Water Exchange of Intermediates in a Non-haem Iron, α-Ketoglutarate Dioxygenase, Deacetoxy-/Deacetylcephalosporin C Synthase

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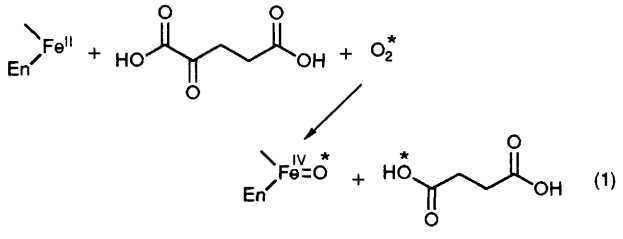
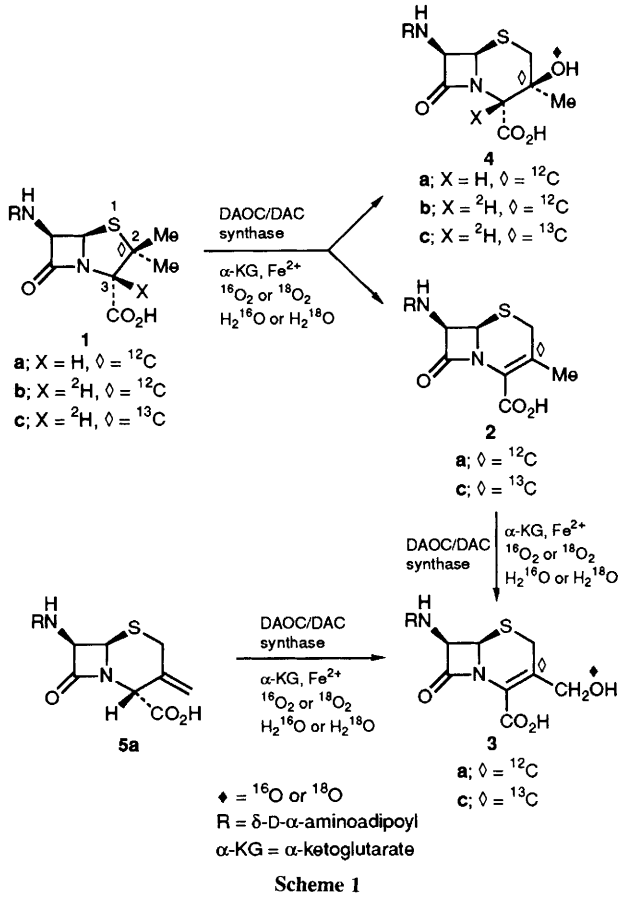
Incubation of [2-^{13}C, 3-^{2}H]penicillin N **1c** and [4-^{2}H]exomethylene cephalosporin C **5b** with the enzyme deacetoxy-/deacetylcephalosporin C synthase (DAOC/DAC synthase) in ^{18}O-labelled water gave oxygenated products showing significant incorporation of ^{18}O; the observed levels of incorporation from ^{18}O-labelled water are complementary to the previously reported incorporations which were achieved from molecular oxygen, and we believe this to be a first example of $H_2{}^{18}O$ incorporation into enzymic products catalysed by a non-haem iron, α-ketoglutarate dependent dioxygenase, and suggest structures for the exchanging intermediates.

Previous studies on the role of molecular oxygen in cephalosporin C biosynthesis [**1a** → **2a** → **3a**] with the enzyme deacetoxy-/deacetylcephalosporin C synthase (DAOC/DAC synthase) revealed incorporation from $^{18}O_2$ into deacetylcephalosporin C **3a** derived from the enzymic conversion of both deacetoxycephalosporin C **2a** (30–40% ^{18}O incorporation)[1–3] and the unnatural substrate, exomethylene cephalosporin C **5a** (30–40% ^{18}O incorporation)[4,5] (Scheme 1). In addition, we have reported[1,2] approximately 50–60% incorporation from $^{18}O_2$ into the hydroxy group of the [4-^{2}H]-3β-hydroxycepham shunt metabolite **4b** produced itself *via* a deuterium isotope induced branching of the enzymic pathway during the enzymic conversion of [3-^{2}H]penicillin N **1b**. These experiments led to the proposed involvement of an iron(IV) oxene derived from ferrous iron and cosubstrates α-ketoglutarate and molecular oxygen [eqn (1)]. When incubations were



carried out with [2-^{13}C]-α-ketoglutarate under an $^{18}O_2$ atmosphere, the succinate formed retained over 90% mono ^{18}O label, whereas the product deacetylcephalosporin C **3a** (from **2a**) again revealed only about 50% ^{18}O incorporation.[6] This result suggested that oxygen exchange was occurring between the iron-oxene oxygen and water, before the oxidation of **2a** to **3a**, and this prompted us to further investigate this process.

We now report the result of labelling studies utilizing ^{18}O-labelled water during the conversion of [2-^{13}C, 3-^{2}H]penicillin N **1c** by the enzyme DAOC/DAC synthase. Thus, [2-^{13}C, 3-^{2}H]penicillin N **1c** (*ca.* 2 mg) and cofactors were prepared in ^{18}O-labelled water (2 ml, 95 atom% ^{18}O supplied by Aldrich Chemical Co.) and then mixed with concentrated DAOC/DAC synthase [0.02 IU, 0.5 ml in 50 mmol dm^{-3} TRIS·HCl buffer pH 7.5; TRIS = tris(hydroxymethyl)methylamine] resulting in a final concentration of $H_2{}^{18}O$ of approxi-



Scheme 1

Table 1 Incorporation of oxygen from $H_2{}^{18}O$ into products derived from incubation of [2-^{13}C, 3-^{2}H]penicillin N **1c** with DAOC/DAC synthase

