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Penicillin Biosynthesis: The Origin of Hydroxy Groups in β-Lactams derived from Unsaturated Substrates

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Two unsaturated peptide substrates were each cyclised by the enzyme isopenicillin N synthase (IPNS) from *Cephalosporium acremonium* CO 728 simultaneously to both desaturated and hydroxylated β-lactam products whose hydroxy groups have been shown to derive their oxygen atom from the cosubstrate dioxygen; a working hypothesis involving iron–oxo species is proposed to explain this phenomenon.

During our investigations on the substrate specificity of the enzyme isopenicillin N synthase (IPNS),[1] responsible for the stoicheiometric conversion of tripeptide (**1**) and dioxygen into isopenicillin N (**2**), probably by way of the enzyme-bound intermediate (**3**),[2] we were able to transform a range of 'unnatural' peptides into bicyclic products containing the β-lactam ring as a common structural feature.[3–5] Generally the observed reaction was a desaturative ring closure, *e.g.* loss of *H, ■H, SH, and NH hydrogens from (**1**), concomitant with the reduction of *one* dioxygen molecule to water.[6] However when unsaturation was introduced, as (**4**) or (**9**), then two series of β-lactams were *simultaneously* formed through desaturative (−4H), path a, and hydroxylative (−2H +1O), path b, manifolds, Schemes 1 and 2. We have now determined the origin of the oxygen atoms which appear as hydroxy groups in the products of path b.

Thus substrates (**4**) and (**9**) were separately incubated with highly purified IPNS[7] from *Cephalosporium acremonium* CO 728 under $^{18}O_2$ (99% excess atom ^{18}O) and after purification by h.p.l.c. to homogeneity the products were analysed by fast

Table 1. Relative (f.a.b.) intensities (obs. and calc.) of ions.

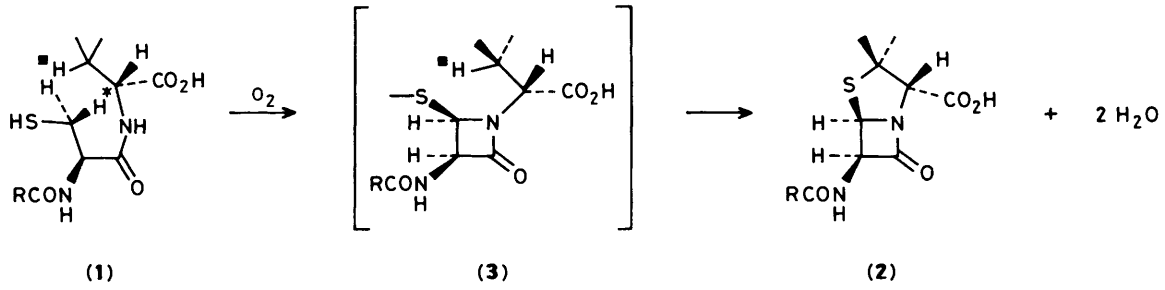
Ion		^{18}O Experiment						^{16}O Experiment						
(**7**) (MH^+)	*m/z*	375	376	377	378	379	380	*m/z*	375	376	377	378	379	
	Obs.	3	17	17	100	25	15	Obs.	25	100	30	15	10	
								Calc.	—	100	18	7	1[a]	
(**8**) (MH^+)	*m/z*	375	376	377	378	379	380							
	Obs.	25	21	33	100	62	42							
(**5**) (MH^+)	*m/z*	356	357	358	359	360	361	*m/z*	356	357	358	359	360	361
	Obs.	1	9	100	31	19	10	Obs.	12	16	100	30	18	14
								Calc.	—	—	100	19	7	1[a]

[a] Calculated assuming no ^{18}O incorporated.

Table 2. Relative (electron impact) intensities (obs. and calc.) of ions.

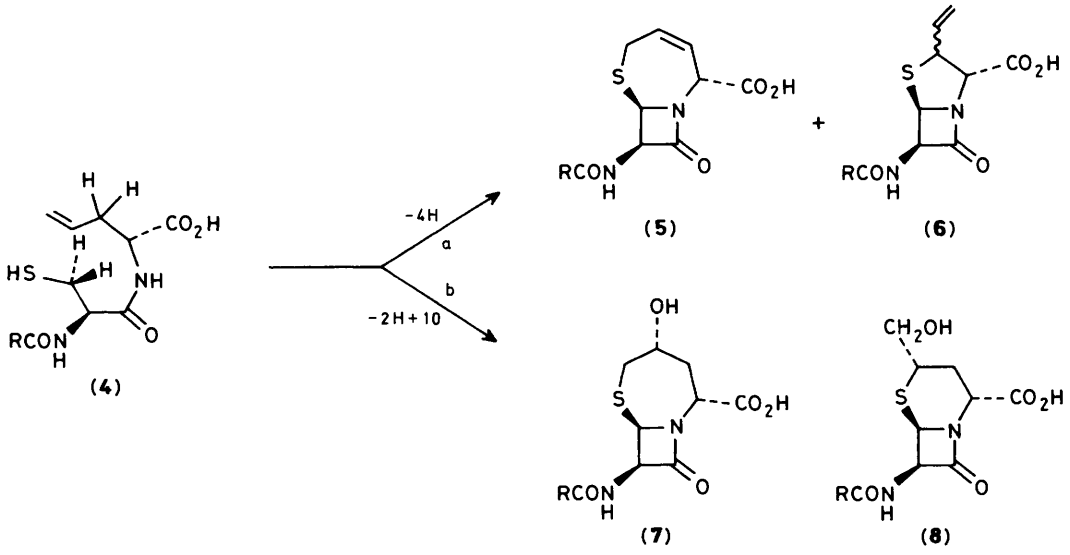
		^{18}O Experiment							^{16}O Experiment					
(**12**) and (**13**)	*m/z*	261	262	263	264	265	266	267	*m/z*	2[illegible]	261	262	263	264
	Obs.	0	17	3	100	14	6	1	Obs.	8	10	100	17	10
									Calc.	—	—	100	13	6[a]

