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ENZYMIC CONVERSION OF DEUTERATED ANALOGUES OF δ-L-α-AMINOADIPOYL-L-CYSTEINYL-D-ALLYLGLYCINE, AN UNNATURAL SUBSTRATE FOR ISOPENICILLIN N SYNTHASE: A UNIFIED THEORY OF SECOND RING CLOSURE.

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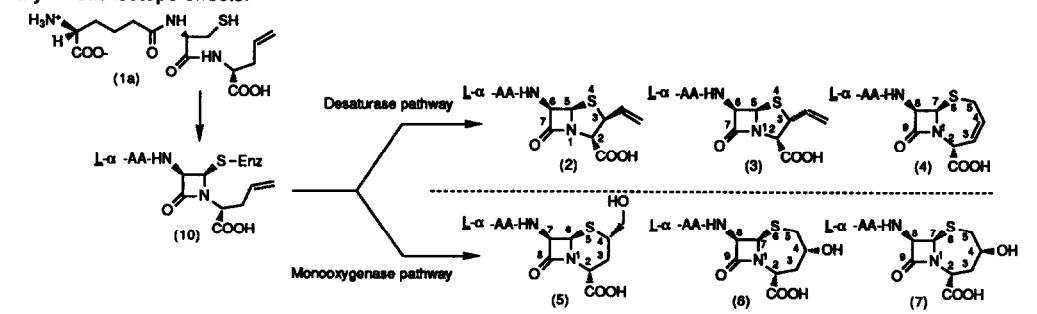
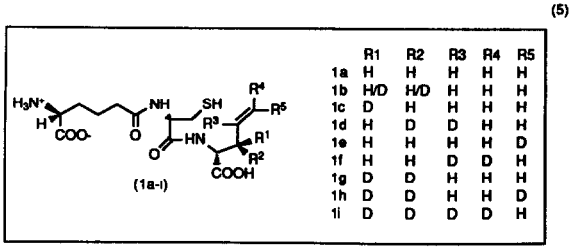
Abstract: The enzyme Isopenicillin N Synthase catalyses the conversion of the modified substrate δ-L-α-aminoadipoyl-L-cysteinyl-D-allylglycine to six β-lactam containing metabolites. Eight tripeptides deuterated in the allylglycine moiety have been prepared and the stereochemical course of their cyclization to the β-lactam containing metabolites has been investigated.

We have reported in communication form[1,2] that the enzyme Isopenicillin N synthase (IPNS) from *Cephalosporium acremonium* CO 728 converted the modified tripeptide substrate δ-L-α-aminoadipoyl-L-cysteinyl-D-allylglycine (1) (LLD-ACAllylglycine) into penam (2,3), homoceph-3-em (4), 4S-hydroxymethylcepham (5), 4R-hydroxyhomocepham (6), and 4S-hydroxyhomocepham (7) type products. (See Scheme 1). Herein we report experimental details of this original incubation of (1a), and report the full results of the incubations of the eight deuterated LLD-ACAllylglycine tripeptides (1b to 1i). These have allowed a thorough investigation of the stereochemistry of the second ring closure of the β-lactam containing products (2-7), while providing us with further evidence for the enzyme's inherent sensitivity to primary kinetic isotope effects.



(1a) (10) (2) (3) (4) (5) (6) (7)

[L-α-AA = L-α-Aminoadipoyl]



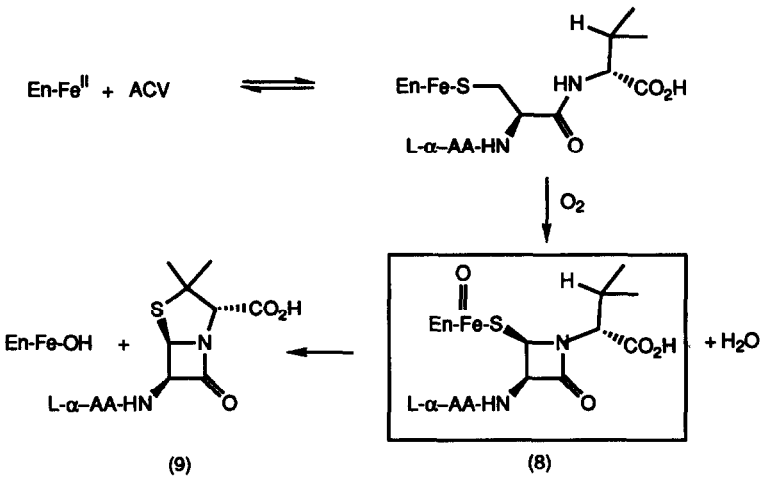
	R1	R2	R3	R4	R5
1a	H	H	H	H	H
1b	H/D	H/D	H	H	H
1c	D	H	H	H	H
1d	H	D	D	H	H
1e	H	H	H	H	D
1f	H	H	D	D	H
1g	D	D	H	H	H
1h	D	D	H	H	D
1i	D	D	D	D	H

(1a-i)

structure 1a gives rise to 2a, 3a, 4a, etc. ...
structure 1b gives rise to 2b, 3b, 4b, etc.

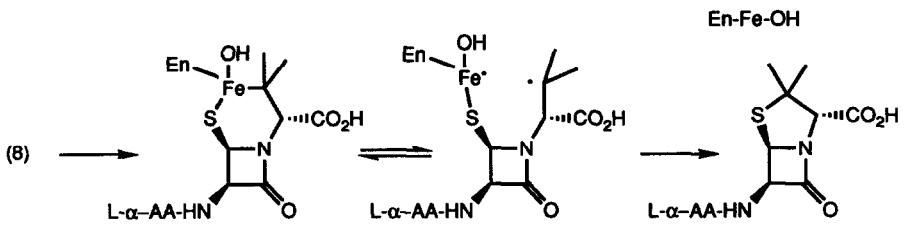
Scheme 1

Before an interpretation of these stereochemical results is presented a brief review of the evidence pertaining to the mechanism of the enzyme IPNS is in order. Experiments have been reported[3] supporting the conclusion that two distinct ring closure steps occur via the intermediacy of a monocyclic intermediate which is bound to the enzyme through a sulphur-iron bond. This contention has recently recieved support from spectroscopic observations on the binding of δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (ACV) to IPNS, which has been shown to involve the formation of such a sulphur-iron bond[4]. Furthermore, as the catalytic cycle requires a stoichiometric equivalent of dioxygen, which in the case of this natural "desaturase" pathway is fully reduced to water[5] and in the "mono-oxygenase" pathway is incorporated[6], (as one atom) into the products, (5), (6), and (7), then we have proposed to account for these results the formation of an iron(IV)-oxo (ferryl) intermediate (8), Scheme 2.



Scheme 2

The second ring closure, to isopenicillin N (9), is then mediated through the highly reactive species (8), possibly via an insertion-homolysis-recombination pathway, Scheme 3:-



Scheme 3

When IPNS is presented with new substrates, such as the allylglycine tripeptide (1), then after the first ring closure the analogous intermediate (10) now has a number of possible modes of reaction, dependent on the conformation of the allylglycine moiety and the chemical reactivity of its double bond verses its methylene carbon-hydrogen bonds. From structural analysis of the reaction products of this reaction, refined by the stereochemical experiments described herein, we can derive a reasonably consistent picture for the mechanism of the second ring closure, as follows. The results are consistent with four basic types of mechanism deriving from the postulated β-lactam intermediate (10):-

(a). Hydrogen atom abstraction/recombination.
(b). Oxy-ene type reaction.
(c). ($2\pi + 2\pi$) cycloaddition, followed by reductive insertion with retention of stereochemistry.
(d). Epoxide formation, followed by epoxide ring opening by sulphur with inversion of stereochemistry.

LLD-ACAllylglycine (1a)

Incubation of the tripeptide (1a) with IPNS under standard conditions[7,8] gave the five β-lactam containing metabolites previously reported[1]. These were purified by reverse phase hplc from the crude incubation mixtures (see experimental for details of individual compounds) to give (2a, 3a, 4a, 5a, 6a) (see figures 1,2,and 3 for ^{1}H-nmr spectra of 4a, 5a, and 6a). These products can be conveniently divided into two categories, arising from two distinct pathways from the postulated common monocyclic β-lactam intermediate (10).

(i) Mono-oxygenase [-2H + O].
(ii) Desaturase [-4H].

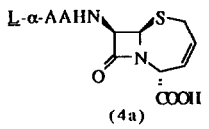
It is upon this basis that we will examine the products derived from the incubation of the tripeptides (1a-i), while in the discussion we will show how these relate to the four distinct mechanisms mentioned above.

Incubation of the tripeptides (1a-i) with denatured enzyme, and of the diastereotopic tripeptide LLL-ACAllylglycine (11) with IPNS gave no conversion to β-lactam containing products as demonstrated by ^{1}H nmr and bioassay, thus providing control reactions for the enzymatic conversion of (1a-i). In order to eliminate the possibility that the 4R-hydroxyhomocepham (6a) could have been derived from a regio- and stereospecific hydration of the double bond of the homoceph-3-em (4a), the tripeptide was incubated with IPNS using D_2O as the solvent (all cofactors, buffers and the enzyme were exchanged into D_2O before use). Purification of the crude incubation mixture by reverse phase hplc, yielded undeuterated 4R-hydroxyhomocepham (6a), with spectroscopic data identical with those for the compound isolated from the normal incubation, suggesting that these compounds did not arise *via* hydration.

