH_3	(2RS,3R)
j	CH_3	CH_3	(2RS,3S)
k	CH_3	CD_3	(2S,$^{13}CH_3$)

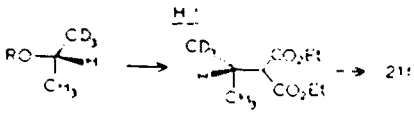


22 23



24 25

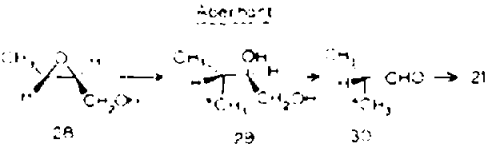
the chiral isopropyl group was generated by reductive cleavage of **22** by lithium in liquid ammonia to yield **23**, which after further transformation gave (2RS,3R)-[4-^{13}C]valine **21a**. Sih *et al.*[62] synthesized (2S,3S)-[4-^{13}C]valine **21b** by a route employing as key step the β-methylaspartase - catalyzed interconversion of **24**→**25**. After further transformations, the C-3 carboxyl of **25** was selectively converted into an unlabelled methyl group. The method was also used by Sih *et al.*[53] to produce the analogous CD_3-labelled chiral valines, (2S,3R)-[4,4,4-d_3]valine **21c** and (2S,3S)-[4,4,4-d_3]valine **21d**. In addition they produced[62] the monodeuterated chiral valine (2S,3R)-[4-d_1]valine, **21e**. The method used would appear to be an excellent route to the preparation of tritiated (3R-^{3}H) valine of high specific activity, although this has not been reported. Hill *et al.*[63] synthesized (2RS,3R)-[4,4,4-d_3]valine **21f** starting with S(+)-2-propanol **26a** previously prepared by Mislow.[64] Displacement of the corresponding tosylate **26b** with diethylsodiomalonate gave **27** which was then trans-



26 a R = H
b R = Ts

27

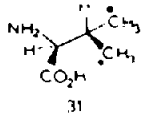
formed to **21f**. Their product was not used for biosynthetic studies on penicillin, but was used in an elegant demonstration of the stereochemistry of valine biosynthesis. In our work,[65,66] chiral valines were generated via cleavage of the chiral epoxyalcohol **28** (or its enantiomer) with labelled methyllithium, to give glycol **29**. After cleavage of **29** with periodate, a Strecker sequence applied to the resultant (unisolated) chiral isobutyraldehyde



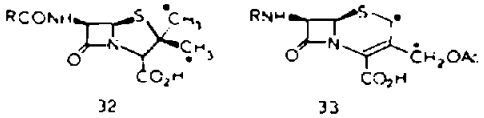
28 29 30

30, gave the corresponding chiral valines. The procedure has been used for the syntheses of (2RS,3S)-[4,4,4-d_3]valine **21g**, and (2RS,3S)-[4-^{13}C]valine **21h** as well as the tritiated analogues (2RS,3R)-[4-^{3}H]valine **21i**, and (2RS,3S)-[4-^{3}H]valine **21j**. The tritiated compounds were required for our studies on the stereochemistry of the catabolism of valine. Furthermore we have recently used this route to chiral isobutyraldehydes for syntheses of (2R)-[3,3,3-d_3]isobutyric acid[67] and (2S)-[3-^{13}C]isobutyric acid.[68] The latter was used to demonstrate that valine catabolism via isobutyryl CoA, in *Pseudomonas putida*,[69] proceeds with dehydrogenation of the *pro*-S methyl group.

The synthetic ^{13}C chiral valines **21a**, **21b** and **21h** were then converted by *P. chrysogenum* into phenoxymethylpenicillin **1b** and by *C. acremonium* into penicillin N **1d** and cephalosporin C **2a**. The ^{13}C NMR spectra of the metabolites showed an enhanced intensity in only one carbon in each case, showing that the biosyntheses proceeded completely stereospecifically. In the case of penicillin, the β-methyl group was labelled by (3R)-[4-^{13}C]valine,[70] while the α-methyl group was labelled by (3S)-[4-^{13}C]valine.[62,66] The assignment of methyl group signals in the ^{13}C NMR spectra of penicillins had been made several years earlier by Archer *et al.*,[71] by correlating the ^{13}C spectrum with the corresponding ^{1}H spectrum through off-resonance decoupling. The ^{1}H signals in turn had been assigned through a study of nuclear Overhauser effects in penicillins and their sulfoxide derivatives. Thus, the penicillin labelling pattern indicates that the incorporation of valine into penicillin proceeds with an overall *retention* of the configuration at C-3 of the precursor **31**→**32**. The results appear to be quite in-



31



32 33

consistent with the hypothesis of Shimi and Iman[40] mentioned earlier, that acetone produced by cleavage of valine in the mycelium might serve as the major source of the methyl groups of the thiazolidine ring of penicillin. Specifically labelled penicillin could not be produced in this manner, unless the acetone did not become separated from the enzyme before incorporation into penicillin.

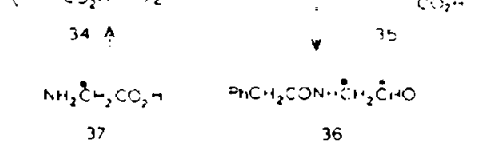
Sih *et al.*[31] also reported the incorporation of their CD_3-labelled chiral valines **21c** and **21d**, into penicillin N. The mass spectra of the corresponding methyl ester of the metabolite supported the conclusion discussed earlier that the original methyl hydrogens of valine remain intact in penicillin biosynthesis.

In the biosynthesis of cephalosporin C, it was shown[53,62,70] that C-2 (–CH_2S–) of the metabolite was specifically labelled by (3R)-[4-^{13}C]valine, while C-17 (–CH_2O) was labelled by (3S)-[4-^{13}C]valine, **31**→**33**. Thus, the biosyntheses of both types of β-lactam antibiotic proceed stereospecifically from valine. It cannot, however, be inferred from the results that both antibiotics are formed from a common intermediate.[24] Moreover, although the results would be consistent with the involvement of an α,β-dehydrovaline intermediate in penicillin and/or cephalosporin C biosynthesis, they do not require such an intermediate. Other mechanisms, to be discussed later, would be consistent with a stereospecific biosynthesis from valine.

