The Role of Molecular Oxygen in Cephalosporin C Biosynthesis

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Cell-free preparations of the deacetoxycephalosporin C/deacetylcephalosporin C synthetase activities from *Cephalosporium acremonium* CO 728 were shown to convert the unnatural substrate methylenecephalosporin C (**1**) in the presence of $^{18}O_2$, into [*hydroxy*-^{18}O]deacetylcephalosporin C (**2b**).

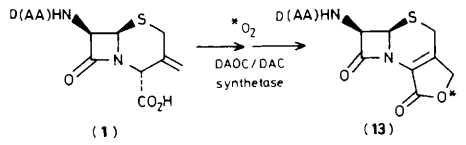
Previous studies showed that protoplast lysates from *C. acremonium* CW19 were able to convert penicillin N (**3**) into deacetoxycephalosporin C (DAOC) (**4a**),[1,2] and (**4a**) into deacetylcephalosporin C (DAC) (**2a**)[3] [the direct precursor of cephalosporin C (**4b**)].[4] More recent studies of the mechanism of action of the DAOC/DAC synthetase activities in both eukaryotic and prokaryotic organisms have been hampered by problems associated with enzyme purification.† Despite these limitations some intriguing observations have been obtained from either intact cell- or semi-purified lysates containing these enzymes. First, both activities have an absolute requirement for α-ketoglutarate, Fe^{2+}, and molecular oxygen and are enhanced by L-ascorbate.[1,3,5,6] Intact cell experiments demonstrated that (2*S*,3*R*)-[^{13}C]valine (**5a**) gave [2-^{13}C]cephalosporin C (**6a**),[7a] while (2*S*,3*S*)-[^{13}C]valine (**5b**) gave [17-^{13}C]cephalosporin C (**6b**).[7b] Intact cell systems have been exploited by Townsend[8,9] and by Abraham *et al.*[10] to demonstrate that use of chiral methylvaline as precursor gave, presumably *via* the 2β-(chiral methyl) penicillin N (**7**), labelled cephalosporin C (**8**), the 2-position of which showed complete tritium scrambling (a substantial primary isotope effect was noted),[8] and also that allylic hydroxylation of chiral 3-methyl-DAOC (**9**) occurred with retention of stereochemistry[9] to give (**10**) (Scheme 1). In our laboratories, a cell-free system has been used to convert the unnatural substrate methylenecephalosporin C (**1**) directly into (**2a**).[11]

The exact role of molecular oxygen in the conversions (**3**) → (**4a**) and (**4a**) → (**2a**) is poorly understood. Stevens *et al.*[12] have reported that the origin of the 3-hydroxymethylene oxygen atom of (**2a**) from (**4a**) was molecular oxygen when an intact cell experiment in the presence of $^{18}O_2$ was performed. However the level of incorporation was not reported.

Recently we have discovered that the related enzyme

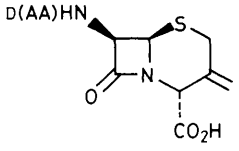
† Scheidegger *et al.*[5a] could not separate the two activities from *C. acremonium* under a variety of protein purification techniques and thus tentatively concluded 'a bifunctional enzyme of molecular weight *ca.* 33 000 was responsible for both DAOC synthetase and DAC synthetase activities.' Recently Wolfe *et al.*[5b] have reported the separation of the two activities from the prokaryotic *Staphylococcus clavigurus*. Molecular weight estimates of 29 500 (DAOC synthetase) and 26 200 (DAC synthetase) were obtained. Our results from *C. acremonium* CO 728 are in agreement with a single bifunctional enzyme.[6]

Table 1. Analysis of incubation products from (**1**).