The origin of the oxygen at C-4 of the hydroxyhomocephams and the hydroxy group of the hydroxymethylcepham was of fundamental importance in understanding the mechanism of the second ring closure in the formation of the oxygenated products. The incorporation of $^{18}O_2$ into the oxygenated products (5,6) has been reported[6], a result that has much precedent in the literature[9,10]. This important result, coupled to many others, has enabled us to propose the existence of an iron-oxene species as the reactive intermediate for the second ring closure in penicillin biosynthesis.[11,12]

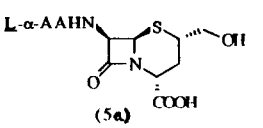
Following these results a thorough investigation of the second ring closure was initiated. We considered the effect of deuteration of the allylglycine moiety at C-3, where a primary kinetic isotope effect should bias the enzyme towards the mono-oxygenase pathway and away from the desaturase pathway. Stereospecific deuteration of the double bond would lead to an understanding of the stereochemistry of the second ring closures, the stereospecificity of the competing dual mono-oxygenase and desaturase pathways, and evidence for the species involved in the oxygenation of the double bond. The tripeptides were prepared according to literature methods[13].

FIGURE 1
^{1}H n m r (500MHz), of Homoceph-3-em (4a)
In D_2O (insert ^{1}H COSY)



(4a)

FIGURE 2
^{1}H n m r (500MHz), of
4S-hydroxymethylcepham (5a)
In D_2O (insert ^{1}H COSY)



(5a)

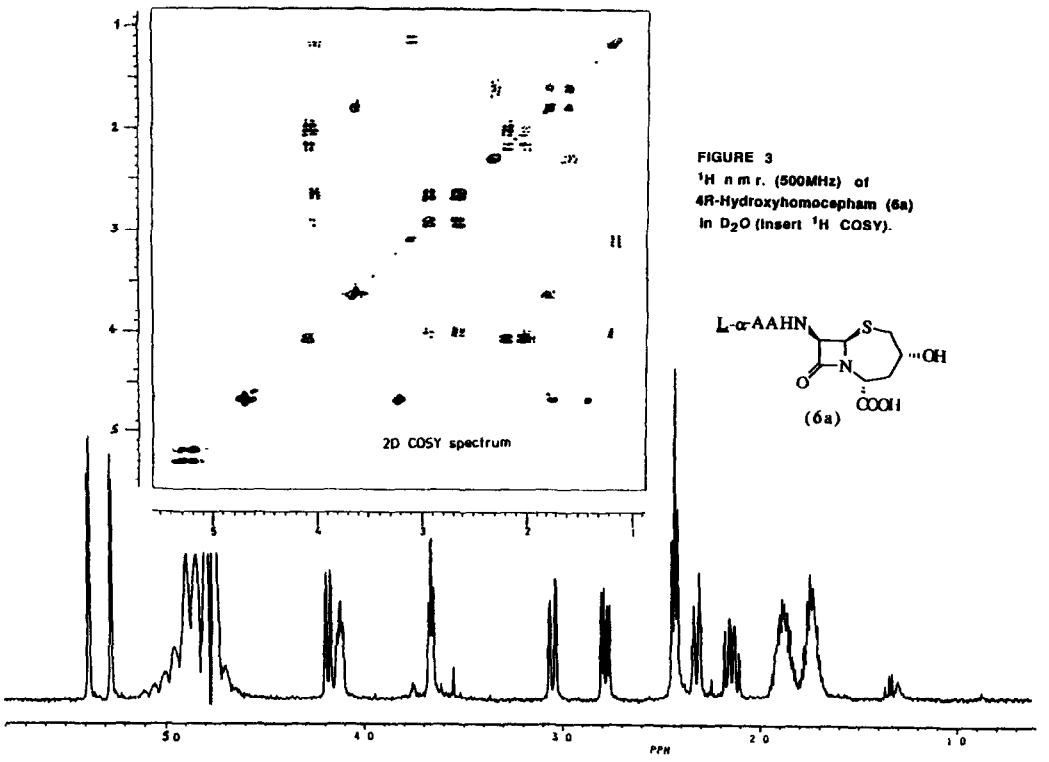


FIGURE 3
^{1}H n m r. (500MHz) of 4R-Hydroxyhomocepham (6a) in D_2O (insert ^{1}H COSY).

Metabolite ratios and HPLC retention times from the tripeptides (1a-i).

	Metabolites	2	3	4	5	6	7
Tripeptide.	1a	4	1	10	2	5	–
	1b	2	-	5	4	10	–
	1c	4	1	10	2	5	–
	1d	-	-	*	8	20	Δ
	1e	4	1	10	2	5	–
	1f	4	1	10	2	5	–
	1g	–	–	*	8	20	1
	1h	–	–	*	8	20	1
	1i	–	–	*	8	20	1
HPLC retention times (min):							
Solvent system A:		8 3	7.1	6.3	5.0	4.5	4.9
Solvent system B		–	–	38.2	18.0	8.2	15.3

(Stationary phase ODS, mobile phase A=10mM ammonium bicarbonate, B=0.05% (aq) formic acid).

Δ Observed, but not isolated.

* <1: observed in crude incubation mixtures. Very weak ^{1}H n.m.r's suggest that they arise predominantly from stereochemical or isotopic impurities in the starting tripeptides.

LLD-AC-(3RS)-[3-$^2H_1$]Allylglycine (1b).

This tripeptide was incubated with the IPNS enzyme under the standard conditions (see experimental). The region of the ^1H n.m.r. spectrum of the crude incubation mixture associated with the β-lactam hydrogen resonances was compared to that obtained from an incubation of the corresponding unlabelled tripeptide (1a), and major differences in the relative ratio of metabolites were observed.

	Relative ratios	
	Non-deuterated (1a)	(3RS)-[3-$^2H_1$] (1b)
β-vinyl penicillin (2)	4	2
α-vinyl penicillin (3)	1	trace
Homoceph-3-em (4)	10	5
Hydroxymethylcepham (5)	2	4
4R-hydroxyhomocepham (6)	5	10

Four of these products (2b, 4b, 5b, 6b) from the deuterated tripeptide (1b) were isolated by repeated reverse phase hplc, and the extent of deuteration in the relevant sites of the products was determined by integration of the ^1H n.m.r resonances (see experimental section).

The preponderance of α- to β-deuteration at C-3 of the oxygenated products (approx. 0.7 to 0.3) can be rationalised by preferential α-abstraction of a hydrogen atom from C-3. Hence α-deuteration will lead to almost total bias of the pathway towards the oxygenated products due to a kinetic isotope effect, whereas β-deuteration will give the same distribution of products as from the undeuterated tripeptide, and these effects working together, after β-lactam formation as the first irreversible event[14], would give the ratios observed.

LLD-AC-3R-[3-$^2H_1$]Allylglycine (1c) and LLD-AC-3S-[3,4-$^2H_2$]Allylglycine (1d).

The two tripeptides (1c) and (1d) were incubated independently with the IPNS enzyme, and the metabolites (2c, 3c, 4c, 5c, 6c) and (2d, 3d, 4d, 5d, 6d) isolated by reverse phase hplc. Integration of the region of the ^1H n.m.r. spectra which contained the β-lactam resonances allowed the relative ratios of metabolites for the two tripeptides (1c) and (1d) to be assessed.

	LLD-AC-3R-[3-$^2H_1$]Ag (1c)	LLD-AC-3S-[3,4-$^2H_2$]Ag (1d)
β-vinyl penicillin (2)	4	–
α-vinyl penicillin (3)	1	–
Homoceph-3-em (4)	10	<1*
Hydroxymethylcepham (5)	2	8
4R-Hydroxyhomocepham (6)	5	20

*mainly from stereochemical impurities.

The ratio of metabolites from the LLD-AC-3R-[3-$^2H_1$]Allylglycine tripeptide (1c) is identical with the ratios of products usually observed from an incubation of the unlabelled tripeptide (1a) with IPNS. The 4R-hydroxyhomocepham (6c) and hydroxymethylcepham (5c) showed the expected incorporation of deuterium at C-3 (^1H n.m.r, m/z). The homoceph-3-em (4c) contained predominantly deuterium at the C-3 site (any proton present was probably due to contaminating fully protiated tripeptide substrate [approx. < 5%]). The 3α-vinyl penicillin (3c) and 3β-vinyl penicillin (2c) were both predominantly deuterated at C-3 (>80% and>95% respectively by ^1H n.m.r.).

The ratio of products from the LLD-AC-3S-[3,4-$^2H_2$]Allylglycine (1d) showed a large primary kinetic isotope effect with almost complete bias of the pathway towards the monooxygenase pathway. This suggests that for the dehydrogenase

pathway to occur with this tripeptide, a carbon-deuterium bond must be broken. Hence it is likely that it is the pro-S hydrogen atom at C-3 of (1a) that is abstracted initially in the second ring closure to form the dehydrogenated products.

Following the results of the incubation of the modified tripeptide substrate LLD-ACAllylglycine (1a) and the three deuterated analogues (1b, 1c, 1d) with the IPNS enzyme, an investigation of the stereochemistry of the second ring closure, and the sensitivity of the dual pathway to a primary kinetic isotope effect was required. These studies with five deuterated tripeptides (1e, 1f, 1g, 1h, 1i) produced some remarkable results, while the incubation of the tripeptides (1g, 1h, 1i) led to the isolation of another novel metabolite, the 4S-Hydroxymethylcepham (7g, 7h, 7i).

LLD-AC-(Z)-[5-$^2H_1$]Allylglycine (1e) and LLD-AC-(E)-[4,5-$^2H_2$]Allylglycine (1f).

The tripeptides (1e) and (1f) were each incubated with the IPNS enzyme under the normal incubation conditions. The enzymatic products were isolated, by hplc, from the crude incubation mixtures, and as expected two classes of metabolites were observed.

Both the 3β-vinyl penicillins (2e) and (2f) and 3α-vinyl penicillins (3e) and (3f) from the incubations of the tripeptides (1e) and (1f) showed the deuterium atoms located at only a single terminal olefinic sites, with the same relative stereochemistry as that of the starting tripeptides (1e, 1f). The two homoceph-3-ems (4e) and (4f) contained a converse ratio of hydrogen to deuterium between the two diastereotopic C-5 sites, that was again retained from the substrate, the tripeptide (1e) (Z-[5-$^2H_1$]) giving Hα:Hβ > 10:1, while the tripeptide (1f) (E-[4,5-$^2H_2$]) gave Hα:Hβ < 1:10. (See figure 4).