[a] Calculated assuming no ^{18}O incorporated.



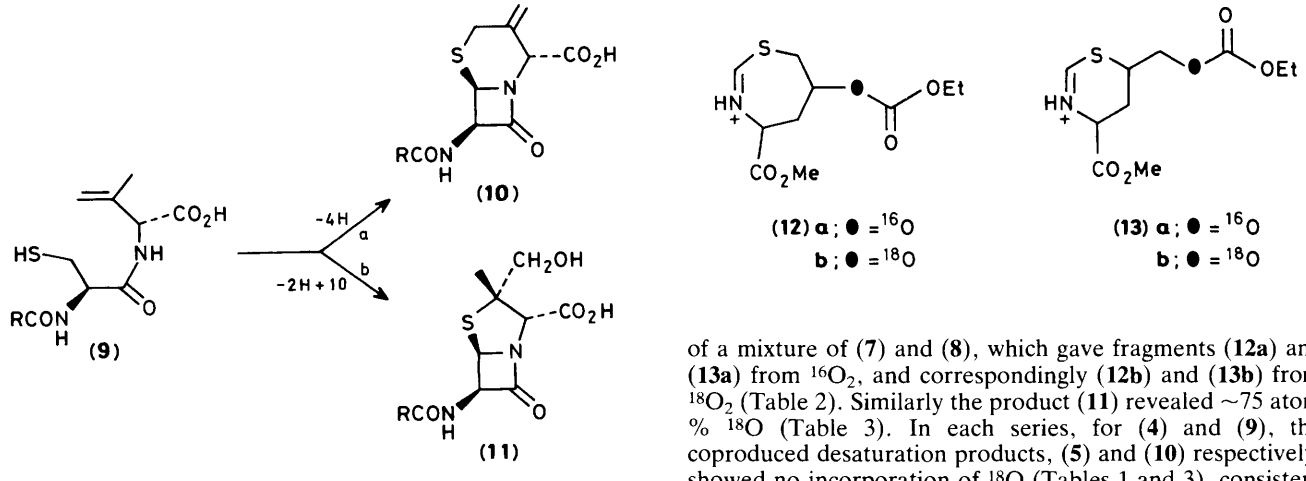
(1) (3) (2)

RCO = δ-(L-α-aminoadipoyl)



(4) (5) (6) (7) (8)

Scheme 1



(9) (10) (11) (12) a; ● = ^{16}O b; ● = ^{18}O (13) a; ● = ^{16}O b; ● = ^{18}O

Scheme 2

atom bombardment (f.a.b.) mass spectroscopy.† The mass spectra of (**7**) and (**8**) both showed incorporation of 80 atom % of ^{18}O (Table 1), and the location of ^{18}O followed from derivatisation to the *N*,*O*-diethoxycarbonyl dimethyl esters[8] of a mixture of (**7**) and (**8**), which gave fragments (**12a**) and (**13a**) from $^{16}O_2$, and correspondingly (**12b**) and (**13b**) from $^{18}O_2$ (Table 2). Similarly the product (**11**) revealed ~75 atom % ^{18}O (Table 3). In each series, for (**4**) and (**9**), the coproduced desaturation products, (**5**) and (**10**) respectively, showed no incorporation of ^{18}O (Tables 1 and 3), consistent with our earlier observations on isopenicillin N (**2**).[9] The difference between the observed incorporation (75—80%) and the $^{18}O_2$ sample (99%) probably results from incomplete removal of $^{16}O_2$ from the enzyme solution,‡ although the possibility of an exchange process in the active complex between some form of bound dioxygen and water cannot be excluded.