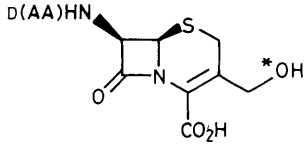


Expt.	Conditions		Relative intensities (%)															
			(**13**)/(MH)$^+$								Fragment (**14**)							
1	$^{16}O_2$	*m/z*	354	355	356	357	358	359	360									
		Found	0	12	100	28	12	3	1									
		Calc.			100	18	7	1										
2	$^{18}O_2$	*m/z*	354	355	356	357	358	359	360	361	*m/z*	154	155	156	157	158	159	160
		Found	4	1	100	23	58	13	8	2	Found	1	1	100	11	53	5	3
											Calc.[a]			100	8	5		

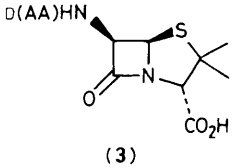
[a] For $C_6H_6N^{16}O_2S$.



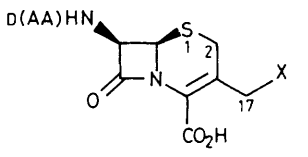
(**1**)



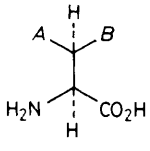
(**2**) **a**; O* = ^{16}O
b; O* = ^{18}O



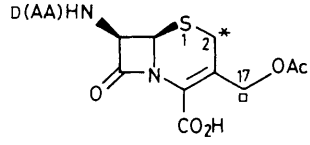
(**3**)



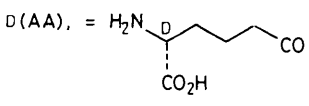
(**4**) **a**; X = H
b; X = OAc

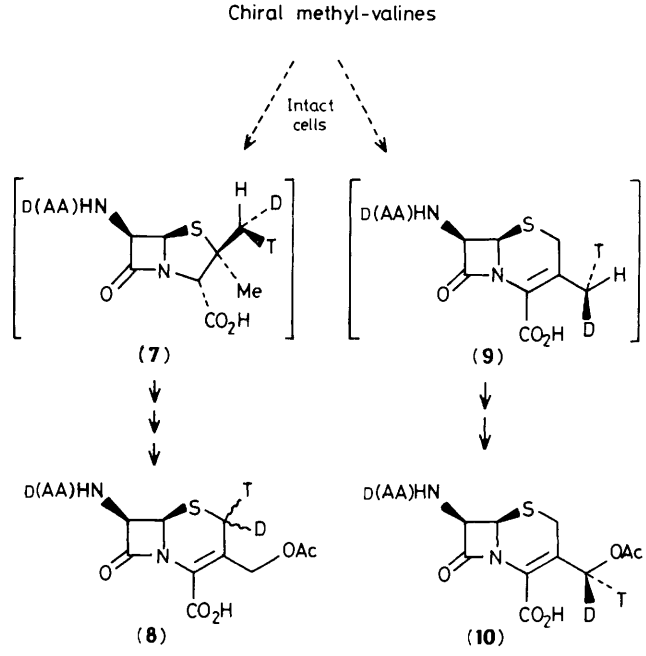


(**5**) **a**; *A* = $^{13}CH_3$, *B* = $^{12}CH_3$
b; *A* = $^{12}CH_3$, *B* = $^{13}CH_3$

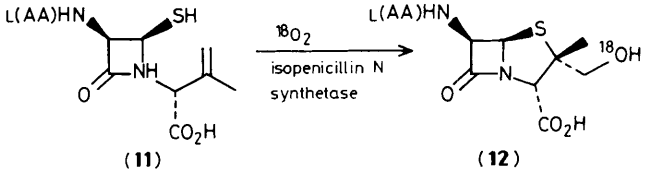


(**6**) **a**; * = $^{13}CH_2$, □ = $^{12}CH_2$
b; * = $^{12}CH_2$, □ = $^{13}CH_2$





Scheme 1

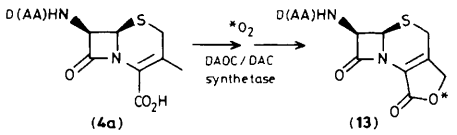


Scheme 2

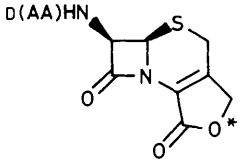
isopenicillin N synthetase is capable of producing hydroxylated β-lactam products from suitably unsaturated substrates[13] and we have now shown[14] that the source of the hydroxy group was molecular oxygen, *e.g.* (**11**) → (**12**) (Scheme 2). As the conversion (**1**) → (**2a**) could arise in a similar way through DAOC/DAC synthetase, we have studied this conversion using a partially purified extract from *C. acremonium* CO 728 under an atmosphere of $^{18}O_2$. We have also observed the cell-free conversion of DAOC (**4a**) into (**2a**) in the presence of $^{18}O_2$.