The above results coupled to those from tripeptides (1b) and (1c) suggest that in the dehydrogenase pathway leading to the penicillins the initial event was the formation of a radical by abstraction of a hydrogen atom[15] from the Pro S position at C-3 of the allylglycine residue. The observation that the penicillins (2c) and (3c) were produced from incubation of tripeptide (1c) in an identical ratio to that seen for the unlabelled substrate (1a), and that both retained a deuterium at C-3, suggests that the β-penicillin (2) was formed directly from this common intermediate, while the α-penicillin was formed after rotation around the C2-C3 bond (with concomitant epimerization). No randomization of the label at C-5 by allylic shift was observed; this suggests either a highly active site constrained allylic radical, very fast interception of the radical once it was formed, or non-randomization due to conjugative considerations.

The homoceph-3-ems (4e, 4f) could be obtained in one of two ways (scheme 4):

(1) Via the capture of the terminal allylic radical formed from allylic rearrangement of an initial intermediate with a radical at C-3. One of the conformers would then give rise to the observed homoceph-3-ems (cis double bond), while the other would be disfavoured as it would give rise to a trans double bond in the seven membered ring. The specificity of the attack for either face of the conjugated allylic radical could in principal control the protium/deuterium distribution at the C-5 centre of the metabolites.

(2) An oxy-ene type reaction or suprafacial-suprafacial [1,3] shift of an iron-carbon σ-bond, formed from the initial radical pair, which would lead to an iron-carbon bond at C-5 of the allylglycine residue. This second ring could then be closed by reductive insertion of the sulphur, with retention of stereochemistry at C-5, analogous to that considered for the LLD-AC-βγ-dehydrovaline tripeptide[16].

LLD-AC[3,3-$^2H_2$]Allylglycine (1g), LLD-AC-(Z)-[3,3,5-$^2H_3$]Allylglycine (1h), and LLD-AC-(E)-[3,3,4,5-$^2H_4$]Allylglycine (1i).

The rationale behind the proposed mechanism for the mono-oxygenase pathway followed from the results of the incubations of the deuterated tripeptides (1g), (1h), and (1i), which led to an almost complete bias towards the mono-

FIGURE 4
^{1}H n m r. (500MHz), of Homoceph-3-ems (4a), (4e) and (4f) in D_2O

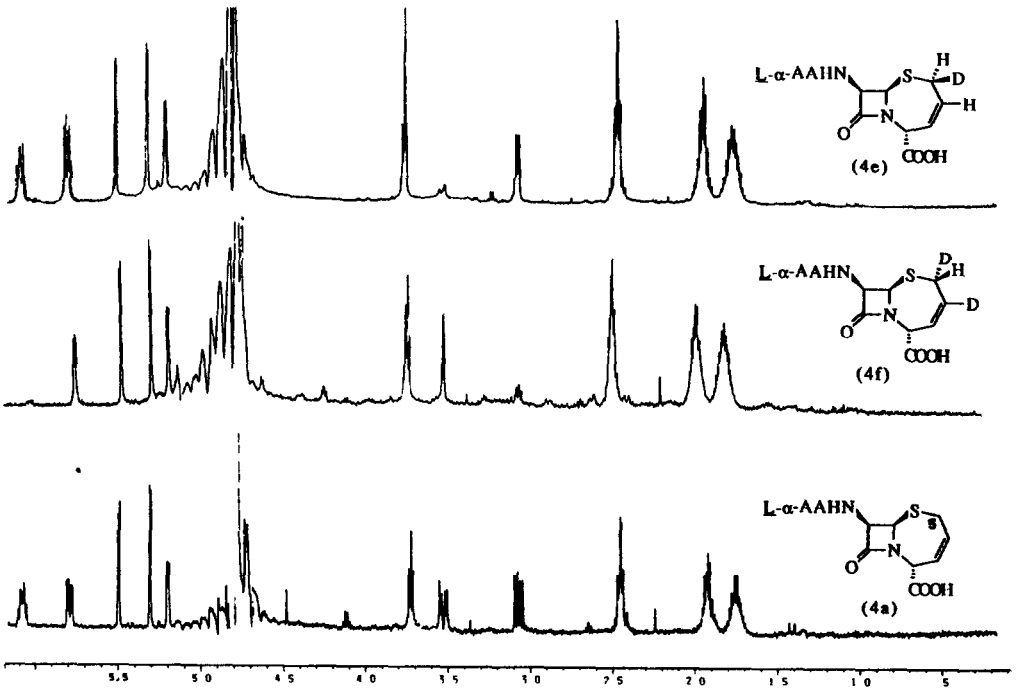
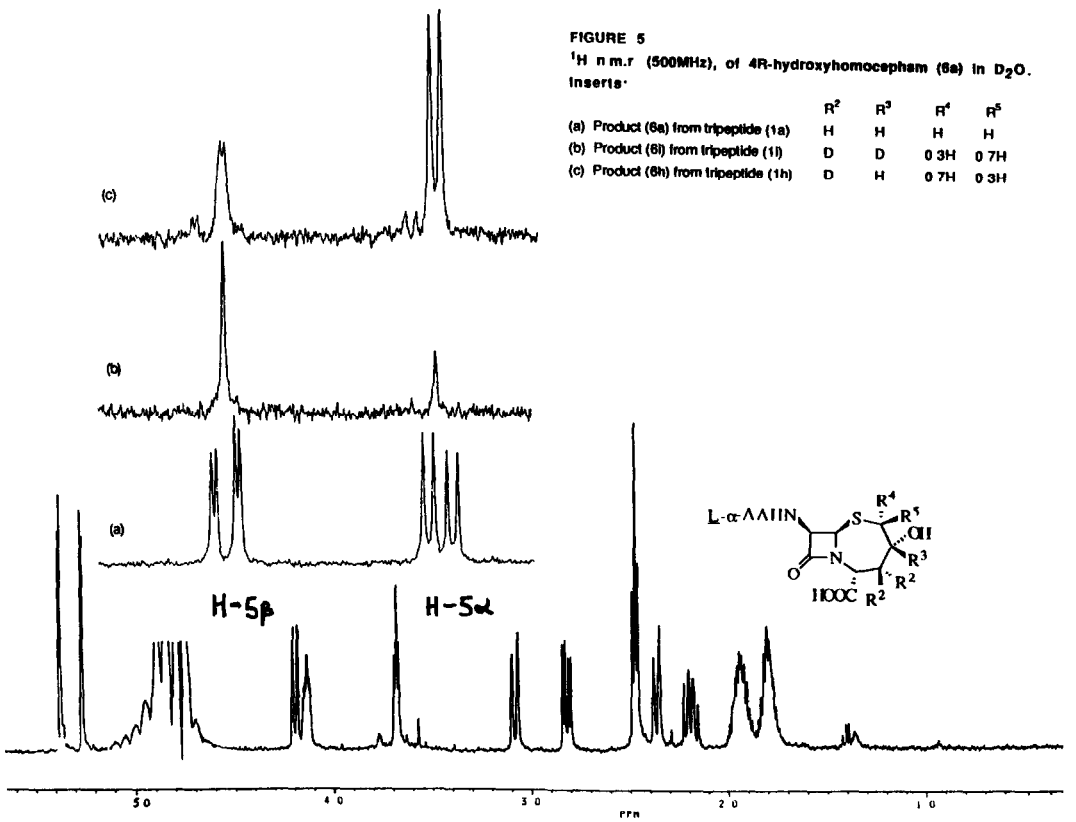


FIGURE 5
^{1}H n m.r (500MHz), of 4R-hydroxyhomocepham (6a) in D_2O. Inserts·

	R^2	R^3	R^4	R^5
(a) Product (6a) from tripeptide (1a)	H	H	H	H
(b) Product (6i) from tripeptide (1i)	D	D	0 3H	0 7H
(c) Product (6h) from tripeptide (1h)	D	H	0 7H	0 3H



oxygenase pathway by a factor of >50 compared to the dehydrogenase pathway.

	Product ratios	
	oxygenated	desaturated
LLD-Allylglycine (1a)	1	2
LLD-AC[3,3-$^2H_2$]Allylglycine (1g)	28	1

This bias allowed greater quantities of the metabolic products from the mono-oxygenase pathway to be isolated than was previously possible, and hence improved the sensitivity of the n.m.r. data we were able to obtain.

The 4R-hydroxyhomocephams (6g), (6h), and (6i), isolated from their respective tripeptides (1g), (1h), and (1i), were purified by reverse phase HPLC from the crude incubation mixtures. This allowed the relative stereochemistry at C-5 to be determined by n.O.e experiments performed on the deuterated compound (6g) (and also from 6a) (δ_H 2.78 H-5α, δ_H 3.05 H-5β). The stereochemical outcome of the cyclizations of (6h) and (6i) was shown to give converse ratios of hydrogen of 7:3 in the two diastereotopic sites at C-5. Thus (6h) gave δ_H 2.74 (0.7H, d, J 7Hz, H-5α), 2.99 (0.3H, d, J 3Hz, H-5β), while (6i) gave δ_H 2.73 (0.3H, s, H-5α), 2.99 (0.7H, s, H-5β), (see figure 5). The converse 7:3 ratio of protium at the two diastereotopic positions at C-5 of the deuterated 4R-hydroxyhomocephams (6h) and (6i), (and also (6e) and (6f)), can be rationalized in the simplest manner in three ways:

(1). The 7:3 ratio reflects partial randomization prior to ring closure.

(2). Two competing parallel processes, one leading to randomization, the other a stereospecific process, prior to stereospecific ring closure.

(3). Two processes, both stereospecific, but giving products of opposite stereochemistries.

The 4S-hydroxymethylcephams (5g, 5h, 5i) were likewise isolated. The stereochemistry of the hydroxymethyl group of the metabolites was assigned as 4S by n.O.e of the metabolite (5g), while the ratio of hydrogen in each of the diastereotopic sites of the hydroxymethyl group from the tripeptides (1h) and (1i) were found to be complementary >10:1 and <1:10 respectively (see figure 6). It has not been possible to determine the absolute stereochemistry at this centre, despite attempts to lactonise or enzymatically oxidise/reduce the hydroxyl group.