Incorporation of cyst(e)ine into penicillins and cephalosporins

Stevens *et al.*[73,74] studied the utilization of various sulphur compounds for the biosynthesis of benzylpenicillin by *P. chrysogenum*. The precursors were evaluated on the basis of their ability to depress the incorporation of $^{35}SO_4^{2-}$ into benzylpenicillin. Thus DL-methionine, L-cysteine, and L-cystine competed effectively with sulphate, while D-cysteine and DL-penicillamine were apparently not utilized. L-Cysteine and glutathione, which releases L-cysteine on hydrolysis, were equally effective. As the authors pointed out,[74] the observed results do not prove that the carbon skeleton of any of these "effective" precursors is actually incorporated, intact or degraded, into penicillin. In another investigation,[31] this group showed that DL-homocysteine, L-cysteic acid, and taurine were less effective than L-cystine in depressing $^{35}SO_4^{2-}$ utilization for penicillin biosynthesis. As mentioned earlier, the sulfide **6** was also ineffective.

In an independent study, Arnstein and Grant[72,75,76] showed that DL-3-^{14}C-cystine **34** effectively labelled the

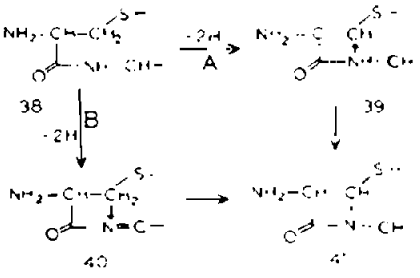


34 35 37 36

β-lactam portion of benzylpenicillin **35**. The labelled penicillin was degraded with acid to benzylpenilloaldehyde **36** which contained all the label. Further degradation of **36** to glycine and decarboxylation of the glycine established that essentially all of the label was originally located at C-5 of penicillin. Arnstein and Grant[75] also reported that L-^{14}C-cystine was utilized for penicillin biosynthesis about five times as efficiently as D-^{14}C-cystine. They further showed that DL-3-^{14}C-serine and 2-^{14}C-glycine led to the formation of labelled penicillin, but not as efficiently as did DL-cystine. The 2-^{14}C-glycine **37**, produced specific labelling of C-6 in penicillin **35**. This is to be expected, since C-2 of glycine serves as the biosynthetic precursor of C-2 of L-serine which in turn is the precursor of L-cysteine.[76] The cyst(e)ine would therefore be labelled as shown in **35**. Cyst(e)ine rather than serine is the actual penicillin precursor, as shown by the fact that labelled serine isolated from mycelial protein biosynthesized in the presence of ^{14}C cystine had a lower specific activity than the penicillin from the same culture.

To establish whether cyst(e)ine is used intact for penicillin biosynthesis, Arnstein and Grant[37] incorporated triply-labelled 3-^{14}C, ^{15}N, ^{35}S-D and L-cystines into benzylpenicillin. The penicillin from the L-cystine incorporation had very nearly the same ^{15}N/^{14}C ratios as the precursor, but the ^{35}S content was substantially enriched with respect to ^{14}C. This enrichment was probably a result of the incorporation of ^{35}S-cystine biosynthesized *de novo* from the catabolic products of the triply-labelled exogenous cystine. Since few metabolic end-products besides cyst(e)ine are possible for the added ^{35}S whereas the labelled nitrogen and carbon can be utilized in a plethora of alternative pathways, it is reasonable that a higher proportion of the ^{35}S of the precursor should end up in penicillin. The results with ^{15}N and ^{14}C, however, strongly indicate that the biosynthesis of penicillin involves the incorporation of the intact cystine molecule. In the experiment with ^{14}C, ^{15}N, ^{35}S-D-cystine, the analysis of the biosynthetic penicillin showed again a substantial enrichment in ^{35}S, and a small loss (25%) of ^{15}N relative to carbon. Thus some, but certainly not all, of the nitrogen appears to be eliminated in the conversion of D-cystine to L-cystine, assuming that this conversion is necessary before the precursor can be converted to penicillin.

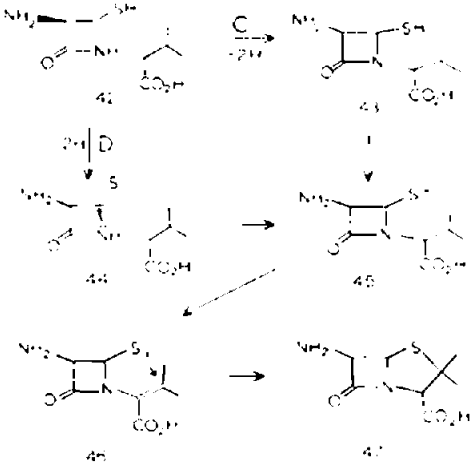
In a later paper, Arnstein and Crawhall[38] presented two hypothetical pathways, **38** → **39** → **41**, and **38** → **40** → **41**, through which cystine (or cysteine) could be



elaborated to yield the β-lactam ring of penicillin. Pathway A requires the loss of the C-2 hydrogen and at least one of the C-3 hydrogens of the cyst(e)ine moiety, while pathway B suggests that the C-2 hydrogen and possibly both C-3 hydrogens would be retained in penicillin. To distinguish between these possibilities, the tritiated precursors, 2-^{3}H-DL-cystine and 3-^{3}H-DL-cystine, were synthesized and incorporated into penicillin. The incorporations were carried out by conducting parallel incubations of the tritiated precursors and either 3-^{14}C-DL- or L-cystine, rather than by incubating the mixed ^{3}H + ^{14}C precursors. Thus the per cent incorporations of the carbon skeletons of cystine from the ^{14}C and from the ^{3}H precursors cannot be assumed to be exactly the same, although they probably were quite similar. Furthermore the amount of added precursor in the case of the 2-^{3}H-cystine (*ca.* 260 mg/l) differed considerably from the amount of added 3-^{3}H-cystine (34–60 mg/l). Thus the extent of utilization of the two precursors cannot be directly compared.

