Evidence for Epoxide Formation from Isopenicillin *N* Synthase

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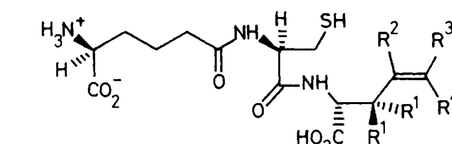
Isolation of a new β-lactam-containing metabolite, from incubation of the three deuteriated tripeptides (**1b**—**d**) with isopenicillin N synthase (IPNS) has provided evidence of an epoxide type intermediate; a unified mechanism is proposed for the formation of products by IPNS from unsaturated precursors.

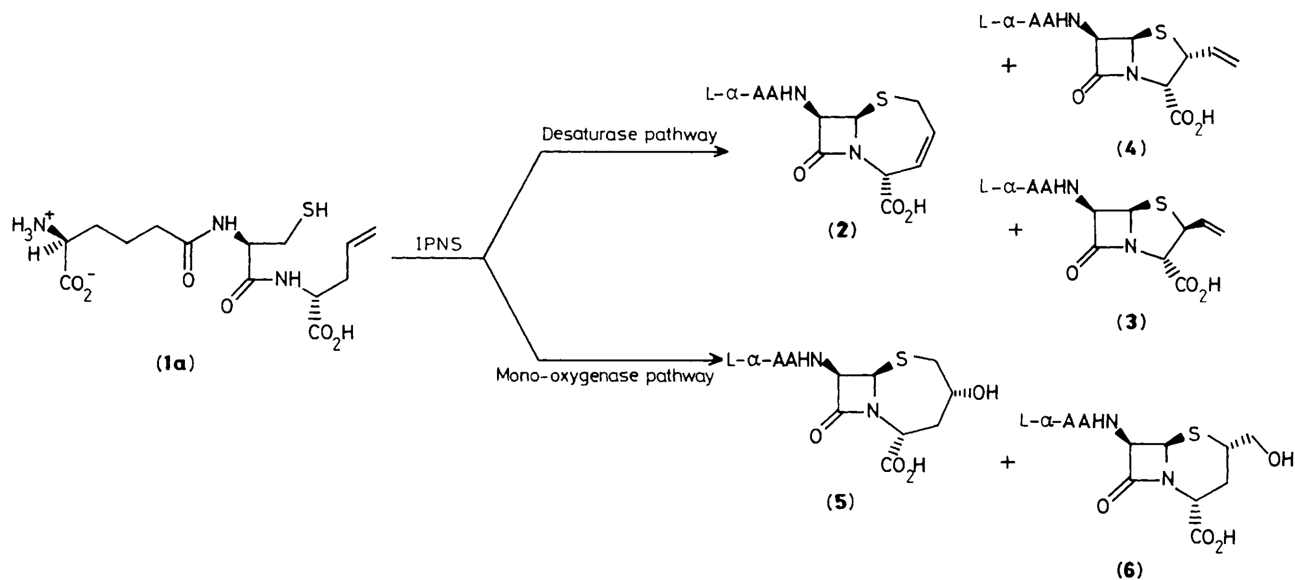
The conversion of the modified natural substrate of isopenicillin N synthase (IPNS), δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine (LLD-ACAg) (**1a**), to 5 distinct β-lactam containing products (**2**)—(**6**), has been reported.[1] This conversion occurs *via* two pathways differing in stoicheiometry (Scheme 1); (i) mono-oxygenase (loss of 2 hydrogens, gain of one oxygen); (ii) desaturase (loss of 4 hydrogens). The oxygen source for the mono-oxygenase products (**5** and **6**) has been shown to be molecular oxygen.[2]

We now report the isolation and characterisation of a sixth β-lactam-containing metabolite (**7**), observed initially in the incubation of the three specifically deuteriated tripeptide analogues of LLD-ACAg (**1b**), (**1c**), and (**1d**)† with IPNS. The deuteriation patterns of these tripeptides has permitted the stereochemistry of carbon–sulphur bond formation to be thoroughly investigated. The ratios of metabolites obtained from incubation of (**1a**) and (**1b**) with IPNS have shown the