Finally incubation of the tripeptide (1g) allowed isolation of the 4S-hydroxyhomocepham (7g). (see experimental for spectroscopic data). The stereochemistry of the protons at C-5 was established by a series of n.O.e experiments. Thus irradiation of the higher field C-5 proton at δ_H 2.87 gave an n.O.e to H-2 (5%), whereas irradiation of the resonance at δ_H 2.97 gave an n.O.e to H-4 (5%) and H-7 (3%). Additionally the β-lactam proton (H-7) gave an n.O.e (2%) to the lower field proton associated with C-5. This suggests the following assignments:

δ_H 2.87 5β-H $\qquad$ δ_H 2.97 5α-H

The two tripeptides (1h) and (1i) likewise gave rise to 4S-hydroxyhomocephams (7h) and (7i). The stereochemistry determined for the diastereotopic hydrogens at C-5 of the metabolite (7g) was utilized to determine the stereochemical pathway for ring closure from the tripeptides (1h) and (1i) upon C-S bond formation. This showed that the ring closure event was stereospecific (>10:1) for each of the metabolites (7h) and (7i), each giving the converse relative stereochemistry at C-5, but giving opposite stereochemical outcomes to the major isomers found in the 4R-hydroxyhomocephams from the same tripeptides. (See figure 7).

Rationalisation of the specificities and stereochemical outcome of the monooxygenase pathway is a more difficult

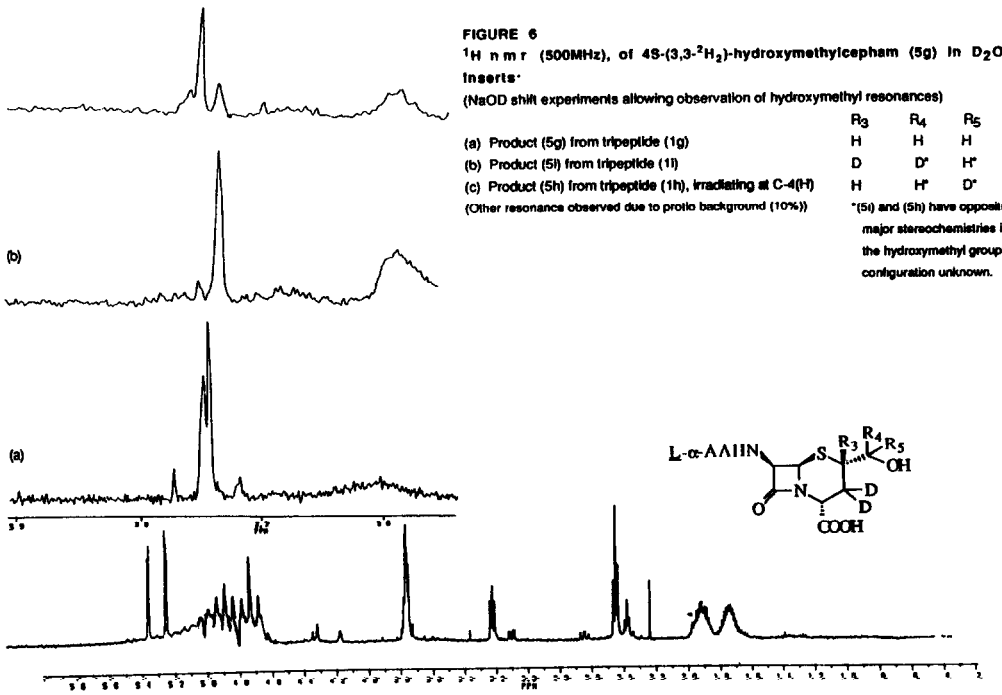


FIGURE 6
^{1}H n m r (500MHz), of 4S-(3,3-$^2H_2$)-hydroxymethylcepham (5g) in D_2O
Inserts*
(NaOD shift experiments allowing observation of hydroxymethyl resonances)

	R_3	R_4	R_5
(a) Product (5g) from tripeptide (1g)	H	H	H
(b) Product (5i) from tripeptide (1i)	D	D*	H*
(c) Product (5h) from tripeptide (1h), irradiating at C-4(H)	H	H*	D*

(Other resonance observed due to protio background (10%))

*(5i) and (5h) have opposite major stereochemistries in the hydroxymethyl group, absolute configuration unknown.

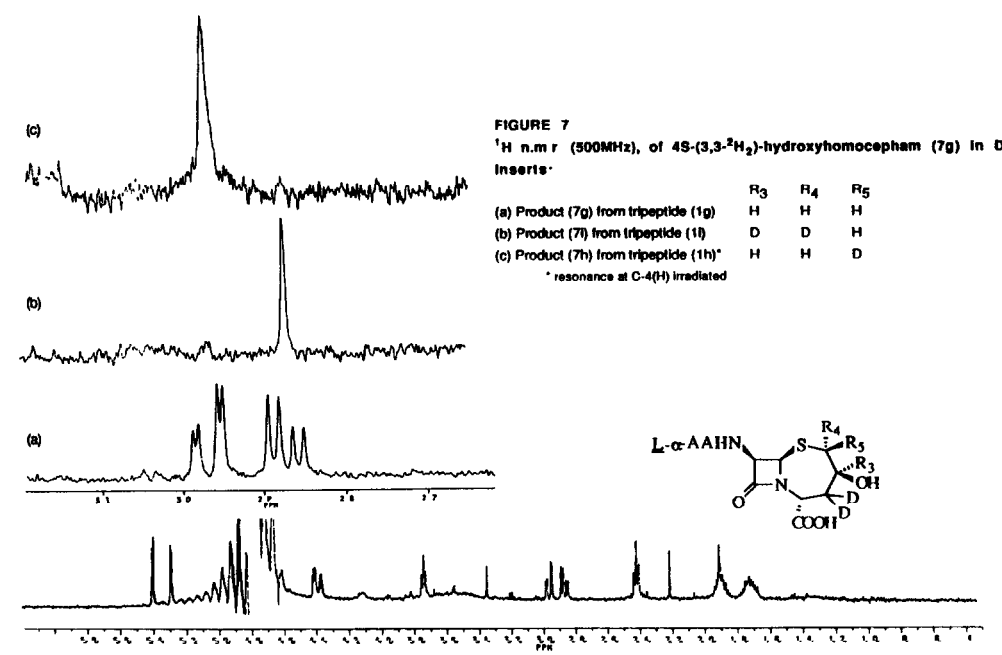


FIGURE 7
^{1}H n.m r (500MHz), of 4S-(3,3-$^2H_2$)-hydroxyhomocepham (7g) in D_2O.
Inserts*

	R_3	R_4	R_5
(a) Product (7g) from tripeptide (1g)	H	H	H
(b) Product (7i) from tripeptide (1i)	D	D	H
(c) Product (7h) from tripeptide (1h)*	H	H	D

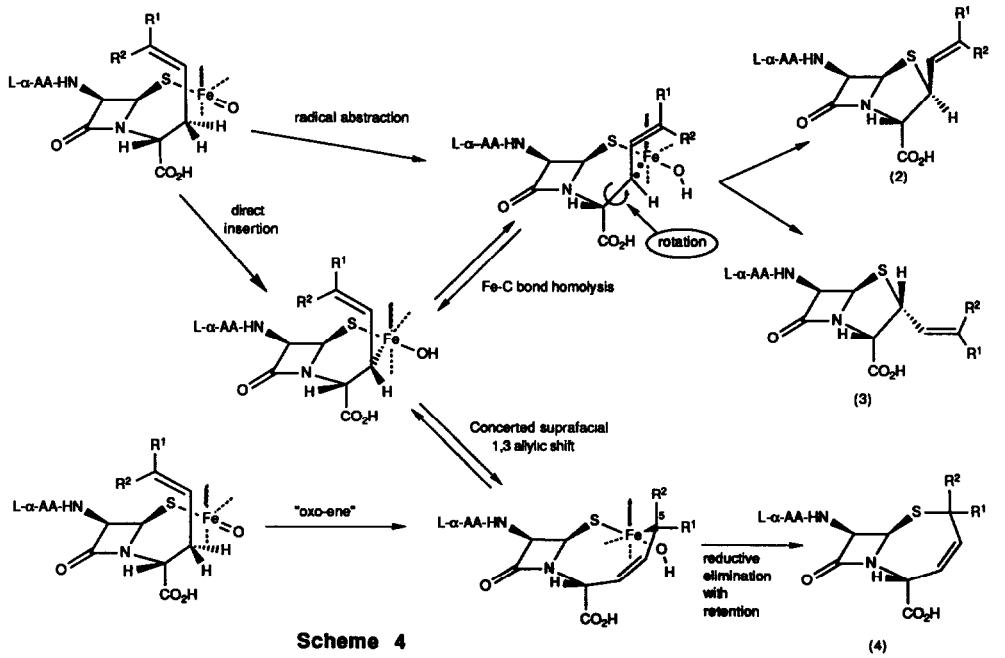
* resonance at C-4(H) irradiated

problem. The major 4R- and the 4S-hydroxyhomocephams arising from the incubation of the tripeptides (1h) and (1i) occur with retention of stereochemistry at C-5 relative to C-4 in the former case and inversion in the latter, implying that they must be derived *via* different mechanisms (fig. 5 and 7). Indeed if they had followed the same pathway we would have perhaps expected the same stereochemical outcome at C-5 for the two products. The stereochemistry of formation of the 4S-hydroxymethylcephams (5h) and (5i) is such that we see predominantly only one diastereoisomer (>10:1 and <1:10) at the hydroxymethyl position (fig. 6). This ratio is the same as that observed for the corresponding 4S-hydroxyhomocephams (7h) and (7i) (fig. 7), and, although it has not been possible to determine the absolute stereochemistry of the hydroxymethyl group, it suggests that they may be derived from a common intermediate, distinct from that which gives rise to the 4R-hydroxyhomocephams (6h) and (6i). However there are other possible interpretations that could be equally valid, and the discussion now presented does not claim to be the only possible one It does however concour with all the facts presented above, and provides a clear rationalization for all the observed products.

