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The studies by our group in this area have been previously reviewed [1]. Since that time great progress has occurred, largely as a consequence of the successful cloning and expression of Isopenicillin N Synthase (IPNS) and Ring Expansion-Hydroxylase (REX). The pathways are shown in Figures 1 and 2 in which those activities shown with question marks have yet to be characterised although cell-free preparations have been prepared. This discussion will focus on two enzymes

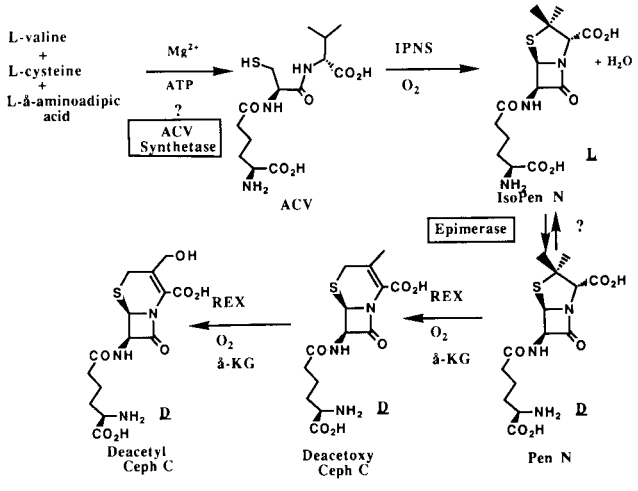

Figure 1

from *Cephalosporium acremonium*, namely IPNS and REX.

Studies on Isopenicillin N Synthase.

The successful purification of this enzyme to homogeneity

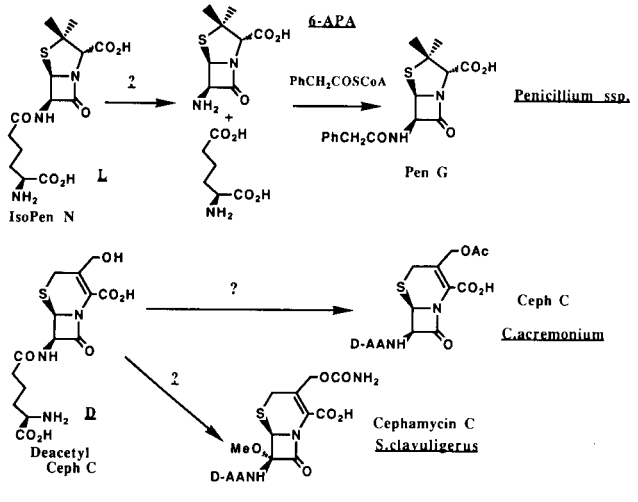

Figure 2

[2], [3] led to its successful cloning and over production in *Escherichia coli* [4]. This revealed the recombinant enzyme as a single polypeptide of 336 aminoacids, containing two cysteins (106 and 255 from tne N-terminus) and probably one iron atom, most likely ferrous [5], in a non-heme environment.

The gene expression in *E. coli* provided soluble recombinant protein [6]. Thus a 25 litre fermentation could provide about 1.0 g of IPNS in an active form, in two steps (lysis and Bioprocess ion exchange Q Fast Flow). Subsequently the enzyme was crystallised and efforts are in hand to obtain suitably sized crystals for diffraction studies. As well as the *C. acremonium* enzyme, IPNS has been obtained and sequenced for two other eukaryotes *i.e. Penicillium chrysogenium* [7] and *Aspergillus nidulans* [8] and two prokaryotes *i.e. Streptomyces lipmanii* [8] and *Streptomyces clavuligeris* [9]. The expected strong sequence homology is observed amongst these enzymes.

Some evidence relating to the folding of the protein has been obtained from the ready formation of a disulphide linkage between Cys 106 and Cys 255 [3], as well as photoaffinity labelling studies which suggest the active site is in the region of these two cysteines [10].

Studies on the mechanism of the penicillin-forming reaction catalysed by IPNS have continued. Thus the overall stoicheometric change involves the loss of four hydrogen atoms

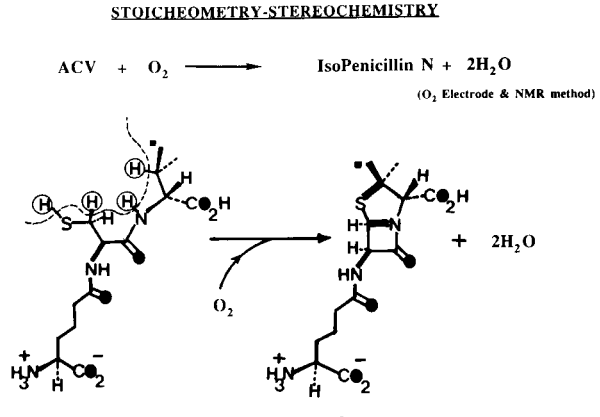

Figure 3

concomitant with the reduction of one molecule of dioxygen to water, Figure 3, both new covalancies being formed with retention of configuration.