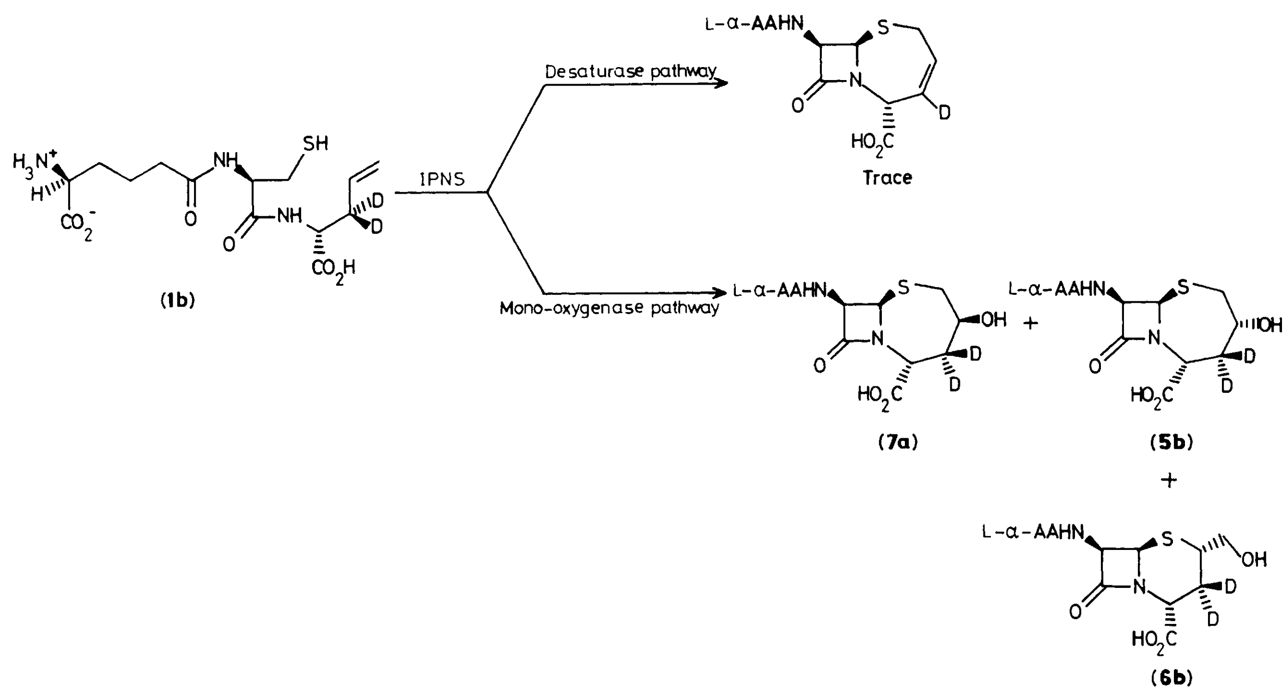
† Full details of the synthesis of these tripeptides will appear elsewhere.



	R^1	R^2	R^3	R^4
(**1a**)	H	H	H	H
(**1b**)	D	H	H	H
(**1c**)	D	D	D	H
(**1d**)	D	H	H	D



Scheme 1. (L-α-AA = L-α-aminoadipoyl) Ratio (**2**) : (**3**) : (**4**) : (**5**) : (**6**) 10 : 4 : 1 : 5 : 2



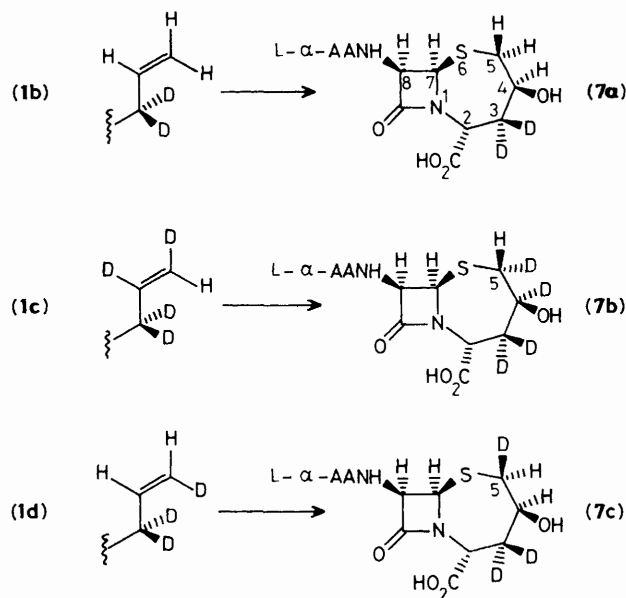
Scheme 2. Ratio (**7a**) : (**5b**) : (**6b**) 1 : 20 : 8

sensitivity of IPNS to primary kinetic isotope effects, the mono-oxygenase pathway operating almost exclusively with tripeptide (**1b**) (see Table 1 and Scheme 2). It was the utilisation of this substantial isotope effect to bias the pathway that allowed the isolation‡ of sufficient quantities [~100 μg