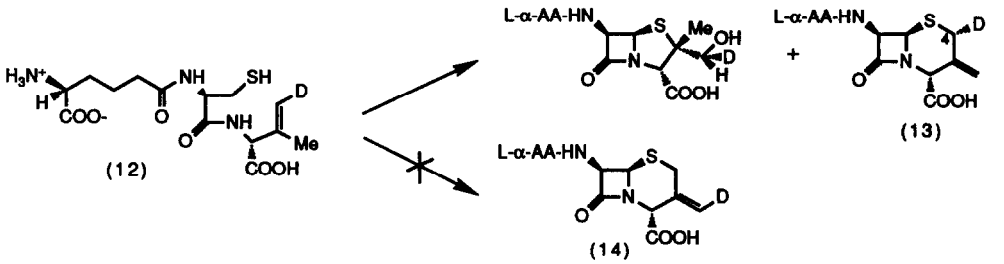
Discussion.

(a) The desaturase pathway.

For the dehydrogenase pathway the mechanism outlined in scheme 4 would seem to offer the best explanation of the products observed. Here the postulated highly reactive iron-oxene is lying on the α-face of the molecule. The β-vinyl penicillins (2) and α-vinyl penicillins (3) can be envisaged as arising from abstraction of a hydrogen from the 3-pro-S position of the allylglycine residue, and from the trapping of the resulting rapidly epimerising carbon radical The homoceph-3-ems (4) could arise from either initial hydrogen abstraction from C-3 of the allyl glycine residue and allylic shift of the radical (thought to be unlikely due to the stereospecificity of the observed homoceph-3-em products), or from a 1,3-suprafacial allylic shift of a 3α-iron-carbon bond, or from a concerted oxy-ene mechanism, similar to that



Scheme 4

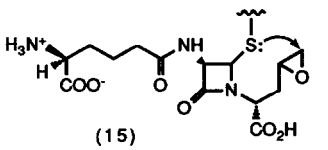


Scheme 5 [L-α-AA = L-α-Aminoadipoyl]

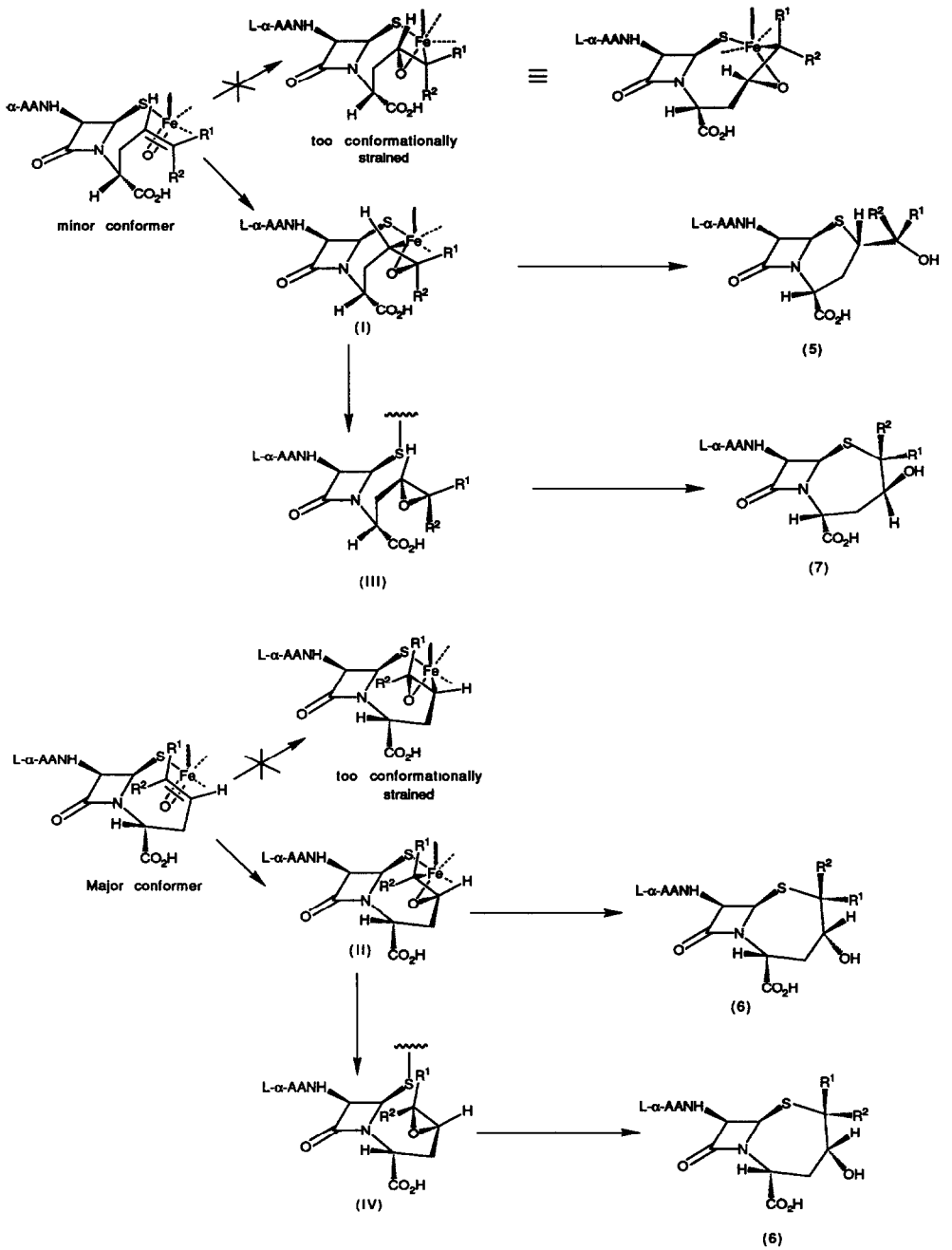
observed for selenium dioxide allylic hydroxylations[17]. The concerted mechanism is favoured considering the product obtained when LLD-AC-(E)-[4-$^2H_1$]-βγ-didehydrovaline (12) was incubated with the IPNS enzyme[18]. The cepham product obtained (13) was regiospecifically and stereospecifically deuterated at the C-4α position (scheme 5). It would be expected that if the cepham product arose from the abstraction of a hydrogen in a non-concerted manner from the methyl group of the LLD-AC-(E)-[4-$^2H_1$]-βγ-didehydrovaline (12) then a mixture of the observed cepham (13) and the cepham with the label situated in the exomethylene group (14), possibly with stereochemical randomisation of the label, would be observed, as both of these products would appear to be precursed by radicals of similar stabilities.

(b) The mono-oxygenase pathway.

For the monooxygenase pathway the mechanism shown in scheme 6 would be in accordance with the results obtained. A $(2\pi+2\pi)$ cycloaddition of the active iron-oxene species to the double bond of the allylglycine from the α-face of the molecule gives, in a similar manner to that postulated for the epoxidation of olefins by cytochrome P_{450}[18], two of the possible four 4-membered oxometallocycles (I and II), the other two being too strained to be formed. Breakdown of the metallocycles (I) and (II) by reductive elimination of the iron with retention would give the observed 4S-hydroxymethyl cephams (5) and the major C-5 diastereomer of the 4R-hydroxyhomocephams (6) respectively The formation of the 4S-hydroxyhomocepham (7) and minor C-5 diastereomer of the 4R-hydroxyhomocepham (6) may be *via* the trapping of an epoxide type intermediate (15), such as (III) and (IV) (as shown below).



Such epoxide intermediates may be formed from the metallocycles (I) and (II) respectively, in a similar fashion to that proposed for cytochrome P_{450}[18] or by a direct epoxidation pathway, the trapping of the epoxide giving only the seven


α-AANH
L-α-AANH
minor conformer
too conformationally strained
Major conformer
(I)
(II)
(III)
(IV)
(5)
(6)
(7)

Scheme 6.

membered rings due to the steric consideratons of nucleophilic attack on the epoxide. Alternatively these results could be explained by the partial homolysis of the weak iron-carbon bond in structure (II), scheme 6, and thus competing randomisation of the label at C-5 prior to ring closure.

Summary.

The above stereochemical results, combined with those we have previously reported on a wide range of substrates[3] provide a rationale for the four basic mechanisms proposed in the introduction of this paper. The results from the nine tripeptides (1a-i) have provided a wealth of complex stereochemical data, allowing a thorough investigation of the second ring closure from the postulated intermediate (10); indeed one can argue that the range of products isolated is evidence in itself for a highly reactive common β-lactam intermediate. The mono-oxygenase pathway may be rationalized in terms of two distinct mechanisms, one of which leads to inversion of the C-5 stereochemistry of the allyl glycine residue of the substrate, while the other leads to retention of the C-5 stereochemistry. We favour a proposal that an epoxide mechanism (to rationalise the inversion events), and a ($2\pi+2\pi$) cycloaddition/reductive elimination (to rationalise the retention events), are in operation for this pathway. The desaturase pathway must also result from two mechanisms (at least) to explain the stereospecificity observed for the substrates reported here, and those reported elsewhere. Thus we propose an "oxo-ene" reaction for the formation of the homocephem metabolites (4), while the penicillins (2) and (3) arise from an allylic radical which is rapidly trapped, allowing epimerization of the C2-C3 bond, but not of the C4-C5 bond.

Experimental.