The results indicated that both 2-^{3}H and 3-^{3}H-cystine produced tritiated benzylpenicillin. The dilution of the tritium was significantly greater than that of ^{14}C in both cases. If one assumes that the per cent incorporations of the carbon skeletons of the ^{14}C and ^{3}H-cystines are equal in the individual experiments, it can be estimated that *ca.* 40% of the 2-^{3}H and 70% of the 3-^{3}H was retained, with respect to the ^{14}C incorporated in each experiment. However, these values are rather insecure for the reasons outlined above and should not be overinterpreted. In any case it is clear that some of the 2-^{3}H of cystine was retained in penicillin and this fact would appear to rule out mechanism A, **38** → **39** for the β-lactam formation. To establish the positions of ^{3}H in the biosynthetic products, the penicillin samples were degraded to penilloaldehyde **36** which was then oxidized and hydrolyzed to glycine. The glycine was in turn oxidized by ninhydrin to carbon dioxide and formaldehyde (from C-2). The penicillin biosynthesized from 2-^{3}H-cystine gave formaldehyde containing practically all the label. Degradation of penicillin from 3-^{3}H-cystine gave inactive formaldehyde. Thus the tritium atoms were located in penicillin in the positions to be expected if cystine is incorporated without hydrogen migration.

Arnstein and Crawhall suggested two possible mechanisms, consistent with the above observations, which could account for the formation of the β-lactam ring of penicillin. In mechanism C, **42** → **43** → **45**, the cysteinyl-



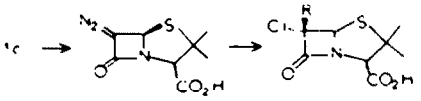
valine dipeptide undergoes oxidation in some unspecified manner to directly produce the β-lactam ring. Alternatively in D, **42** → **44** → **45**, an intermediate thioaldehyde may be formed which then is attacked by the amide nitrogen. Subsequent steps via the dehydrovaline intermediate **46** lead eventually to penicillin. The mechanisms imply that the β-lactam ring is the first to be formed, although in fact to this date no evidence obtained in an *in vitro* experiment supports this feature. Although thioaldehydes such as **45** are unprecedented in living systems, these theories, especially D, served as useful working hypotheses for a large amount of subsequent work in this field.

In a study of the ability of cysteine analogues to replace cyst(e)ine in penicillin biosynthesis, Arnstein and Margreiter[39,40] found that 2-methyl or 3-methylcystine could not substitute for cystine in the biosynthesis of penicillin. Penicillin formation was inhibited by 2-methylcystine and 3,3-dimethylcystine, and also by S-ethylcysteine.[40] However, S-methyl-L-cysteine, S-benzyl-L-cysteine, N-methyl-L-cysteine, and the 3-methylcystines did not inhibit penicillin formation. It thus appears that only L-cyst(e)ine can serve as a direct penicillin precursor to any significant extent.

Later, Abraham and coworkers showed that cysteine served as precursor of penicillin N and cephalosporin C

in *C. acremonium*.[41,42,81] In 1972, it was shown by a Lilly group[82] that the new cephalosporin C analogue **2f** from *Streptomyces clavuligerus* could be efficiently labelled by ^{14}C cysteine, valine and α-aminoadipic acid. The 7α-methoxyl carbon was formed, as usual, from methionine. These investigators also found that incorporation of a mixture of 3,3'-^{14}C-DL-cystine and 3,3'-^{3}H-DL-cystine into **2f** proceeded with only minor (20%) loss of tritium, rendering it unlikely that the introduction of the methoxyl group proceeds via a dehydrocyst(e)ine similar to **39**.

After a considerable hiatus, the experiments of Arnstein and Crawhall[7] on the incorporation of 2-^{3}H and 3-^{3}H-cystine into penicillin were reinvestigated by double label techniques in two laboratories. After incubation of (U-^{14}C)-2-^{3}H-L-cystine with a high-yielding strain of *P. chrysogenum*, Bycroft[83] and coworkers isolated benzylpenicillin having a $^{3}H/^{14}C$ ratio 84% of that of the precursor. The tritium retention is somewhat greater than that observed by Arnstein and Crawhall,[7] but does not change their conclusions. Bycroft also showed that the retained tritium was located, as expected, at C-6. The biosynthetic product was degraded by a resin bound acylase to 6-aminopenicillanic acid **1c**, which upon diazotization in hydrochloric acid solution gave, via **48**, the chloride **49a** which was devoid of tritium. When un-



48 49a R = H, b R = D

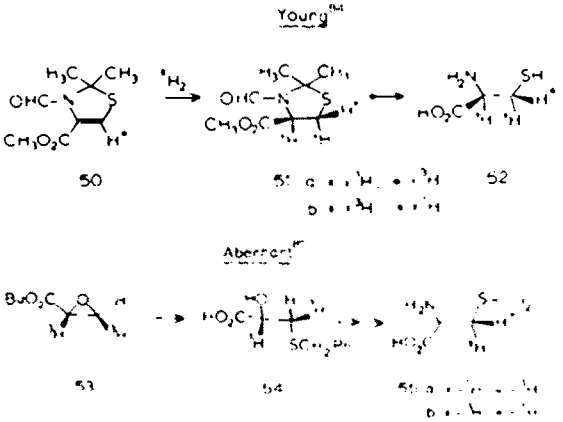
labelled 6-aminopenicillanic acid was subjected to the same treatment except that DCl in D_2O replaced HCl in H_2O, the product **49b** was formed. Thus hydrogen exchange occurred only at C-6 in the degradation to **49**. Furthermore 2-$^{2}H_1$-L-cystine was incorporated into benzylpenicillin which contained deuterium only at C-6, as shown by ^{2}H NMR spectroscopy.

Vanderhaege *et al.*[51] incubated the doubly-labelled 3,3-^{3}H-(U-^{14}C)-L-cystine with *P. chrysogenum* and isolated phenoxymethylpenicillin having a $^{3}H/^{14}C$ ratio 45% of that of the added cystine. Thus the tritium retention was very close to the expected amount if one C-3 hydrogen of cystine is eliminated. Again, the results agree in general with those observed earlier by Arnstein and Crawhall,[7] although the 45% retention of tritium is rather easier to explain than Arnstein's observed *ca.* 70%.

Stereochemistry of cyst(e)ine incorporation into penicillin

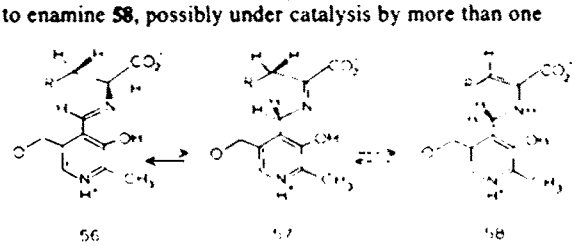
In view of the fact that, apparently, one of the C-3 hydrogens of L-cysteine is retained and one eliminated in penicillin biosynthesis, it was of interest to investigate the stereochemical fates of these diastereotopic hydrogens. This problem has been examined in two independent studies, by Morecombe and Young,[84] and in our laboratory.[85] The required stereospecifically labelled precursors were synthesized by different routes, outlined below **50**→**52** and **53**→**55**. Chiral cysteines have also recently been prepared[86] by an enzymatic procedure, using the O-acetylserine sulphydrase-catalyzed conversion of chirally labelled O-acetylserines into cysteines.