‡ *Reverse phase h.p.l.c.* Stationary phase: ODS hypersil C_{18}. Mobile phase: (i) primary purification 25 mM aqueous ammonium hydrogencarbonate; (ii) subsequent purification, 0.05% aqueous formic acid.

Table 1. Product ratio from incubation of (**1a**) and (**1b**) with IPNS.

	Product ratio		
	Mono-oxygenase		Dehydrogenase
LLD-ACAg (**1a**)	1	:	2
LLD-AC[3,3-$^2H_2$]Ag (**1b**)	>28	:	1



Scheme 3

Table 2. Mechanisms for second ring closure.

Mechanistic type	Product[a]
1: Hydrogen atom abstraction/recombination[4]	(3), (4)
2: Oxo-ene reaction[3]	(2)
3: $[2\pi + 2\pi]$ cycloaddition/reductive elimination[3]	(5), (6)
4: Epoxide formation/inverting displacement	(7)

[a] Full details of the stereochemical course to products (2)—(6) from (1a—d) will appear elsewhere.

from 5 mg of the tripeptides (**1b**)–(**1d**)] and hence characterisation of the new metabolite (**7a,b,c**), by ^{1}H n.m.r. and mass spectrometry§ (see Scheme 3). These products were absent in a denatured enzyme control and do not arise from epimerisation of the 4α-hydroxyhomocepham.

The stereochemistry at C-5 of the labelled 4β-hydroxyhomocepham metabolites (**7b**) and (**7c**) was established by comparison of their ^{1}H n.m.r. spectra with that of the 4β-hydroxyhomocepham (**7a**), obtained from the incubation of tripeptide (**1b**) (see Figure 1), while the 5α-H and 5β-H ^{1}H n.m.r. resonances in (**7a**) were determined by nuclear Overhauser enhancement (n.O.e.) experiments.§ The stereochemistry of the deuterium label at C-5 in the metabolites (**7b**) and (**7c**) shows clearly that these products arise with effective inversion of the stereochemistry at C-5 relative to C-4, and hence we propose that they arise *via* an 'epoxide type' intermediate or equivalent (see Scheme 4), which has not previously been proposed for IPNS. This stereochemical result is in direct contrast to that observed in the formation of the hydroxymethylpenam metabolite (**9**) from the incubation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-isodehydrovaline (**8**),[3] which was ascribed to a $[2\pi + 2\pi]$ cycloaddition reaction

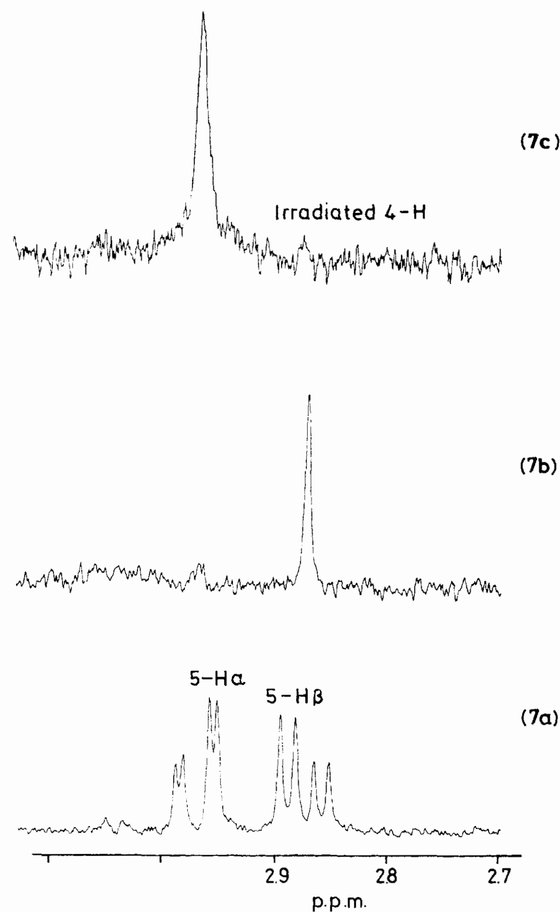
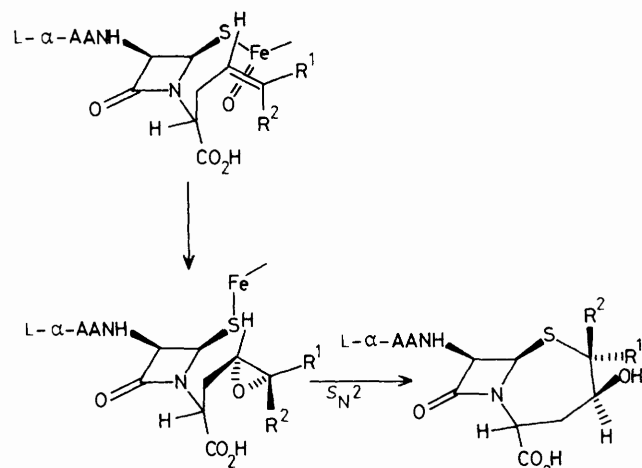
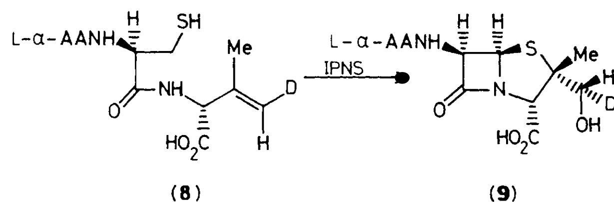