General Experimental

^{1}H nuclear magnetic resonance spectra were recorded on a Bruker AM 500 (500MHz) and all chemical shifts (δ) are expressed in parts per million (ppm). All solutions were referenced internally to TSP (δ_H 0.00), and were in the pH range 6-7. Samples for n.O.e. experiments were degassed in the solution state under high vacuum (<0.1mmHg). HPLC was performed on two Waters model 510 pumps, Rheodyne 7125 injector (200ml loop), Waters model 441 absorbance detector (λ=214nm) with a 250x4 6 i.d. column packed with Zorbax hypersil ODS, and a pre column. Flow rates were typically 1ml/min and were controlled with a Waters automated gradient controller. All solutions were rigouously degassed and filtered (0.2μm) before use. Mass spectrometry was performed on a V.G. Micromass ZAB-IF in FAB mode (+ve argon fast atom bombardment).

General incubation procedure[7,8].

The IPNS was typically available in 50mM Tris-HCl buffer, and was exchanged into 50mM ammonium bicarbonate before use by passage through a Pharmacia PD-10 column equilibrated with 50mM ammonium bicarbonate.

Incubations were performed by dissolving the tripeptide[13] (approx. 1mg) in 50mM ammonium bicarbonate (3.5ml), followed by the sequential addition of DTT (0.1ml, 100mM), ascorbic acid (0.1ml, 5mM), ferrous sulphate (0.1ml, 5mM), catalase (50μl, 1/10 dilution Sigma standard solution), and the IPNS solution (1.5ml, 5-8 IU). The reaction was adjusted to pH 7.8, divided into two equal portions and shaken at 27°C at 250 rpm., for 1 hour. After 20 and 40 minutes additional DTT(0.1ml, 100mM) and ferrous sulphate (0.1ml, 5mM), was added. On completion the enzyme was precipitated by the addition of acetone to the incubation mixture (to give approx. 70% v/v), and removed by centrifugation (16,000g, 4°C. 10 minutes). The acetone was removed *in vacuo*, the remaining aqueous solution freeze dried, and the residue

examined by ^{1}H n.m.r. for the prescence of characteristic β-lactam resonances. These conditions gave 90–100% conversion of the substrate by ^{1}H n.m.r. The products were purified by reverse phase HPLC (Stationary phase ODS hypersil, mobile phase A=25mM ammonium bicarbonate, B=0.05% formic acid).

(2S,3S,5R,6R)-6-[(5S)-5-amino-5-carboxypentanamido]-3-vinyl-7-oxo-1-aza-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (2a) [3S-3-Vinyl penicillin].

δ_H (500 MHz, D_2O), 1.70 to 1.93 (4H, m, $CH_2CH_2CH_2CO$), 2.43 to 2.45 (2H, m, CH_2CO), 3.74 to 3.76 (1H, m, $CHCH_2$), 4.63 (1H, d, J 3 Hz, H-2), 5.18 (1H, d, J 11 Hz, $CH=CH_2$), 5.36 (1H, d, J 17 Hz, $-CH=CH_2$), 5.45 and 5.47 (2H, ABq, J 4 Hz, H-5 and H-6), 5.88 to 5.95 (1H, m, $CH=CH_2$), (H-3 obscured by HOD). δ_H (500 MHz, D_2O:CD_3CN 1:3, 4-6 ppm) 4.27 (1H, dd, J 3,7 Hz, 3-H), 4.57 (1H, d, J 3Hz, 2-H), 5.10 (1H, d, J 11 Hz, $CH=CH_2$), 5.25 (1H, d, J 17 Hz, $CH=CH_2$), 5.42 and 5.49 (2H, ABq, J 4 Hz, 5-H and 6-H), 5.73 to 5.80 (1H, m, $CH=CH_2$) m/z (+ve Ar FAB), 358 (MH^+). The 3 Hz coupling constant between H-2 and H-3 was used to assign the stereochemistry at C-3 (*cf* 6 Hz for structure 3a)[19]

3S-[3-$^2H_1$]-3-vinyl penicillin (2b).

The deuterium labelling at C-3 assumed from the multiplicity of the signal at δ5.88 to 5.93.

δ_H (500 MHz, D_2O), 1.70 to 1.92 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.42 to 2.45 (2H, m, CH_2CO), 3.72 to 3.76 (1H, m, $CHCH_2$), 5.18 (1H, d, J 11 Hz, $-CH=CH_2$), 5.36 (1H, d, J 17 Hz, $-CH=CH_2$), 5.45 and 5.47 (2H, ABq, J 4 Hz, H-5 and H-6), 5.88 to 5.93 (1H, dd, J 11,17 Hz, $CH=CH_2$), (H-2 obscured by HOD)

3S-[3-$^2H_1$]-3-vinyl penicillin (2c).

δ_H (500 MHz, D_2O) 1.70 to 1 93 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.43 to 2.45 (2H, m, CH_2CO), 3.74-3.78 (1H, m, $CHCH_2$), 5.18 (1H, d, J 11 Hz, $-CH=CH_2$), 5.35 (1H, d, J 17 Hz, $-CH=CH_2$), 5.46 and 5.48 (2H, ABq, J 4Hz, H-5 and H-6), 5.89 (1H, dd, J 10,17 Hz, $CH=CH_2$). δ_H(500 MHz, D_2O:CD_3CN 1:3, 4-6 ppm) 4.63 (1H, s, H-2), 5.03 (1H, d, J 11 Hz, $CH=CH_2$), 5.24 (1H, d, J 17 Hz, $CH=CH_2$), 5.32 and 5.42 (2H, ABq, J 4 Hz, 5-H and 6-H), 5.79 (1H, dd, J 11,17 Hz, $CH=CH_2$). [H-3 essentially absent (<5%)].

3S-(Z-[2'-$^2H_1$]-Vinyl) penicillin (2e).

δ_H (500 MHz, D_2O), 1.70 to 1.93 (4H, m, $CH_2CH_2CH_2CO$), 2.43 to 2.45 (2H, m, CH_2CO), 3.75 to 3.79 (1H, m, $CHCH_2$), 4.63 (1H, d, J 3 Hz, H-2), 5.17 (1H, d, J 11 Hz, $CH=CHD$), 5.45 and 5.47 (2H, ABq, J 4 Hz, H-5 and H-6), 5.89 to 5.92 (1H, m, $CH=CHD$), (H-3 obscured by HOD).

3S-(E-[1',2'-$^2H_2$]-Vinyl) penicillin (2f).

δ_H (500 MHz, D_2O), 1.70 to 1.93 (4H, m, $CH_2CH_2CH_2CO$), 2 43 to 2 45 (2H, m, CH_2CO), 3.74-3.78 (1H, m, $CHCH_2$), 4.63 (1H, d, J 3 Hz, H-2), 5.35 (1H, s, $CD=CHD$), 5.45 and 5.47 (2H, ABq, J 4Hz, H-5 and H-6), 5.89 to 5.92 (1H, m, $CH=CHD$). (H-3 obscured by HOD)

(2S,3R,5R,6R)-6-[(5S)-5-amino-5-carboxypentanamido]-3-vinyl-7-oxo-1-aza-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (3a) [3R-3-Vinyl penicillin].

δ_H (500 MHz, D_2O), 1.71 to 1 93 (4H, m, $CH_2CH_2CH_2CO$), 2.42 to 2.44 (2H, m, CH_2CO), 3.75 to 3.77 (1H, m, $CHCH_2$), 5.20 (1H, d, J 11 Hz, $-CH=CH_2$), 5.34 (1H, d, J 17 Hz, $-CH=CH_2$), 5.47 and 5.58 (2H, ABq, J 4Hz, H-5 and H-6), 5 75 to 5.81 (1H, m, $CH=CH_2$), (H-2 and H-3 obscured by HOD). δ_H (500 MHz, D_2O:CD_3CN 1:3, 4-6 ppm) 4.38 (1H, d, J 6 Hz, H-2), 4 62 (1H, dd, J 6, 9Hz, H-3), 5.03 (1H, d, J 11 Hz, $CH=CH_2$), 5.24 (1H, d, J 17 Hz, $CH=CH_2$), 5.32 and 5.42 (2H, ABq,

J 4 Hz, 5-H and 6-H), 5.77 to 5.83 (1H, m, CH=CH_2).m/z (+ve Ar FAB), 358 (MH^+). Stereochemistry at C-3 assigned by J=6 Hz for 2-H to 3-H (see structure 2a)[19].

3R-[3'-$^2H_1$]-3-vinyl penicillin (3c).

The deuterium labelling at C-3 assumed from the multiplicity of the signal at δ5.80.

δ_H (500 MHz, D_2O), 1.70 to 1 92 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.42 to 2.44 (2H, m, CH_2CO), 3.72 to 3.76 (1H, m, $CHCH_2$), 5.20 (1H, d, J 11 Hz, CH=CH_2), 5.36 (1H, d, J 17Hz, CH=CH_2), 5.45 and 5.57 (2H, ABq, J 4Hz, H-5 and H-6), 5.80 (1H, dd, J 11,17Hz, CH=CH_2), (H-2 obscured by HOD).

3R-(Z-[2'-$^2H_1$]-vinyl) penicillin (3e).

δ_H (500 MHz, D_2O), 1.70 to 1.93 (4H, m, $CH_2CH_2CH_2CO$), 2.42 to 2.44 (2H, m, CH_2CO), 3.74 to 3.76 (1H, m, $CHCH_2$), 5.20 (1H, d, J 11 Hz, CH=CHD), 5.47 and 5.58 (2H, ABq, J 4 Hz, H-5 and H-6), 5.77 to 5.80 (1H, m, CH=CHD), (H-2 and H-3 obscured by HOD).

3R-(E-[1',2'-$^2H_2$]-vinyl) penicillin (3f).

δ_H (500 MHz, D_2O), 1.70 to 1 93 (4H, m, $CH_2CH_2CH_2CO$), 2 42 to 2.44 (2H, m, CH_2CO), 3.74 to 3.78 (1H, m, $CHCH_2$), 5.32 (1H, br s, CD=CHD), 5.47 and 5.58 (2H, ABq, J 4 Hz, H-5 and H-6), (H-2 and H-3 obscured by HOD).