The stereospecifically labelled cyst(e)ines **52** or **55**



were then mixed with ^{14}C-cyst(e)ine and then converted into benzylpenicillin by *P. chrysogenum*. In both laboratories it was observed that most of the tritium from (3R-^{3}H)-cyst(e)ine, **52b** or **55b**, was retained in the biosynthetic product, while most of the tritium from (3S-^{3}H)-cyst(e)ine, **52a** or **55a**, was eliminated. Thus, the ring closure leading to the formation of the β-lactam ring, whatever the mechanism may be, must take place with retention of the configuration at C-3 of the cysteine part of the precursor. The analogous experiment in the case of cephalosporin C biosynthesis has not yet been reported.

In the incorporation experiments, both groups observed an appreciable amount of non-stereospecificity in the overall biosynthetic process. In the Young[84] experiment, up to 14% non-stereospecificity (retention of 3S-^{3}H) was observed, whereas in our work up to 21% non-stereospecificity was found. In our case there is reason to believe that 5–10% of the precursor may have been non-stereospecifically labelled due to some hydrogen migration in the catalytic tritium reduction step leading to the precursor of **53**. However, this would not be sufficient to account for the observed results. The most reasonable explanation of the observed results is that competing biochemical reactions in the mycelium cause a partial loss of tritium or a scrambling of its stereochemistry. Such unexpected losses of C-3 hydrogens of amino acids have recently been observed in studies on the biosyntheses of gliotoxin[87] and cytochalasin C.[88] The results can be explained, as proposed by Tamm,[88] by postulating as a metabolic side-process the involvement of a reversible transamination process leading, via **56**, to **57**. This may then reversibly isomerize to enamine **58**, possibly under catalysis by more than one



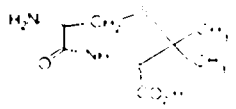
56 57 58

enzyme with differing stereochemistries of hydrogen elimination at C-3. The observed non-stereospecificity could also be explained by reversible conversion of the cyst(e)ines to the α-ketoacid, which might undergo enzymic or non-enzyme-catalyzed enolization with concomitant C-3 hydrogen loss. It is quite probable that the actual penicillin biosynthesis itself is entirely stereospecific with regard to the C-3 hydrogen elimination.

Further work will be required to evaluate the reasons for the observed lack of complete stereospecificity with the synthetic tritiated chiral cyst(e)ines.

Biosynthetic intermediates: the tripeptide theory

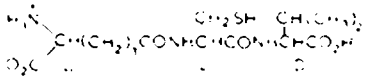
As mentioned earlier, several postulated intermediates which bear a structural resemblance to penicillin, e.g. **5** and **6**, had been eliminated from consideration as penicillin precursors due to their failure to be incorporated into penicillin or to depress the utilization of valine or cysteine.[87,88] Arnstein *et al.*[89] had also excluded phenylacetylcysteine as a biosynthetic intermediate in benzylpenicillin formation. Arnstein and Clubb[90] later reported the cyclic dipeptide **59** did not depress the utiliza-



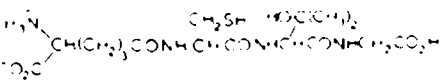
59

tion of L-1-^{14}C-L-valine. However, since the material used was a mixture of isomers containing D and L cysteine and valine, the significance of the negative result is lost. Also it is not clear whether any of this cyclic dipeptide was taken up by the mycelium. Arnstein and Morris[91,93] then reported that synthetic L-cystinyl-L-1-^{14}C-L-valine led to the formation of labelled penicillin. The corresponding L-cystinyl-D-valine was not utilized for antibiotic or for protein synthesis. The observation that the LL-dipeptide gave penicillin of higher specific activity than the specific activity of labelled protein suggested that the dipeptide was not completely hydrolyzed before incorporation. However, since the specific activity of penicillin was considerably lower than that of the added dipeptide, it was suggested that this dipeptide is not the major source of penicillin *in vivo*. Furthermore, endogenous cysteinylvaline could not be detected in penicillin-producing cultures of *P. chrysogenum*. Arnstein *et al.*[91,94] then reported the isolation from the mycelium of this organism of a new tripeptide, identified as δ(α-aminoadipyl)cysteinylvaline (ACV). The absolute configurations of the component aminoacids were not determined at that time. No other peptides containing both cysteine and valine would be detected in this organism. Therefore the ACV tripeptide was nominated as a reasonable intermediate in penicillin biosynthesis. The N-terminal residue of the ACV tripeptide corresponded to the side-chain found in penicillin N **1e**, from *C. acremonium*. It was postulated that at some unknown point in the biosynthesis of penicillins such as benzylpenicillin **1a**, side-chain interchange occurs with incorporation of the non-polar side-chain. Support for the view that penicillin biosynthesis involves the mandatory formation of an *N*-terminal α-aminoadipyl tripeptide can be found in the fact that lysine inhibition of penicillin biosynthesis can be reversed by addition to *P. chrysogenum* of α-aminoadipic acid. As discussed by Demain,[96] the lysine may be acting as a competitive inhibitor of the incorporation of α-aminoadipic acid into the ACV tripeptide. Alternatively lysine may be interfering with the normal synthesis of α-aminoadipic acid in the mycelium. Genetic evidence also exists which supports the view that the α-aminoadipic acid is an obligatory precursor of all penicillins. Lysine auxotrophs of *P. chrysogenum*[97] and *C. acremonium*[98] have been developed which do not produce ACV or penicillins when grown on a lysine-containing medium, but do produce both ACV and penicillins when grown in the presence of α-aminoadipic acid. Other mutants have been obtained which appear to be blocked before and after ACV synthesis.