Such a process is best described as "Desaturative Cyclisation", in that the loss of hydrogen is coupled to cyclisation of ring structures. As such it is without precedent, though it is conceivable that the formation of the typical Z-olefins by desaturation of fatty acids may be similar to the IPNS catalysed

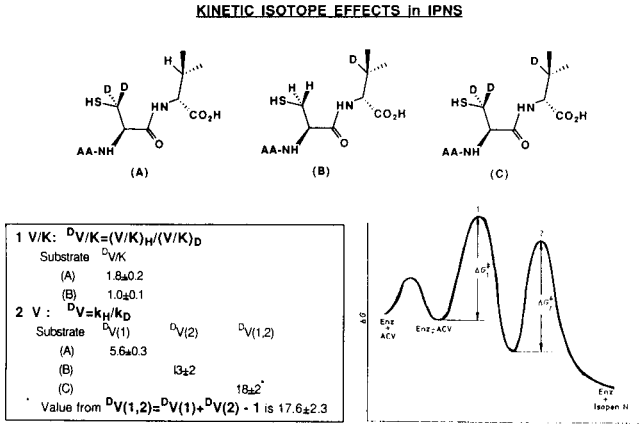

Figure 4

process. The order of ring formation has been revealed through a study of deuterium kinetic isotope effects [11]. These results are presented in Figure 4 and their interpretation has resulted in the energy profile shown. In this picture the enzyme-bound intermediate is proposed to be the monocyclic lactam shown in Figure 5 and experiments were designed, based on isotope effects, to reveal this species. It was found

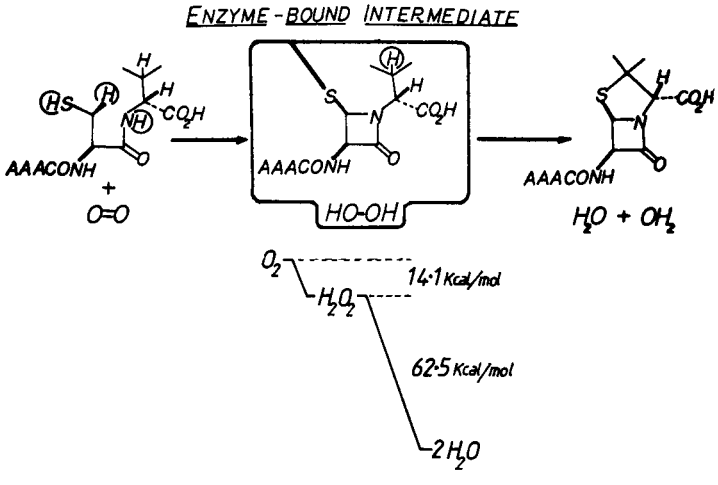

Figure 5

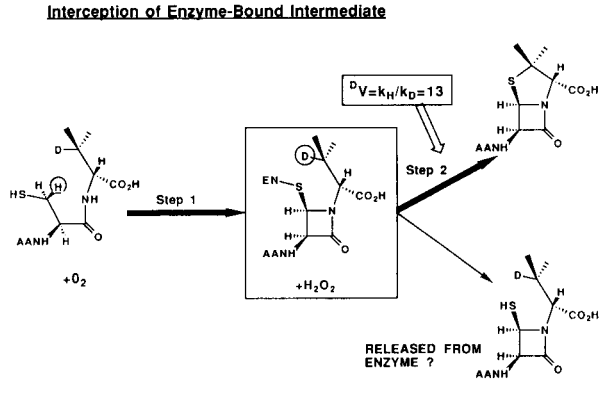

Figure 6

that the presence of a deuterium atom at the valine-3 position caused the release of an intermediate into solution which was presumed to be the monocyclic mercapto-azetidinone, Figure 6. Subsequently it was found to be hydrated aldehyde, Figure 7, containing *NO* sulphur [12]. This result can be understood in the following manner. Thus in the normal pathway if the thiol of the precursor tripeptide is directly bound to the ferrous iron in IPNS then at the intermediate stage this will be

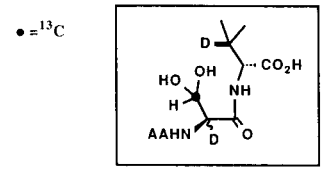

Figure 7

ferryl (i.e. 4-valent iron oxide) [13] attached directly to the thiolazetidinone Figure 8. Normally attack on the valine-hydrogen by this species occurs to give penicillin. However a deuterium isotope effect, measured on v_{max} as (13 ± 2) slows sufficiently this step so as to allow a new and competing process, *i.e.* the fragmentation to an acyl iminium ion along with atomic sulphur and divalent iron. In this way the enzyme is restored to its original iron(II) state for the next catalytic cycle. Trapping of the iminium ion would give the hydrate Figure 7. If this picture is correct then one can predict that an acyl iminium ion in a five-membered lactam would be of greater stability than the four-membered case and hence a tripeptide containing homocysteine in place of cysteine would be expected to be processed by IPNS largely by this route. In fact this was found to be so [14], Figure 9. The isolated hydroxy-γ-lactam also indicated that the source of the quenching