(2R,7R,8R)-8-[(5S)-5-amino-5-carboxypentanamido]-9-oxo-1-aza-6-thiabicyclo[5.2.0]non-3-ene-2-carboxylic acid (4a) [Homoceph-3-em].

δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.43 to 2.47 (2H, m, CH_2CO), 3.06 (1H, dd, J 8,16 Hz, H-5α), 3.53 (1H, dd, J 6,16 Hz, H-5β), 3.73 to 3.76 (1H, m, $CHCH_2$), 5.20 (1H, br d, J 5 Hz, H-2), 5.30 and 5.48 (2H, ABq, J 4Hz, H-7 and H-8), 5 78 (1H, dd, J 5,12 Hz, H-3), 6.04 to 6.10 (1H, m, H-4). Irradiation of the resonance associated with the proton at δ_H 3.06 gave an n.O.e. to H-7 (3%) to H-4 (8%) and a large geminal enhancement to H-5 (29%), while irradiation of the resonance associated with the lower field H-5 at δ_H 3.53 gave enhancements to H-2 (1%), H-4 (5%) and H-5 (25%). A ^{1}H-COSY nmr spectra established the connectivity S-CH_2-CH=CH-CH(COOH) m/z (+ve Ar FAB), 358 (MH^+).

[3-$^2H_1$]-Homoceph-3-em (4b).

δ_H (500 MHz, D_2O), 1.70 to 1.98 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.45 to 2.48 (2H, m, CH_2CO), 3.07 (1H, dd, J 8,16 Hz, 5α-H), 3.53 (1H, dd, J 6,16 Hz,5β-H), 3.69 to 3.73 (1H, m, $CHCH_2$), 5 23 (1H, br s, H-2), 5.29 and 5.49 (2H, ABq, J 4Hz, H-7 and H-8), 6 05 to 6.10 (1H, br m, H-4), [signal at δ_H 5.78 for H-3 <2%], m/z (+ve Ar FAB), 359 (MH^+).

[3-$^2H_1$]-Homoceph-3-em (4c).

δ_H (500 MHz, D_2O), 1.68 to 1.98 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.43 to 2.48 (2H, m, CH_2CO), 3.05 (1H, dd, J 8,16 Hz, 5α-H), 3.55 (1H, dd, J 6,16 Hz, 5β-H), 3.73 to 3.76 (1H, m, $CHCH_2$), 5.21 (1H, br s, H-2), 5.28 and 5.48 (2H, ABq, J 4 Hz, H-7 and H-8), 6.05 to 6.10 (1H, br m, H-4).

5S-[5-$^2H_1$]-Homoceph-3-em (4e).

δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.44 to 2.47 (2H, m, CH_2CO), 3.04 (1H, d, J 8Hz, 5α-H) (5α-H.5β-H > 10.1), 3.75 to 3.78 (1H, m, $CHCH_2$), 5.22 (1H, br d, J 5 Hz, H-2), 5.30 and 5.48 (2H, ABq, J 4 Hz, H-7 and H-8), 5.78 (1H, dd, J 5,12 Hz, H-3),6.05 to 6.09 (1H, m, H-4). m/z (+ve Ar FAB), 359 (MH^+).

5R-[4,5-$^2H_2$]-Homoceph-3-em (4f).

δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.44-2.48 (2H, m, CH_2CO), 3.52 (1H, br s, 5β-H) (5β-H:5α-H>10.1), 3 75 to 3.78 (1H, m, $CHCH_2$), 5.22 (1H, br d, J 5 Hz, H-2), 5 28 and 5.48 (2H, ABq, J 4 Hz, H-7 and H-8), 5.76 to

5.79 (1H, m, H-3).

(2R,4S,6R,7R)-7-[(5S)-5-amino-5-carboxypentanamido]-4-hydroxymethyl-8-oxo-1-aza-5-thiabicyclo[4.2.0]octane-2-carboxylic acid (5a) [4S-Hydroxymethylcepham].

δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.14 to 2.22 (1H, m, H-3α), 2.35 to 2.40 (1H, m, H-3β), 2.43 to 2.46 (2H, m, CH_2CO), 3.21 to 3.26 (1H, m, H-4), 3.73 to 3.76 (3H, m, $CHCH_2$ and CH_2OH), 4.36 to 4.38 (1H, m, H-2), 5.24 and 5.34 (2H, ABq, J 4Hz, H-6 and H-7). Irradiation of the resonance at δ2.17 gave an n.O.e. to δ2.38 (12%) (C-3 geminal proton), δ3.23 (4%) (H-4), δ4.35 (3%) (H-2), and δ5.26 (6%) (6-H). Irradiation at δ2.38 gave an n.O.e to δ2.17 (11%), (C-3 geminal proton), δ3.23 (8%) (H-4), and δ4.35 (10%) (H-2) but not to H-6. In order to cleanly irradiate the hydroxmethyl protons, the solution was adjusted to pH11 (NaOD) to shift the signal due to the α-aminoadipoyl α-proton which lies under this signal at pH 7. The chemical shifts of the other peaks of interest were not significantly affected. Irradiation at δ3.75 gave an n.O.e. to δ3.23 (12%) (H-4) , and δ5.26 (10%) (H-6). A ^{1}H-COSY nmr spectra established the connectivity S-CH(CH_2OH)-CH_2-CH(COOH).m/z (+ve Ar FAB), 376 (MH^+).

(3RS,4S)-[3-$^2H_1$]-4-Hydroxymethylcepham (5b).

δ_H (500 MHz, D_2O), 1.68 to 1.95 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.13 to 2.16 (0.3H, m, H-3α), 2.30 to 2.36 (0.7H, m, H-3β), 2.45 to 2.49 (2H, m, CH_2CO), 3.21 to 3.26 (1H, m, H-4), 3.73 to 3.76 (3H, m, $CHCH_2$ and CH_2OH), 4.35 to 4.39 (1H, m, H-2), 5.24 and 5.34 (2H, ABq, J 4 Hz, H-6 and H-7).

3S,4S-[3-$^2H_1$]-4-Hydroxymethylcepham (5c).

δ_H (500 MHz, D_2O), 1.70 to 1 96 (4H, m, $CH_2CH_2CH_2CO$), 2.13 (0.8H, d, J 7 Hz, H-3α), 2.36 (0.2H, br s, H-3β), 2.42 to 2.46 (2H, m, CH_2CO), 3 22 to 3.27 (1H, m, H-4), 3.70 to 3.75 (3H, m, $CHCH_2$ and CH_2OH), 4.35 (1H, d, J 7 Hz, H-2), 5.24 and 5 34 (2H, ABq, J 4 Hz, H-6 and H-7) (Deuteration levels at C(3) reflects stereochemical impurity in tripeptide).

3R,4S-[3,4-$^2H_2$]-4-Hydroxymethylcepham (5d).

δ_H (500 MHz, D_2O), 1.69 to 1.95 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.13 (1H, t, J 7 Hz, H-3α), 2.42 to 2.46 (2H, m, CH_2CO), 3.70 to 3.75 (3H, m, $CHCH_2$ and CH_2OH), 4.35 (1H, d, J 7 Hz, H-2), 5 23 and 5.33 (2H, ABq, J 4 Hz, H-6 and H-7). (H-3β essentially absent ≤5%)

4S-4-(Hydroxy-[$^2H_1$]-methyl)cepham (5e).

δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.14 to 2.22 and 2.35 to 2.40 (2H, 2xm, H-3), 2.43 to 2.46 (2H, m, CH_2CO), 3.22 to 3 25 (1H, m, H-4), 3.72 to 3.74 (1H, m, $CHCH_2$), 3.74 (1H, d, J 6 Hz, CHDOH), 4.34 to 4.37 (1H, m, H-2), 5.24 and 5.34 (2H, ABq, J 4 Hz, H-6 and H-7).

4S-[4-$^2H_1$]-4-(Hydroxy-[$^2H_1$]-methyl)cepham (5f).

δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.15 to 2.21 and 2.35 to 2.40 (2H, 2xm, H-3), 2.43 to 2.46 (2H, m, CH_2CO), 3 73 to 3.75 (1H, m, $CHCH_2$), 3.75 (1H, d, J 7 Hz, CHDOH), 4.34 to 4.38 (1H, m, H-2), 5.24 and 5.34 (2H, ABq, J 4 Hz, H-6 and H-7).

4S-[3,3-$^2H_2$]-4-Hydroxymethylcepham (5g).

δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.43 to 2.45 (2H, m, CH_2CO), 3.20 (1H, t, J 7Hz, H-4), 3.73 to 3.76 (1H, m, $CHCH_2$ and 2H, AB of ABX, CH_2OH) (addition of NaOD shifted the resonance due to the $CHCH_2$ proton to higher field so that it was possible to clearly observe the resonances of the CH_2OH group), 4.35 (1H, br s, H-2), 5.24 and 5 35 (2H, ABq, J 4 Hz, H-6 and H-7)

4S-[3,3-$^2H_2$]-(Hydroxy-[$^2H_1$]-methyl)cepham (5h).
δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.36 to 2.45 (2H, m, CH_2CO), 3.20 (1H, d, J 7 Hz, H-4), 3.73 to 3.77 (2H, m, $CHCH_2$ and CHDOH) (addition of NaOD shifted the resonance due to the $CHCH_2$ proton to higher field so that it was possible to clearly observe the resonances of the CHDOH group, d, J 7 Hz), 4.35 (1H, s, H-2), 5.24 and 5.35 (2H, ABq, J 4 Hz, H-6 and H-7).

4S-[3,3,4-$^2H_3$]-4-(Hydroxy-[$^2H_1$]-methyl)cepham (5i).
δ_H (500 MHz, D_2O), 1.71 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.36 to 2.45 (2H, m, CH_2CO), 3.72-3.76 (2H, m, $CHCH_2$ and CHDOH) (addition of NaOD shifted the resonance due to the $CHCH_2$ proton to higher field so that it was possible to clearly observe the resonance of the CHDOH group), 4.35 (1H, s, H-2), 5.24 and 5.35 (2H, ABq, J= 4 Hz, H-6 and H-7).