Thus incubation of the substrates (**1**) and (**4a**) using a cell-free preparation of the DAOC/DAC synthetase activi-

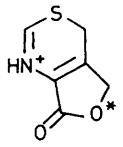
Table 2. Analysis of incubation products from (**4a**).



Expt.	Conditions		Relative intensities (%) (13) $(MH)^+$						Fragment (14)						
1	$^{16}O_2$	*m/z*	355	356	357	358	359	360	*m/z*	155	156	157	158	159	160
		Found	2	100	30	14	7	4	Found	1	100	13	8	3	2
		Calc.		100	18	7	1		Calc.		100	8	5		
2	$^{18}O_2$	*m/z*	355	356	357	358	359	360	*m/z*	155	156	157	158	159	160
		Found	4	100	25	72	27	22	Found	4	100	14	70	10	6



(**13**) **a**; O* = ^{16}O
b; O* = ^{18}O



(**14**) **a**; O* = ^{16}O
b; O* = ^{18}O

ties[6] from *C. acremonium* CO 728 under an atmosphere of $^{18}O_2$ gas (99%)‡ and the necessary co-factors§ gave crude labelled (**2a** and **b**), which was purified by reverse-phase h.p.l.c. (octadecylsilane column; mobile phase 25mM-ammonium hydrogen carbonate) and treated with formic acid to give the homogeneous lactones (**13**), which were directly analysed by positive ion thermospray h.p.l.c. mass spectrometry (reverse phase octadecylsilane column; 0.05 M-ammonium acetate containing 1% acetonitrile adjusted to pH 5 with formic acid as eluant). The results (Tables 1 and 2) indicate a significant (30—40%) incorporation of ^{18}O into the lactone (**13**) [*via* DAC (**2**)] from both methylenecephalosporin C (**1**) and DAOC (**4a**) substrates. The most probable explanation for the less than stoicheiometric level of incorporation is the presence of residual $^{16}O_2$ in the enzyme preparation that could not be removed. An alternative explanation, namely that the source of the hydroxylated oxygen atom was derived partly from water, cannot at this stage be eliminated.

In summary, the formation of the same hydroxylated product (**2**), with incorporation of oxygen derived from dioxygen, by attack on a methyl group, as in (**4a**), or on a double bond, as in (**1**), is mechanistically reminiscent of the action of isopenicillin N synthetase, wherein this same duality was observed.[13,14] It is possible that both of these non-haem iron-dependent enzymic activities are mediated through similar reactive species.

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‡ The Tris solution (pH 7.4) containing Fe^{2+}, ascorbate, α-ketoglutarate, and ammonium sulphate was degassed and pre-saturated with $^{18}O_2$ gas. The enzyme preparation could not be similarly treated without loss of activity.

§ Partially purified DAOC/DAC synthetase (2 ml; *ca.* 0.5 International Units) in Tris-HCl buffer (pH 7.4; 50mM) was preincubated for 5 min at 27 °C and 250 rev min^{-1} with 200 μl of co-factor solution prepared from α-ketoglutarate (14.6 mg), L-ascorbate (17.6 mg), dithiothreitol (30.8 mg), iron(II) sulphate (1.4 mg), and ammonium sulphate (1.32 g) in distilled water (10 ml). The substrate (1 mg) in Tris-HCl (pH 7.4; 1.8 ml; 50mM) was added and the pH adjusted to 7.4 (NaOH). The resulting solution was incubated at 27 °C and 250 rev. min^{-1} for 2 h, after which the protein was precipitated by the addition of acetone (to 70% v/v). After centrifugation (10 krev. min^{-1}, 2 min at °C) the supernatant was evaporated to dryness.

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