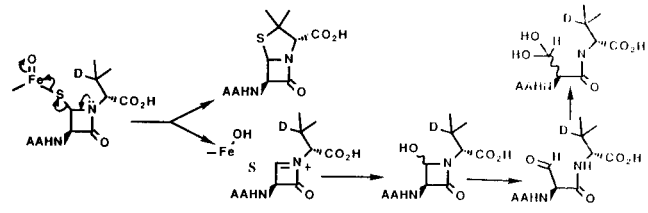

Figure 8

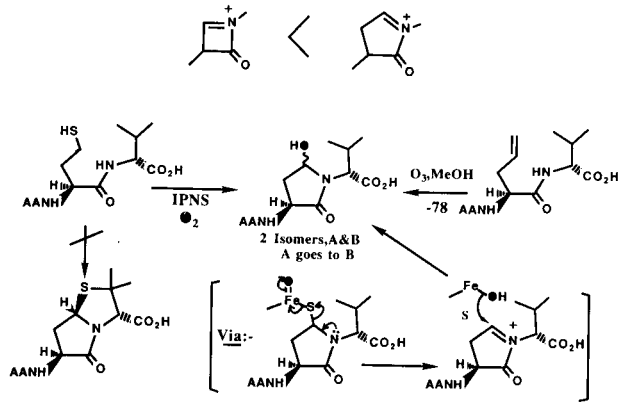

Figure 9

hydroxyl was molecular oxygen, as shown by ^{18}O isotopic studies. These experiments give strong support to the idea of the cysteine thiol is directly attached to a ferryl ion at the intermediate stage, which after collapse to the iminium ion, donates the ferrous bound hydroxyl to the positive centre. Further evidence for this concept was found in the reaction of the aminoadipoyl homocysteinyl cysteine peptide, Figure 10, which cyclised to the bicyclic disulphide. This result proves that the sulphur of the cysteine, or homocysteine, is directly

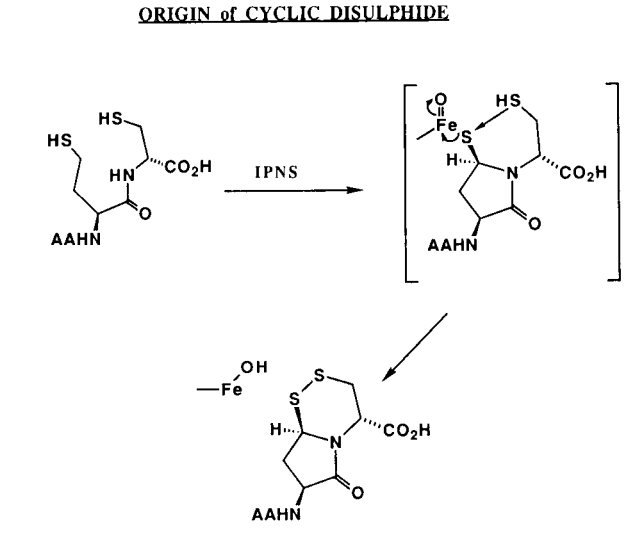

Figure 10

coupled to a strongly electron withdrawing element, conferring the properties of a sulphenyl halide on this functionality.

The evidence for homolytic character in the second C-S bond forming step was described earlier [1] and resulted in the description of this stage shown in Figure 11. Further evidence

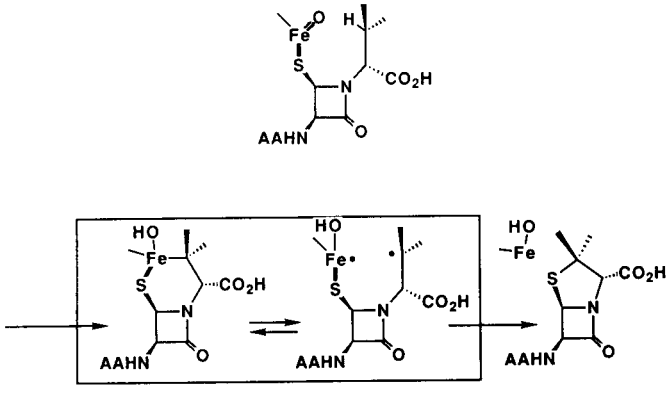

Figure 11

in support of this view has been obtained by the use of the "cyclopropyl carbinyl test", which is based on the known rapid ring opening of cyclopropyl carbinyl radicals (*ca.* 108 per.sec.). The test is shown in Figure 12 and the new product is the eight membered ring species, deriving from opening of the cyclopropane [15].