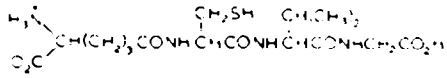
In 1967, Abraham *et al.*[99] established that ^{14}C-L-α-aminoadipic acid in *Cephalosporium* sp. served as precursor of cephalosporin C and penicillin N, both having a D-α-aminoadipyl side-chain. Although D-α-aminoadipic acid caused labelling in penicillin N and cephalosporin C, the isotopic dilution was high and it was not clear that the D-precursor was incorporated before isomerization to the L-isomer. This group[100] later found that D- and L-α-aminoadipyl-L-cysteine-^{14}C both produced labelled penicillin N and cephalosporin C in *Cephalosporium* sp. The LL-dipeptide was used more efficiently than the DL-dipeptide, and both dipeptides were extensively hydrolyzed in the mycelium. However, the ACV tripeptide was not hydrolyzed. Later, Abraham and Loder[24,101] isolated from *Cephalosporium* sp., by precipitation as cupric mercaptides, a mixture of three peptides, designated P1, P2 and P3. Peptide P3 was the previously obtained ACV tripeptide, which was further characterized in this work as the LLD isomer **60**. The probable structures of peptides P1 and P2 are shown in **61** and **62**. Their role in antibiotic



60 (P3)



61 (P1)



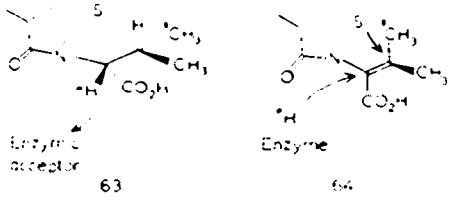
62 (P2)

biosynthesis remains unknown. Experiments on the biosynthesis[24] of the LLD-ACV tripeptide, using particulate fractions of sonicated cells of *Cephalosporium* sp., showed that the synthesis of ACV started at the N-terminal, probably with the formation of L-α-aminoadipyl-L-cysteine, which then combines with L-valine rather than D-valine.[102] Thus at some later state, the configuration of the valine residue must become inverted to form the final product LLD-ACV which accumulates. No synthesis of β-lactam antibiotic occurred with this system, however. At approximately this time, Bauer[103] also found that ACV could be biosynthesized from ^{14}C-L-valine or ^{14}C-DL-cysteine in a cell-free system obtained from mycelial extracts of *P. chrysogenum*. The absolute configuration of the amino-acid constituents of the resultant tripeptide were not established. However, simultaneously in 1976, Sih *et al.*[104] and Vanderhaege *et al.*[104] reported that the ACV tripeptide obtained from *P. chrysogenum* has the LLD-configuration, like that of P3 from *Cephalosporium* sp. No LLL-ACV tripeptide could be detected in *P. chrysogenum*. Thus, the configurations of this peptide are identical to those found at the corresponding positions of isopenicillin N from *P. chrysogenum*. Assuming that LLD-ACV is a direct isopenicillin N precursor, it therefore became unnecessary to postulate elaborate intermediates, e.g. dehydrovalines, to explain the inversion

of an L-valine precursor to the D-configuration found in the penicillins.

Attempts by Abraham *et al.*[24] to demonstrate the conversion of LLD-ACV (peptide P3) into β-lactam derivatives in intact cells, or with broken cell preparations, of *Cephalosporium acremonium* were not successful. Recently, however, metabolically active protoplasts have been obtained from *P. chrysogenum* which are capable of synthesizing penicillins from ^{14}C-valine.[24] Furthermore, a cell-free system, prepared by osmotic lysis of the protoplasts obtained from *Cephalosporium acremonium*,[107] was able to effect the conversion of synthetic tritiated LLD-ACV into penicillin N (detected chromatographically but not isolated) in *ca.* 0.1% yield. This yield is considerably higher than the radiochemical yield of penicillin N from ^{14}C-L-valine, suggesting that the tripeptide is incorporated intact into penicillin. The tritiated LLL or DLD-ACV tripeptide analogues did not produce labelled penicillin or other detected metabolites.

It was also shown in this work that the incorporation of tritium into penicillin N from L - α - aminoadipyl - L-cysteinyl - D - 2 - ^{3}H - valine was approximately the same as the retention of tritium from L - α - aminoadipyl - L - cysteinyl - D - 4 - ^{3}H - valine. Since it has been shown[53,54] that all six of the valine methyl hydrogens are retained in penicillin biosynthesis, this result appears to indicate that the C-2 hydrogen of the valine residue of LLD-ACV is also retained in the biosynthesis of penicillin N. As the incorporations of the two tritiated tripeptides were performed in two separate, albeit identical, experiments, it would be of value to repeat the experiment using a ^{14}C tripeptide (preferably ^{14}C cysteine) as co-tracer in a double-label experiment to confirm the *complete* retention of the valine C-2 hydrogen. The results appear to eliminate the long-accepted α,β-dehydrovaline-type intermediates from consideration as intermediates in the formation of the thiazolidine ring of penicillin. As mentioned by Fawcett *et al.*,[101] such an intermediate would be consistent with the above results only if the dehydrovaline were enzyme-bound, and regained the same hydrogen during ring formation as was eliminated in the dehydrogenation step, **63**→**64**.



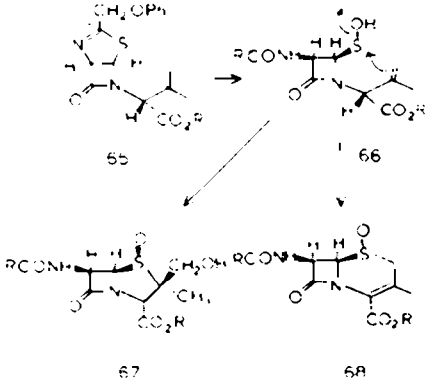
It had previously been established,[50,51] however, that the C-2 hydrogen of L-valine itself must be lost at some stage in the biosynthesis of penicillin. To evaluate the possible role of a dehydrovaline in the biosynthesis of the LLD-ACV tripeptide, Sih *et al.* incubated L-valine-2,3-^{3}H with starved, washed cells of *C. acremonium* and isolated LLD-ACV in *ca.* 1% radiochemical yield. Degradation of the product established that tritium was retained only at C-3, although it is difficult to evaluate the percent retention of tritium at this position in the absence of a simultaneous ^{14}C-valine incorporation. Assuming that the C-3 hydrogen is completely retained, the result eliminates the possibility of involvement of non-enzyme-bound dehydrovalines at any stage in penicillin biosynthesis. In another experiment by this group, the deuterated, ^{15}N-chiral valine **21k**, was incorporated by washed cells of *C. acremonium* into penicillin N and cephalosporin C. The ratio of ^{15}N/^{2}H in these metabolites indicated that they had been biosynthesized with approximately equal amounts of nitrogen exchange (25%). This fact provides circumstantial evidence that both penicillin N and cephalosporin C are formed from a common intermediate.