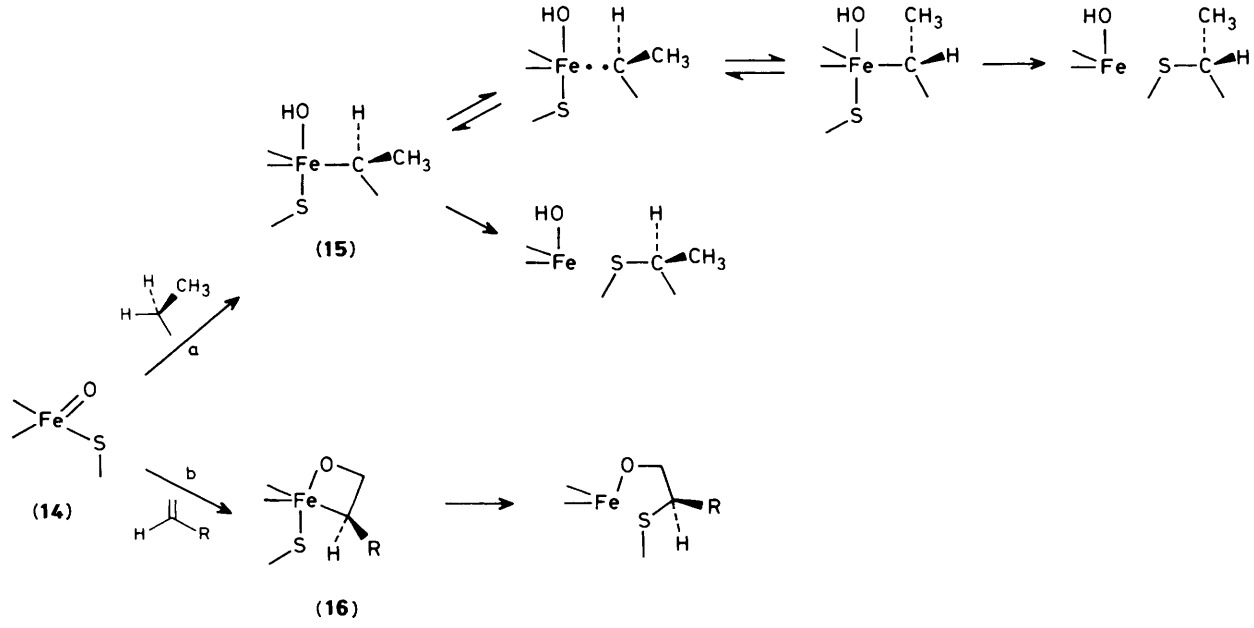
† A low level background ion intensity was observed to variable degrees in all f.a.b. analyses; an averaged background intensity was subtracted from the observed ion intensities for the ^{18}O experiments in order to calculate the relative ^{16}O : ^{18}O intensities.

‡ Experiments with exhaustive degassing of the enzyme solution, prior to $^{18}O_2$ introduction, led to enzyme denaturation.

Table 3. Relative (f.a.b.) intensities of ions in ^{18}O experiment.

Ion								
(**11**) (MH^+)	*m/z*	375	376	377	378	379	380	381
	Obs.	6	33	24	100	29	24	10
	Calc.[b]	—	—	—	100	18	7	1
(**11**) (MH^+-H_2O)	*m/z*	357	358	359	360	361	362	363
	Obs.	1	24	15	100	26	16	9
	Calc.[b]	—	—	—	100	18	7	1
(**11**) ($MHCO^+$)[c]	*m/z*	403	404	405	406	407	408	409
	Obs.	4	24	11	100	24	14	5
	Calc.[b]	—	—	—	100	19	8	1
(**10**) (MH^+)	*m/z*	356	357	358	359	360	361	
	Obs.	4	7	100	32	13	7	
	Calc.[a]	—	—	100	18	7	1	

[a] Calculated assuming no ^{18}O incorporated. [b] Calculated assuming one full ^{18}O atom incorporated. [c] Formic acid was used as solvent to transfer to f.a.b. probe.



Scheme 3

Since IPNS is an iron-dependent desaturase utilising one dioxygen molecule per molecule of penicillin synthesised,[6] the preliminary formation of the enzyme-bound monocycle (**3**)[2] requires that subsequent ring closure to (**2**) be coupled to reduction of hydrogen peroxide, or equivalent. The observation of competing hydroxylation (+1O) and desaturation (−2H) suggests the cyclisation site contains a reactive species which can abstract hydrogen (path a: desaturation) or donate oxygen, from dioxygen, (path b: hydroxylation) concomitant with C–S bond formation. As a working hypothesis we suggest an iron–oxo species, *e.g.* (**14**), as a useful device to explain this mechanistic duality. As shown in Scheme 3 such a species (**14**) could undergo insertion into C–H bonds, path a, leading to an iron–carbon species (**15**) which by reductive elimination would provide the C–S bond, with overall desaturation. If the intermediate organoiron species (**15**) underwent homolysis and recombination then the stereochemical outcome of C–S bond formation could be controlled by the active site's topology and hence accommodate the previous observation of both retention and inversion with this enzyme.[10,11] Alternatively, cycloaddition of (**14**) to an olefin, path b, would lead to a metallocycle (**16**), and subsequently by reductive elimination to a C–S bond, with concomitant hydroxylation.

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