Studies with a tripeptide substituted with allylglycine in place of valine revealed a new aspect of IPNS, namely its capacity to add oxygen, from dioxygen, to the substrate, thereby becoming a mixed desaturative-hydroxylating system [16] Figure 13. The streochemistry of this process was of some considerable interest and is shown for one of the dideuterated allylglycines in Figure 14. All of these results and many others obtained by changing the valine moiety for other aminoacids can be rationalised on the basis of the S-ferrylazetidinone intermediate referred to above. The reaction of the ferryl

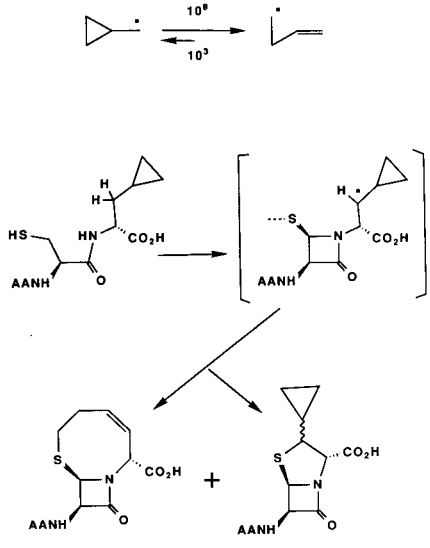

Figure 12

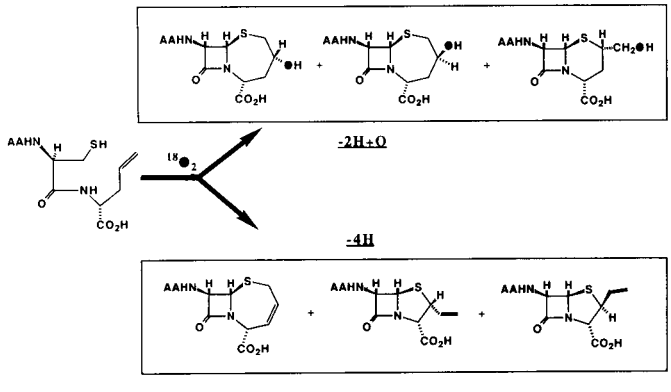

Figure 13

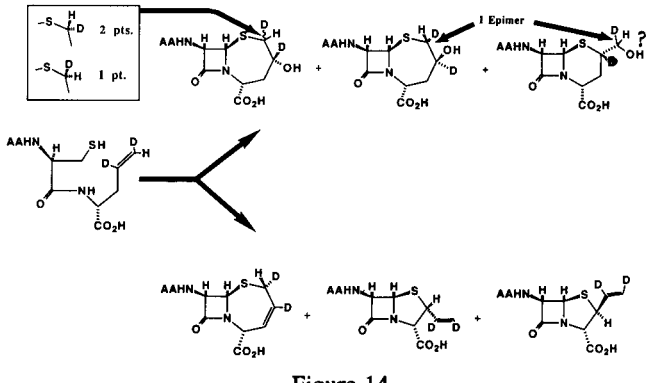

Figure 14

species with the side chain appears to be controlled in part by the conformation of the side chain. Four types of reaction are necessary to explain the stereochemical results, Figure 15.

(1) Insertion-homolysis for saturated side chains
(2) (2 + 2) cycloaddition
(3) Ene reaction for unsaturated
(4) Epoxidation side chains

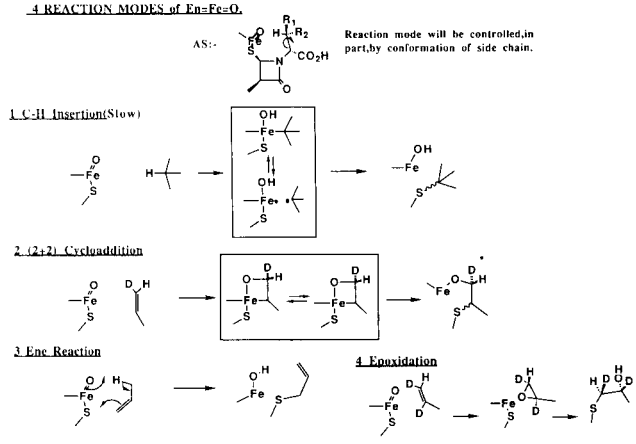

Figure 15

The application of these reactions to the cyclisation of the allylglycine substrate are shown in Figures 16 and 17 for the desaturative (-4H) and mixed desaturative-hydroxylative