Biosynthesis of penam and cephem ring systems

A problem of major concern to workers in this field from the outset has been the elucidation of the detailed mechanisms through which the bicyclic ring systems of the penicillins and cephalosporins are formed *in vivo*. The problem has been an extremely difficult one to study experimentally, since apparently no intermediates between the ACV tripeptide and the fully-formed ring systems accumulate to any detectable extent. Thus, for example, it has been impossible to establish experimentally the sequence of ring formations. The tentative assumption has been made[3] that the 4-membered ring is formed first, but, as mentioned earlier, this has not been proven experimentally.

In their efforts to shed light on the biosyntheses of these ring systems, a number of investigators have recently chosen to study chemical model systems related to proposed biosynthetic intermediates. Through such studies, the chemical feasibility of certain proposed steps could be evaluated. It should be kept in mind that nearly all of these studies were performed under the assumption that dehydropeptides, especially those containing dehydrovaline residues, were acceptable, if unproven, biosynthetic intermediates. The tritium labelling studies of Abraham[104] and Sih[108] had not been published. Thus the model studies will be described in the light of the biosynthetic theories which prevailed at the time of the experiments.

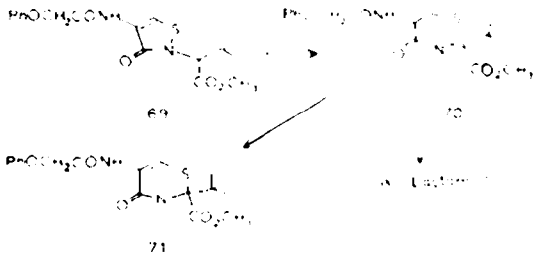
In 1972, Cooper[109] suggested that the thiazoline-azetidinone derivative **65**, obtained via the rearrangement and reduction of a penicillin sulfoxide,[110] might serve as a reasonable chemical model of a cysteinyldehydrovaline, proposed as an intermediate in penicillin biosynthesis. Cooper found that upon treatment of **65** with *m*-chloroperbenzoic acid, a mixture containing the penam derivative **67** and cephem **68** was formed. These products are



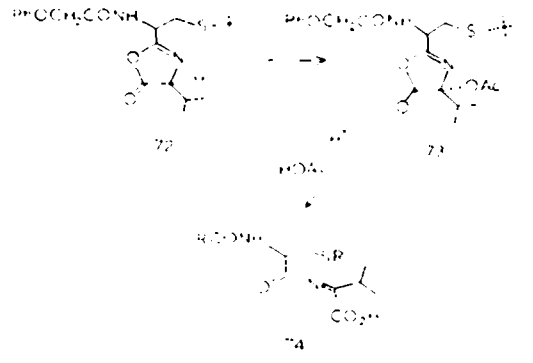
probably formed via a sulphenic acid intermediate **66**, which undergoes nucleophilic displacement at sulphur by the double bond. Thus, the transformations do not give chemical precedent for Arnstein's[3] mechanism involving a Michael-type addition of thiolate anion to the double

bond of an α,β-dehydrovaline di- or tri-peptide. However, this work indicated that such dehydrovalines could generate the β-lactam antibiotic ring systems by another mechanism. Recent extensions of Cooper's approach have resulted in what might be considered biogenetic-type syntheses of penicillins and cephalosporins.[111-113]

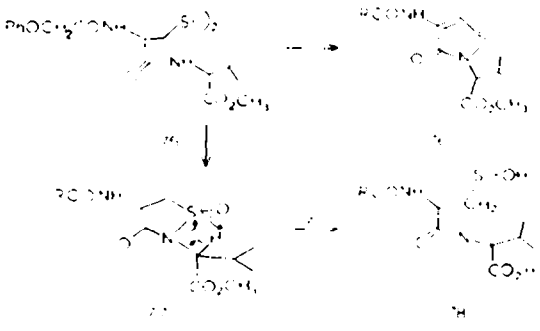
Morin *et al.*[114-117] investigated a variety of chemical models of reactions which might be involved in the oxidation steps leading to dehydrovaline peptides (or other oxidized biosynthetic intermediates). It was shown[114] that the isothiazolone intermediate **69** could be converted into the α-oxidized derivative **71**, by treatment



with a non-nucleophilic base, presumably via the acylimine **70**. An intermediate such as **70** could be considered a structure capable of leading, under the appropriate enzymic control, to the β-lactam ring system. Morin also showed[115] that oxazolones formed from simple dipeptides, e.g. the doubly-protected cysteinylvaline-derived oxazolone **72**, could be easily oxidized by $Pb(OAc)_4$ or $Hg(OAc)_2$ to C-terminal oxidized products **73**. Such a product might be envisioned as being capable under acidic catalysis of leading to an imine, e.g. **74**,

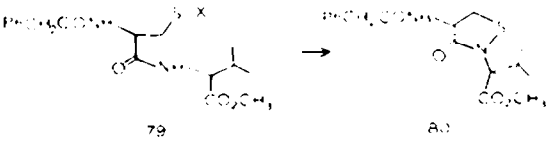


which would be a chemically reasonable intermediate for β-lactam synthesis. However, the latter interconversion was not reported. Morin later reported[116] that the oxidation of an acylcysteinylvaline dipeptide **75** with N-chlorosuccinimide produced the isothiazolidone **76**, while

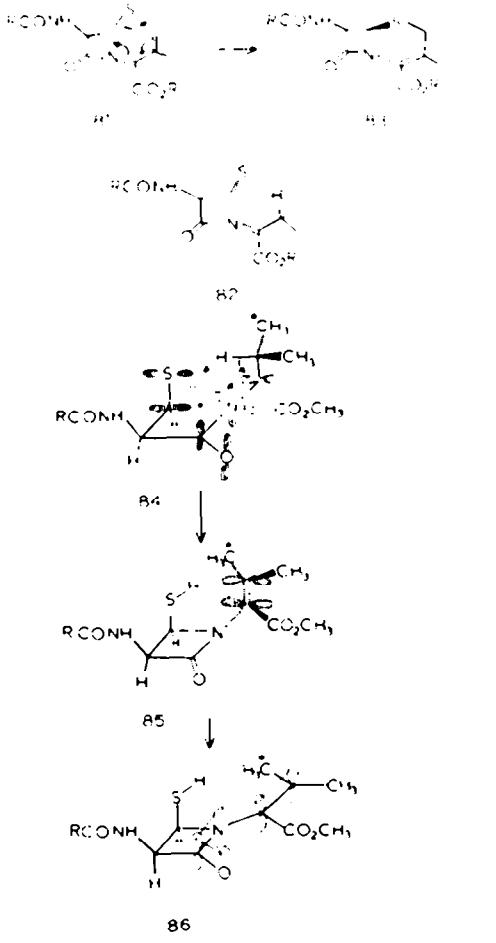


bromination of **75** with bromine in the presence of silver oxide and pyridine yielded a mixture of **76** plus **77**. Thermolysis of oxides such as **77** might be expected to yield, by an ene reaction, acylimine **78** which could constitute a reasonable intermediate for β-lactam biosynthesis.