(2R,4R,7R,8R)-8-[(5S)-5-amino-5-carboxypentanamido]-4-hydroxy-9-oxo-1-aza-6-thiabicyclo[5.2.0]nonane-2-carboxylic acid (6a) (4R-Hydroxyhomocepham.)
δ_H (500 MHz, D_2O), 1.70 to 1.97 (4H, m, $CH_2CH_2CH_2CO$), 2.09 to 2.17 (1H, m, H-3α), 2.30 to 2.34 (1H, m, H-3β), 2.42 to 2.45 (2H, m, CH_2CO), 2.76 to 2.80 (1H, m, H-5α), 3.03 to 3.06 (1H, m, H-5β), 3.73 to 3.76 (1H, m, Cα-H of α-AA), 4.09 to 4.14 (1H, m, H-4), 4.18 (1H, dd, J 3,13 Hz, H-2), 5.26 and 5.38 (2H, ABq, J 4 Hz, 7-H and 8-H). Irradiation of the resonance at δ_H 2.13 gave an n.O.e. to H-7 (5%) and to H-4 (3%), whereas irradiation of the resonance at δ_H 2.32 gave an enhancement to H-4 (8%), and H-2 (3%). Additionally irradiation of the resonance at δ_H 2.78 gave an n.O.e. to H-7 (8%), whereas irradiation of the resonance at δ_H 3.05 gave an enhancement to H-2 (14%), and H-4 (15%) (large geminal n.O.e's were observed (20-25%) in all of these experiments). A ^{1}H-COSY nmr spectra established the connectivity S-CH_2-CH(OH)-CH_2-CH(COOH). m/z (+ve Ar FAB), 376 (MH^+).

3RS,4R-[3-$^2H_1$]-4-Hydroxyhomocepham (6b).
δ_H (500 MHz, D_2O) 1.67 to 1.94 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.10 to 2.12 (0.3H, dd, J 10,11 Hz, H-3α), 2.30 to 2.33 (0.7H, m, H-3β), 2.40 to 2.45 (2H, m, CH_2CO), 2.76 to 2.80 (1H, m, H-5α), 3.02 to 3.06 (1H, m, H-5β), 3.73 to 3.76 (1H, m, Cα-H of α-AA), 4.10 to 4.13 (1H, m, H-4), 4.17 to 4.18 (1H, m, H-2), 5.27 and 5.38 (2H, ABq, J 4 Hz, 7-H and 8-H). m/z (+ve Ar FAB), 377 (MH^+).

3S,4R-[3-$^2H_1$]-4-Hydroxyhomocepham (6c).
δ_H (500 MHz, D_2O)1.70 to 1.95 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.10 to 2.13 (0.8H, br m, H-3α), 2.29 to 2.32 (0.2H, br m, H-3β), 2.40 to 2.44 (2H, m, CH_2CO), 2.77 (1H, dd, J 6,15 Hz, H-5α), 3.05 (1H, dd, J 3,15 Hz, H-5β), 3.73 to 3.76 (1H, m, Cα-H of α-AA), 4.13 (1H, dd, J 3,6 Hz, H-4), 4.17 (1H, br s, H-2), 5.28 and 5.40 (2H, ABq, J 4 Hz, 7-H and 8-H) m/z (+ve Ar FAB), 378 (MH^+).

3R,4R-[3,4-$^2H_2$]-4-Hydroxyhomocepham (6d)
δ_H (500 MHz, D_2O) 1.72 to 1.96 (4H, 2xm, $CH_2CH_2CH_2CO$), 2.29 (1H, br s, H-3β), 2.43 to 2.47 (2H, m, CH_2CO), 2.78 and 3.04 (2H, ABq, J 15 Hz, 5-H_2), 3.73 to 3.77 (1H, m, Cα-H of α-AA), 4.18 (1H, br s, H-2), 5 27 and 5.38 (2H, ABq, J 4 Hz,7-H and 8-H). [Signals at δ2.13 (H-3α), and δ4.13 (H-4) essentially absent ≤5%].

4R-[5-$^2H_1$]-4-Hydroxyhomocepham (6e).
δ_H (500 MHz, D_2O), 1.70 to 1.97 (4H, m, $CH_2CH_2CH_2CO$), 2.09 to 2.34 (2H, 2xm, H-3), 2.42 to 2.46 (2H, m, CH_2CO), 2.77 (0.7H, d, J 7 Hz, H-5α), 3.03 (0.3H, d, J 3 Hz, H-5β), 3.75 to 3.78 (1H, m, Cα-H of α-AA), 4.11 to 4.14 (1H, m, H-4), 4 18 (1H, dd, J 3,13 Hz, H-2), 5.26 and 5 38 (2H, ABq, J 4 Hz, 7-H and 8-H). m/z (+ve Ar FAB), 377 (MH^+).

4R-[4,5-$^2H_2$]-4-Hydroxyhomocepham (6f).
δ_H (500 MHz, D_2O), 1.70 to 1.97 (4H, m, $CH_2CH_2CH_2CO$), 2.09 to 2.34 (2H, 2xm, H-3), 2.42 to 2.47 (2H, m, CH_2CO), 2 76 (0.3H, br s, H-5α), 3.03 (0.7H, br s, H-5β), 3.74 to 3.76 (1H, m, C_α-H of α-AA), 4.18 (1H, dd, J 3,13 Hz, H-2), 5.26 and 5.38 (2H, ABq, J 4 Hz, 7-H and 8-H). m/z (+ve Ar FAB), 378 (MH^+).

4R-[3,3-$^2H_2$]-4-Hydroxyhomocepham (6g).
δ_H (500 MHz, D_2O), 1.70 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.41 to 2 44 (2H, m, CH_2CO), 2 77 (1H, dd, J 7,15 Hz, H-5α), 3.03 (1H, dd, J 3,15 Hz, H-5β), 3.71 to 3.76 (1H, m, C_α-H of α-AA), 4.10 (1H, dd, J 3,7 Hz, H-4), 4.16 (1H, s, H-2), 5.25 and 5.36 (2H, ABq, J 4 Hz, 7-H and 8-H). m/z (+ve Ar FAB), 378(MH^+).

4R-[3,3,5-$^2H_3$]-4-Hydroxyhomocepham (6h).
δ_H (500 MHz, D_2O), 1.70 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.41 to 2.44 (2H, m, CH_2CO), 2.74 (0.7H, d, J 7 Hz, H-5α), 2 99 (0.3H, d, J 3 Hz, H-5β), 3.71 to 3.76 (1H, m, C_α-H of α-AA), 4.10 (1H, 2xd, J 3,7 Hz, H-4), 4.16 (1H, s, H-2), 5.25 and 5 36 (2H, ABq, J 4 Hz, 7-H and 8-H). m/z (+ve Ar FAB), 379(MH^+).

4R-[3,3,4,5-$^2H_4$]-4-Hydroxyhomocepham (6i).
δ_H (500 MHz, D_2O), 1 70 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.38 to 2.43 (2H, m, CH_2CO), 2.73 (0.3H, s, H-5α), 2.99 (0.7H, s, H-5β), 3.72 to 3.76 (1H, m, C_α-H of α-AA), 4.15 (1H, s, H-2), 5 24 and 5.35 (2H, ABq, J 4 Hz, 7-H and 8-H). m/z (+ve Ar FAB), 380 (MH^+).

(2R,4S,7R,8R)-[3,3-$^2H_2$]-8-[(5S)-5-amino-5-carboxypentanamido]-4-hydroxy-9-oxo-1-aza-6-thiabicyclo[5.2.0]nonane-2-carboxylic acid [3,3-$^2H_2$]-(4S)-4-Hydroxyhomocepham (7g).
δ_H (500 MHz, D_2O), 1.70 to 1 95 (4H, m, $CH_2CH_2CH_2CO$), 2.40 to 2 44 (2H, m, CH_2CO), 2.87 (1H, dd, J 7,15 Hz, H-5α), and 2 97 (1H, dd, J 3,15 Hz, H-5β), 3.73 (1H, t, J 7 Hz, C_α-H of α-AA), 4.37 (1H, s, H-2), 4.40 (1H, dd, J 3,7 Hz, H-4), 5.28 and 5.40 (2H, ABq, J 4 Hz, 7-H and 8-H). Irradiation of the resonance associated with δ_H 2.87 gave an enhancement to H-2 (5%), while irradiation of the resonance at δ_H 2.97 gave a n.O.e to H-4 (5%) and H-7 (3%).Additionally, irradiation of the β-lactam protons (H-7 and H-8) gave an n.O.e (2%) to δ_H 2.97 (H-5α). m/z (+ve Ar FAB), 378 (MH^+).

4S-[3,3,5-$^2H_3$]-4-Hydroxyhomocepham (7h).
δ_H (500 MHz, D_2O), 1 70 to 1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.41 to 2.43 (2H, m, CH_2CO), 2.97 (1H, d, J 3 Hz, H-5α), 3.73 to 3.75 (1H, t, J 7 Hz, C_α-H of α-AA), 4.37 (1H, s, H-2), 4.40 (1H, d, J 3 Hz, H-4), 5.28 and 5.40 (2H, ABq, J 4 Hz,7-H and 8-H). m/z (+ve Ar FAB), 379 (MH^+).

4S-[3,3,4,5-$^2H_4$]-4-Hydroxyhomocepham (7i).
δ_H (500 MHz, D_2O), 1.68 to 1.91 (4H, m, $CH_2CH_2CH_2CO$), 2.41 to 2.43 (2H, m, CH_2CO), 2 87 (1H, s, H-5β), 3.72 to 3 75 (1H, t, J 7 Hz, C_α-H of α-AA), 4.38 (1H, s, H-2), 5.28 and 5.40 (2H, ABq, J 4 Hz, 7-H and 8-H). m/z (+ve Ar FAB), 380 (MH^+)

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