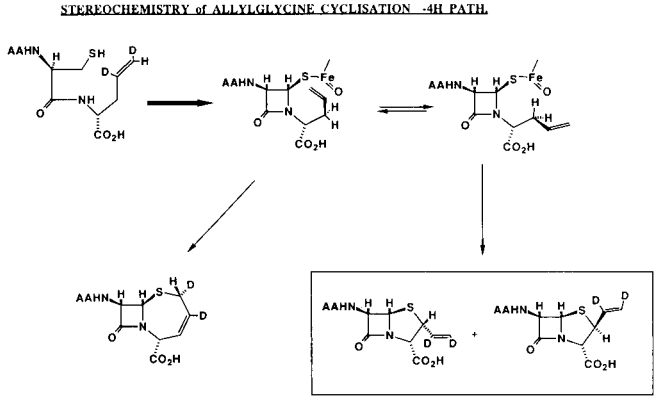

Figure 16

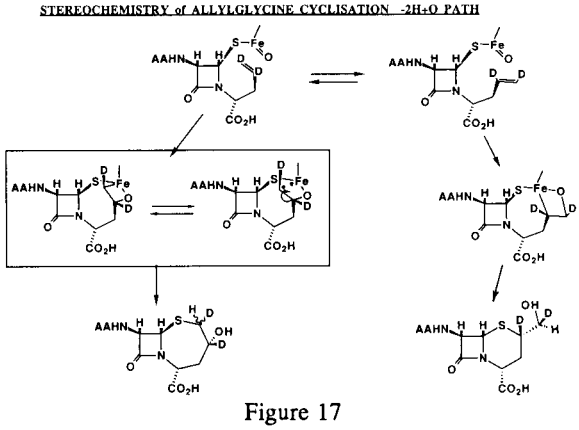

Figure 17

pathways (-2H + O), respectively. The stereochemistry of formation of a minor product from allylglycine requires an epoxidation followed by inverting ring opening, Figure 18.

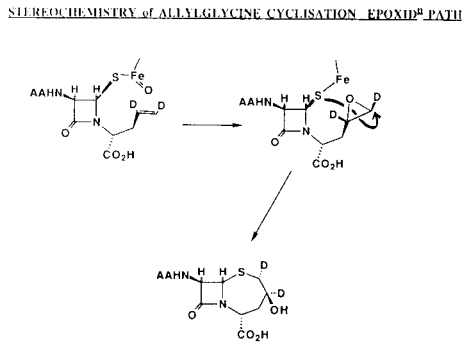

Figure 18

That the selection between the four modes of reaction depends on the steric relationship between the ferryl entity and the side chain was nicely shown in the case of a tripeptide terminating in isodehydrovaline (isopropenylglycine). In this case only (2 + 2) and Ene pathways were evident, reflecting the highly favourable steric relationship between the ferryl centre and the isopropenyl group, Figure 19.

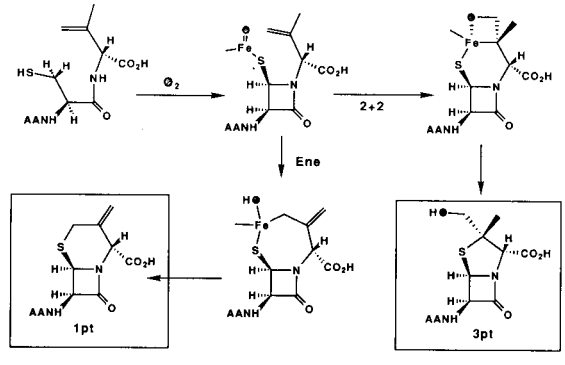

Figure 19

In this last case a complete analysis of the stereochemistry was achieved [17] and the results are shown in Figure 20. As is apparent the two rotational conformations of the deuterium

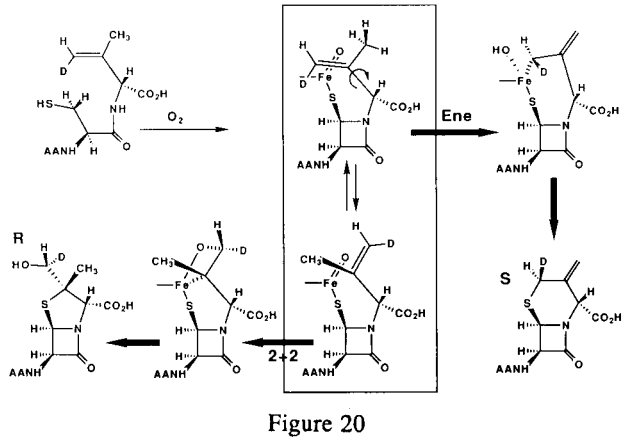

Figure 20

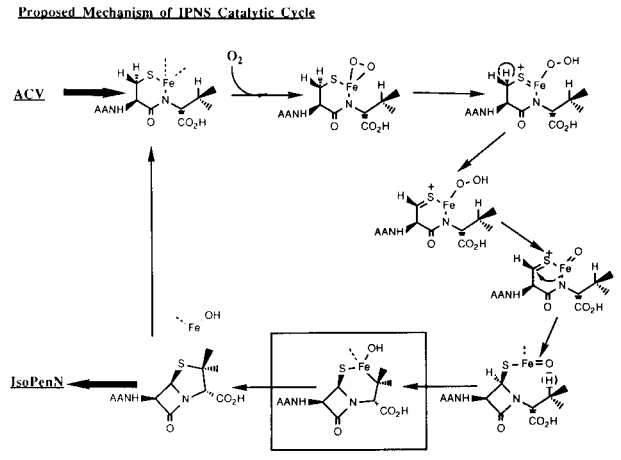

Figure 21

labelled isopropenyl group provide the branching point for formation of Ene and (2 + 2) cycloaddition paths.