In attempts to prepare thioaldehydes from cysteinyl peptides, Baldwin *et al.*[118] found that treatment of cysteinylvaline or cysteinyldehydrovaline[119] derivatives



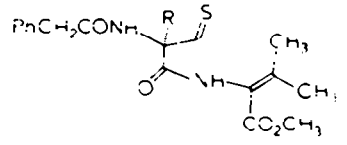
such as **79** with mild oxidizing reagents led instead to the isothiazolidone analogue **80**. The ease of these interconversions suggested that such structures as **80** might be involved in the biosynthesis of β-lactam antibiotics. Further oxidation, *in vivo*, of **80** could give (possibly via **77** and **78**) the thioaldehydes **81** and **82**. An intramolecular Diels–Alder reaction of **81** could yield the 3-cephem system **83**. Alternatively **82** might be expected to yield, by a suprafacial ene reaction **84**→**85**, the β-lactam system. However, an analogue of **85** was synthesized by Baldwin *et al.*,[120] and it failed to undergo ring closure to form the penam ring system. The failure of this ring closure is probably the result of an unfavourable alignment of the pertinent orbitals in this system. Presumably the compound adopts a conformation **86** allowing



continuous overlap of the p orbitals of the carbon–carbon double bond with those of the β-lactam system. In the absence of this constraint, the ring closure would ordinarily be expected to proceed, as shown, for example by the transformation **87** → **88**.[121]

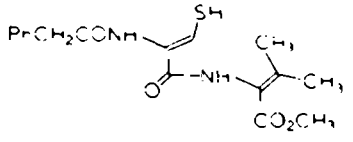
87 88

Baldwin's ene mechanism **84** → **85** for β-lactam synthesis was subjected to an experimental test by Scott and colleagues.[122,123] This group reported the synthesis of **89a** which is the enamine tautomer of the imine **82**. The photochemical synthetic route to **89a** required that it be generated as the thioaldehyde rather than as the thioenol tautomer **90**. No evidence of the formation of β-lactam compounds resulting from **89a**, under the conditions of its formation, could be obtained. The major reaction appeared to be the formation of the tautomer **90** which,



89 a R = H

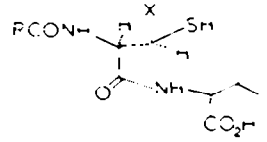
b R = CH₃



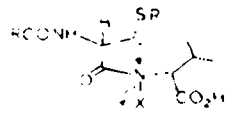
90

as reported earlier by Leonard,[119] is unstable and undergoes polymerization. To avoid this competing tautomerization, the α-methyl analogue **89b** was synthesized. However, this product did not lead to β-lactam formation even though ample opportunity for the formation of the imine tautomer analogous to **82** existed.

As discussed earlier, it now seems likely from the results of labelling studies[105,108] that dehydrovaline intermediates do not play a role in the biosynthesis of penicillins. The model studies described above appear to render the involvement of free thioaldehyde intermediates less than likely in the formation of the β-lactam ring, although the sp^3 equivalent[124] **91** of a thioaldehyde is not necessarily excluded by these results. Scott *et al.*[124] have recently examined an alternative suggestion[125] for the formation of this ring. In this hypothesis, the valine nitrogen of a cysteinylvaline precursor is oxidized to a hydroxamic acid analogue **92** (where X is

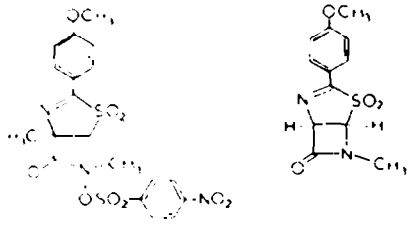


91 (X = OR, SR, etc.)



92

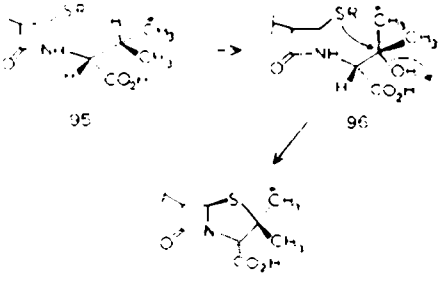
a good leaving group). Displacement of the hydroxyl or 'X' group by an anion generated at C-3 of the cysteine residue would form the β-lactam ring. Indeed such hydroxamic acid derivatives are well-known microbial metabolites: e.g. the mycobactin group of iron-complexing hydroxamic acid derivatives,[126] or the antibiotic actinonin.[127] Chemical analogy for such a process was found by Scott[124] in the transformation of the hydroxamate sulphone **93** into **94** upon treatment with potassium t-butoxide. It will be of considerable interest to attempt the interconversion of analogous hydroxamic acid derivatives into β-lactams *in vivo*.



93 94

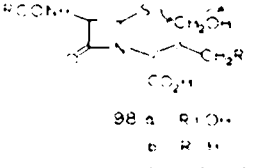
In a consideration of intermediates alternative to dehydrovalines for the formation of the thiazolidine ring of the penicillins, it should be recalled that Abraham *et al.*[101] had isolated from the mycelium of *C. acremonium* a tetrapeptide (P1) **61** containing a 3-hydroxyvaline residue. Although Stevens[36] had shown that 3-hydroxyvaline did not compete with L-valine for the biosynthesis of

L-L-D-ACV tripeptide could be hydroxylated after the biosynthesis of this tripeptide **95** → **96**. Displacement of the hydroxyl group by the sulphur atom could then generate the thiazolidine ring. It would be expected that such a displacement **96** → **97** would proceed with inversion of the



95 96

97



98 a R = OH

b R = H

configuration at the C-3 position of valine. In order that this route could be compatible with the observed stereochemistry of the incorporation of chiral valines into

penicillin,[61,62,66] it would be necessary to establish that the hydroxylation reaction also proceeded with inversion of configuration. Although in general hydroxylations of apparently inactivated carbons *in vivo* proceed with retention of configuration,[59,60] there are several known exceptions.[128-130] It would therefore be of considerable value to investigate the resultant chirality at the valine C-3 carbon of peptide P1, when biosynthesized from a chiral valine.