Figure 1. ^{1}H N.m.r. spectra of (**7a**), (**7b**), and (**7c**) (irradiated at 4-H).



Scheme 4

§ *Data for metabolites.* (**7a**) ^{1}H n.m.r. δ_H (500 MHz, D_2O, sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$]propionate, TSP = 0.00) 5.40 and 5.28 (2H, ABq, *J* 4 Hz, H-7 and H-8), 4.40 (1H, dd, *J* 3,7 Hz, H-4), 4.37 (1H, s, H-2), 3.73 (1H, t, *J* 7 Hz, $CHCH_2$), 2.97 (1H, dd, *J* 3,15 Hz, H-5), 2.87 (1H, dd, *J* 7, 15 Hz, H-5), 2.44—2.40 (2H, m, CH_2CO), 1.95—1.70 (4H, m, $CH_2CH_2CH_2CO$); *m/z* (+ve argon fast atom bombardment, f.a.b.) 378 (MH^+). Irradiation of the proton at δ_H 2.97 gave a n.O.e. to H-4 (5%) and to H-7 (3%), whereas irradiation of the proton at δ_H 2.87 gave a n.O.e. to H-2 (5%). Additionally, irradiation of the β-lactam protons (H-7 and H-8) gave a n.O.e. to the proton at δ_H 2.97. Thus the assignments were made as 5α-H at δ_H 2.97 and 5β-H at δ_H 2.87. (**7b**) Data as for (**7a**) except H-4 absent and δ_H 2.87 (1H, s, H-5β); *m/z* (+ve argon f.a.b.) 380 (MH^+). (**7c**) Data as for (**7a**) except δ_H 4.40 (1H, d, *J* 3 Hz, H-4), and 2.97 (1H, d, *J* 3 Hz, H-5α); *m/z* (+ve argon f.a.b.) 379 (MH^+).



between the double bond of the substrate and the reactive iron–oxene species, followed by reductive elimination of the iron (with retention of configuration) to give the bicyclic product (**9**).

The above stereochemical results, combined with those we have previously reported on the unsaturated substrates,[1,3] provide a rationale for the formation of all six products, (**2**)—(**7**), from the allylglycine precursor (**1**). The simplest view is that four distinct and competing mechanisms are available for the second ring closure (Table 2). Mechanistic types 1 and 2 constitute the desaturase pathway, whereas 3 and 4 are the basis of the mono-oxygenase paths. The balance between these four processes, in the case of substrate (**1**), probably reflects the different geometric relationships between the allyl group and the iron–oxene intermediate. The high chemical reactivity of such an intermediate would be expected to provide successful ring closures over a range of transition state geometries. This indiscriminate character may be widely occurring in iron–oxygen utilizing enzymes. It is of note that numerous model systems proposed to utilise an iron-oxene species form epoxides on reaction with alkenes.[5,6]

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