The experiments described above, along with others, give rise to a working hypothesis for the IPNS catalytic cycle, Figure 21, in which the substrate tripeptide is directly attached to the ferrous iron in the binding site [13]. In this scheme the sulphur atom of the cysteinyl moiety provides the conducting bridge for electron flow from the peptide into the dioxygen molecule. In this scheme the removal of the 3-*pro-S* cysteinyl hydrogen is seen as an acid-base reaction mediated by a suitably placed basic site in the enzyme. Following β-lactam formation, *via* a 1,3-sigmatropic shift, the *S*-ferryl moiety mediates the final ring closure through insertion to an iron-carbon bonded metallocycle and subsequent reductive elimination to isopenicillin N. The lack of discrimination established by IPNS towards substrate structure is remarkable. By modifying the structural features of the tripeptide through synthesis a whole range of unusual bicyclic β-lactams have been generated in our laboratory through the use of IPNS as a "synthesising machine", Figure 22.

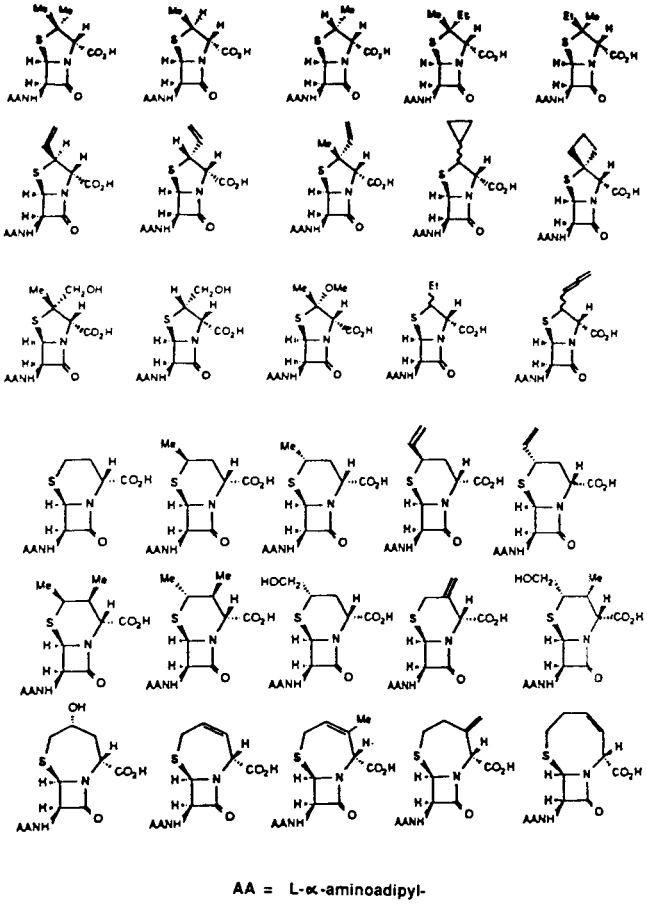

Figure 22

Studies on Ring Expansion-Hydroxylase (REX).

This enzyme (REX) from *C. acremonium* which mediates the conversion of Penicillin N into Cephalosporins, is bifunctional [18] in that it catalyses the sequential ring expansion to deacetoxycephalosporin C and it's subsequent hydroxylation to deacetylcephalosporin C, Figure 23. Each step requires stoicheometrically one dioxygen molecule and one molecule of α-ketoglutarate [19]. The enzyme from *C. acremonium* has been cloned [20] and over-expressed in *E. coli*, in which host it is produced as inclusion bodies. However treatment of these bodies with high molar urea dissociates them into active enzyme which shows the same substrate specificity as wild-type enzyme [21]. The first step in this sequence involves the loss of 1 hydrogen atom from the β-methyl

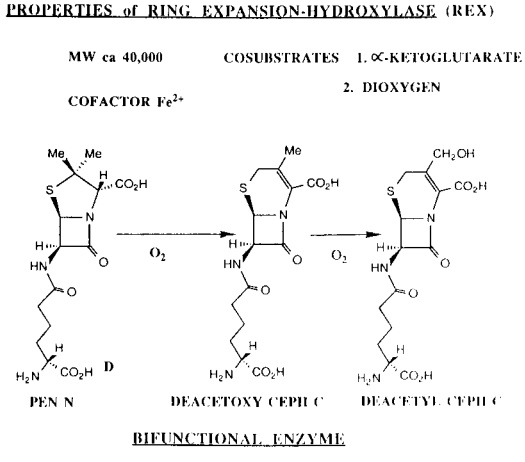

Figure 23

group and from the C-3 proton of penicillin N [22]. To ascertain the order of these events it was decided to conduct isotopic studies, in particular deuterium kinetic isotope effects on the parameter V_{max}/K_m. The specifically deuteriated penicillin N derivatives were readily synthesized from the corresponding tripeptides using IPNS, since this enzyme will accept the *D*-α-aminoadipoyl side chain [23] and provide thereby penicillin N directly Figure 24. On the other hand REX will not accept isopenicillin N as a substrate [24]. The initial experiments, with D_6-Pen N, gave as expected the two cephalosporins, but in this case a new substance X was detected, at a level of about 1-2% of the major pathway, Figure 25. Also, appreciable loss of deuterium was observed, over that expected for