Whether α,β-dehydrovalines are involved in the biosynthesis of cephalosporin C and its analogues is an entirely open question, and a rather difficult one to test since the final product lacks both hydrogens originally present at C-2 and C-3 of the valine precursor. However, it seems clear that an isodehydrovaline is not involved, since Sih[53] has shown that the valine methylhydrogens are incorporated without exchange. These results also show that the formation of the 3-cephem system does not proceed via a 2-cephem system. It has been suggested[131,132] that the biosynthesis of this ring might take place via a 4-hydroxy-dehydrovaline intermediate, such as **98a** or **98b**. However, no evidence in support of this theory has been obtained to date. An alternative theory[24] suggests that the cephalosporins could be biosynthesized by rearrangement of a penicillin precursor, such as penicillin N. Such a rearrangement has been effected chemically via the penicillin sulphoxide.[133] Recently the first evidence that such an interconversion is possible *in vivo* was reported by Demain and Kohsaka.[134] Protoplast lysates were obtained from *C. acremonium* by a modification of the procedure of Fawcett *et al.*[105] This modified cell-free system was capable of synthesizing very small quantities of β-lactam antibiotics. The products were not isolated but were assayed by their inhibition of the growth of *E. coli* ESS, a mutant sensitive only to β-lactam antibiotics. Upon treatment of the antibiotic mixture from the cell-free system with penicillinase, which deactivates penicillin N but not cephalosporin C, antibiotic activity was retained. However, the antibiotic activity was destroyed upon incubation with cephalosporinase, which deactivates both penicillin N and the cephalosporins. Furthermore the production of cephalosporin-like antibacterial activity by this system was stimulated by the addition of penicillin N, but not by the addition of benzylpenicillin or 6-aminopenicillanic acid. It is reasonable to conclude tentatively that penicillin N was directly converted to a cephalosporin (not necessarily cephalosporin C) by this system. However, it would be of value to actually isolate the product or to demonstrate the interconversion using labelled penicillin N.

Late steps in the biosyntheses of penicillins and cephalosporins

A long-standing problem concerning the biosynthesis of the penicillins bearing more common side-chains, e.g. benzylpenicillin **1a**, phenoxymethylpenicillin **1b**, has been the identification of the stage at which the new acyl group is introduced and the determination of the role (if any) played by penicillin N **1e**, isopenicillin N **1d**, and 6-aminopenicillanic acid **1c**, in the pathway. Circumstantial evidence for the intermediacy of 6-APA is found in the fact that a 6-APA-acyl transferase is present in extracts of *P. chrysogenum*. This enzyme catalyzes the synthesis of the "common" penicillins from 6-APA and an acyl CoA.[135,136] Acyl group transfer between penicillins and 6-APA can also be catalyzed, apparently, by the same enzyme. However, Vanderhaege *et al.* reported that neither penicillin N nor isopenicillin N was hydrolyzed to 6-APA by a penicillin acylase from *P. chrysogenum* which did hydrolyze benzylpenicillin to 6-APA.[139] The occurrence of isopenicillin N in cultures of *P. chrysogenum* makes isopenicillin N a more likely precursor of the common penicillins than penicillin N. Certain *P. chrysogenum* mutants are unable to synthesize benzylpenicillin unless α-aminoadipic acid is added to the medium.[140]

Fawcett *et al.*[141,142] have recently provided more direct evidence of the synthesis of benzylpenicillin from isopenicillin N and 6-APA in *P. chrysogenum*. Incubation of *2β-methyl*-tritiated isopenicillin N or 6-APA with an extract of *P. chrysogenum* led to the formation of isolable tritiated "solvent-soluble" penicillin, which co-crystallized and corresponded on radiochromatograms with benzylpenicillin. In contrast, tritiated penicillin N did not produce benzylpenicillin in this manner. The results strongly support the contention that isopenicillin N is a normal precursor of penicillins with non-polar side-chains. It remains unclear, however, whether 6-APA is a mandatory intermediate, although this is rendered less likely by Vanderhaege's results.[139]

In connection with the biosynthesis of β-lactam antibiotics in Cephalosporium or Streptomyces species, it remains unknown at what stage or by what mechanism the configuration of the LLD-ACV precursor becomes inverted in the α-aminoadipyl residue to the D-configuration. Demain[143] and Abraham[13,131,132] have proposed biosynthetic pathways for cephalosporin C which proceed via deacetoxycephalosporin C **2g**, and deacetylcephalosporin C **2b**. The proposed intermediate **2b** has been known for quite some time.[144] Recently, the deacetoxy analogue **2g** was isolated,[145] and mutants of *C. acremonium* have been obtained which produce either deacetoxycephalosporin C alone, or deacetoxy and deacetylcephalosporin C together.[146,147] Furthermore, using disrupted mycelia of these mutants, the activity of two enzymes was detected. One of these catalyzes the conversion of deacetoxycephalosporin C to deacetylcephalosporin C and the other catalyzes acetylation, by acetyl CoA, of the hydroxyl to form cephalosporin C. The hydroxylation reaction required O_2 and a reduced pyridine nucleotide as well as manganous ion, in common with the class I oxido-reductases.[148] This finding is in good agreement with the observations that the yield of cephalosporin C in *C. acremonium* is markedly dependent on the oxygen content of the medium,[149,150] and the fact that cyanide ion prevented the conversion of penicillin N to cephalosporin C in the cell-free system developed by Demain.[134] The above results thus strongly support the proposed sequence for the final steps in cephalosporin C biosynthesis. Since the isolated intermediates **2b** and **2g** possess a D-α-aminoadipyl side-chain, it is clear that the inversion of configuration in this side-chain must take place at a stage preceding the introduction of the C-17 hydroxyl group. Hopefully, further work with cell-free systems will shed light on the mechanism of this inversion (as well as other unsolved problems in the biosynthesis of these metabolites).

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