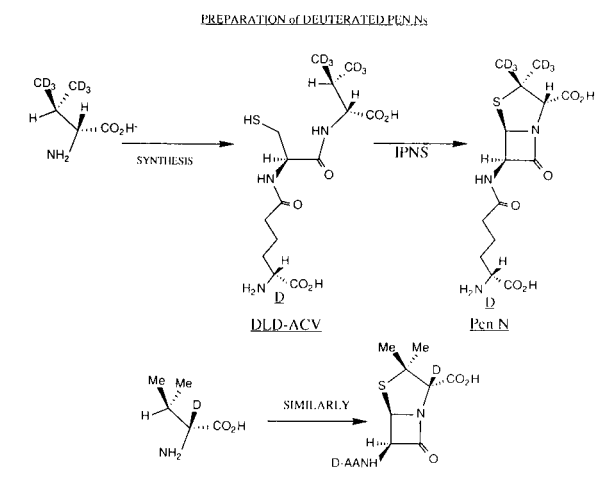

Figure 24

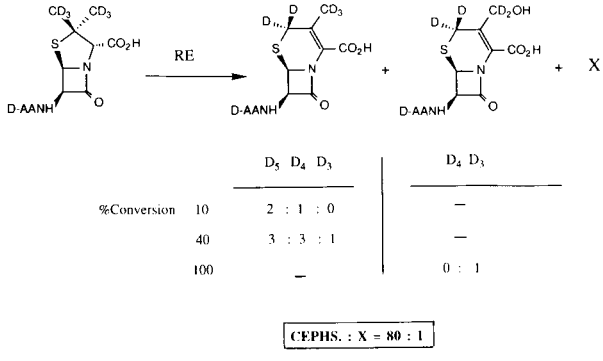

Figure 25

the conversion. When the experiment was repeated with C-3 deuterated penicillin N the previously unknown minor product X now became a major product and was readily isolated and identified as the 3-β-hydroxy cepham [25], Figure 26. Clearly the change in ratio of products was due to a primary kinetic isotope effect. The origin of the hydroxyl group was shown to be molecular oxygen by the appropriate labelling experiment. The competitive (V_{max}/K_m) isotope effects from the deuteriated substrates were determined and an isotope effect observed only on the methyl labelled species, Figure 27.

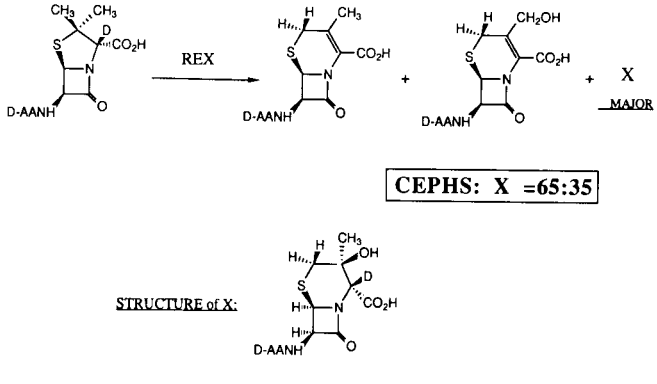

Figure 26

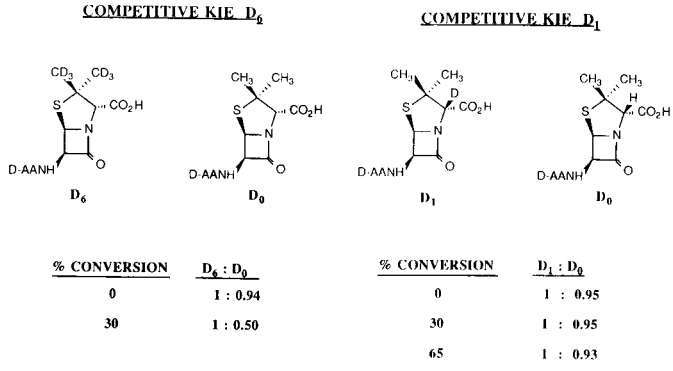

Figure 27

Since a V_{max}/K_m isotope effect will be observed up to and including the first irreversible step, these results, coupled with the observation of an isotopically dependent production of a 3-β-hydroxy cepham, can only be explained by a sequence involving an initial loss of the 2-β-methyl hydrogen to form an intermediate, which can either lose the 3-hydrogen in the normal path [22], [25] to deacetoxycephalosporin C, or in the presence of a 3-deuterium atom can be deflected to the 3-β-hydroxycepham Figure 28. The nature of the intermediate immediately preceding the branch point is not known but a bridged cation or radical is possible, Figure 28. The latter possibility is supported by the finding that a chemically generated free radical, at the 2-β-methyl group of a penicillin undergoes very rapid ring enlargement to a cepham system [26].

It is apparent that the detailed chemistry of the ring expansion step bears a strong relationship to that of IPNS and suggests that both enzymes might be derived from an ancient common precursor. In keeping with this suggestion is the presence of significant homologes in the aminoacid sequences in the two *C. acremonium* enzymes [20]. A major

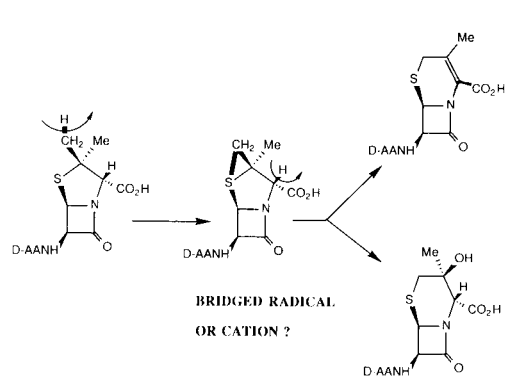

Figure 28

difference in the stoicheometric utilisation of dioxygen does exist between these two enzymes. In IPNS an overall 4 electron oxidation of the tripeptide substrate is achieved, with conversion of one molecule of dioxygen into water. However in REX, for each of its consecutive steps, only a two electron oxidation of the substrate is achieved for each molecule of dioxygen, since in each step one molecule of α-ketoglutarate is transformed into carbon dioxide and succinate [27]. Thus the

REX enzyme utilises only the ferryl state in its attack on the substrate, having produced this reactive species by the initial splitting of α-KG, Figure 29.

The subsequent functionalisation of this methyl group to deacetylcephalosporin C is envisaged as a second attack of ferryl on this centre, followed by reductive elimination to the hydroxy compound.

Finally, on a more general note, it is of considerable interest that IPNS is able to successfully cyclise to bicyclic lactams such a wide variety of precursor structures Figure 30. Since little is yet known in detail about the biosynthesis enzymes of secondary metabolism, perhaps this will be found to

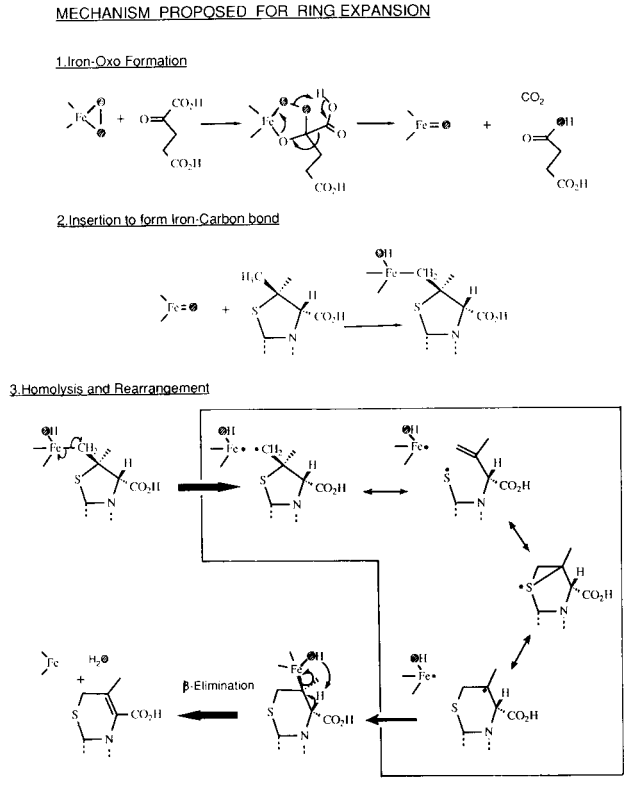

Figure 29

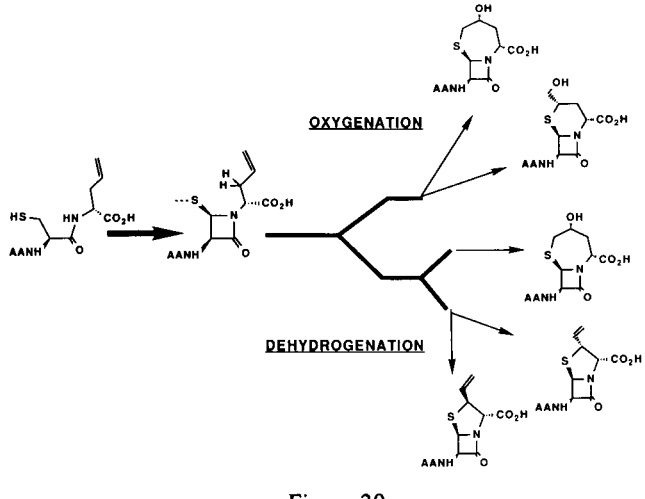

Figure 30

be a more general phenomenon. If so, it offers many possibilities for the use of such enzymes and their mutants for synthetic purposes. In the case of IPNS the ability to cyclise such a variety of structures certainly is connected with the high reactivity of the postulated ferryl species. As has been demonstrated above, once such a reactive entity has been generated in the active site, chemical reaction follows irrespective of the conformation of the substrate. Since the active site can clearly accommodate several conformations, particularly for "unnatural substrates" then the multiplicity of products found follows directly. It is in fact possible that the wide range of oxidative patterns frequently found among natural products, particularly at the more highly oxidised end of the pathways, may result from the action of a relatively few enzymes which are capable of producing several product types from a single precursor.

Acknowledgements.

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