Product	Conditions		*m/z* (MH$^+$)						^{18}O (%) Incorporation
2c			358	359	360	361	362	363	
	$H_2{}^{16}O/{}^{16}O_2$	Found (%)	14	100	20	9	4	—	—
	$H_2{}^{18}O/{}^{16}O_2$	Found (%)	14	100	22	9	2	—	0
4c			377	378	379	380	381	382	
	$H_2{}^{16}O/{}^{16}O_2$	Found (%)	15	100	26	10	2	—	—
	$H_2{}^{18}O/{}^{16}O_2$	Found (%)	18	100	23	26	7	3	21[a]
3c			374	375	376	377	378	379	
	$H_2{}^{16}O/{}^{16}O_2$	Found (%)	14	100	20	11	3	—	—
	$H_2{}^{18}O/{}^{16}O_2$	Found (%)	17	92	37	100	21	10	66[a]

[a] Incorporation values take into account the level of isotope enrichment used.

Table 2 Incorporation of oxygen from $^{18}O_2$ into products derived from incubation of [4-^{2}H]exomethylene cephalosporin C **5b** with DAOC/DAC synthase

Product	Conditions		*m/z* (MH$^+$)						^{18}O (%) Incorporation
6			375	376	377	378	379	380	
	$^{16}O_2/H_2{}^{16}O$	Found (%)	100	20	8	2	—	—	—
	$^{18}O_2/H_2{}^{16}O$	Found (%)	9	12	100	19	8	2	94[a]
	$^{18}O_2/H_2{}^{16}O$	Found (%)	7	9	100	20	8	2	95[a]
7			356	357	358	359	360	361	
	$^{16}O_2/H_2{}^{16}O$	Found (%)	100	20	8	2	—	—	—
	$^{18}O_2/H_2{}^{16}O$	Found (%)	100	24	88	22	7	2	46[a]
	$^{18}O_2/H_2{}^{16}O$	Found (%)	77	22	100	19	7	1	56[a]

[a] See footnote [a] in Table 1.

Table 3 Incorporation of oxygen from $H_2{}^{18}O$ into products derived from incubation of [4-^{2}H]exomethylene cephalosporin C **5b** with DAOC/DAC synthase

Product	Conditions		*m/z* (MH$^+$)						^{18}O % Incorporation
6			375	376	377	378	379	380	
	$H_2{}^{16}O/{}^{16}O_2$	Found (%)	100	20	8	2	—	—	—
	$H_2{}^{18}O/{}^{16}O_2$	Found (%)	100	20	22	5	2	1	17[a]
7			356	357	358	359	360	361	
	$H_2{}^{16}O/{}^{16}O_2$	Found (%)	100	20	8	2	—	—	—
	$H_2{}^{18}O/{}^{16}O_2$	Found (%)	100	25	50	11	4	1	40[a]

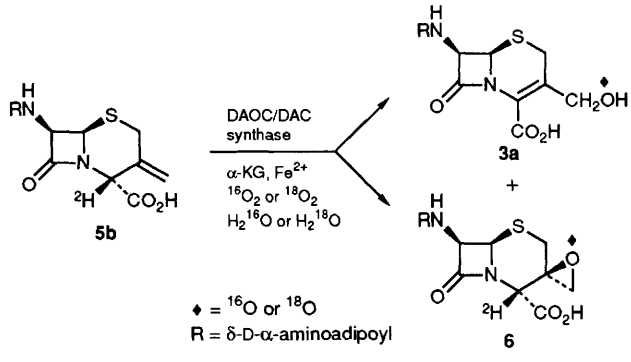
[a] See footnote [a] in Table 1.

mately 76%. The mixture was then incubated under standard conditions[7] and the products isolated by HPLC. Analysis of the purified products, [3-^{13}C]DAOC **2c**, [3-^{13}C]DAC **3c** and [3-^{13}C, 4-^{2}H]-3β-hydroxycepham **4c** by electrospray mass spectroscopy revealed significant oxygen incorporation from labelled water into both hydroxylated enzymic products (see Table 1, Scheme 1).†

In order to probe further such oxygen incorporation, we established the origin of the epoxide oxygen atom (H_2O* *vs.* *O_2) of the novel spirocepham **6** itself derived from **5b** by deuterium isotope induced branching of the natural enzymic pathway.[8] Thus, [4-^{2}H]exomethylene cephalosporin C **5b** (*ca.* 8 mg) was incubated firstly with DAOC/DAC synthase (1.2 IU, 0.5 ml in 50 mmol dm^{-3} TRIS·HCl buffer pH 7.5) under an atmosphere of $^{18}O_2$ gas (98 atom% ^{18}O supplied by MSD Isotopes, 100 ml) in the presence of the usual cofactors (Scheme 2).

Analysis indicated 94% incorporation of oxygen from $^{18}O_2$ into the spiro-epoxide **6** and 46% into DAC **3a** (isolated and analysed as lactone **7**,[2] (see Table 2). Incubation was then repeated with a second sample of **5b** and the degree of incorporation determined to be in good accord with the earlier result (see Table 2). Both experiments indicate that the epoxide oxygen is largely derived from molecular oxygen whereas incorporation into DAC **3a** was less effective.‡

Secondly, **5b** (ca. 3 mg) was incubated with DAOC/DAC synthase (0.3 IU, 0.5 ml in 50 mmol dm^{-3} TRIS·HCl buffer pH 7.5) in the presence of $H_2{}^{18}O$ (76 atom%). Analysis by electrospray mass spectroscopy again revealed incorporation of labelled water into both enzymic products (see Table 3), although the level of incorporation into the spiroepoxide **6** was much lower than that observed with molecular oxygen as the label source.



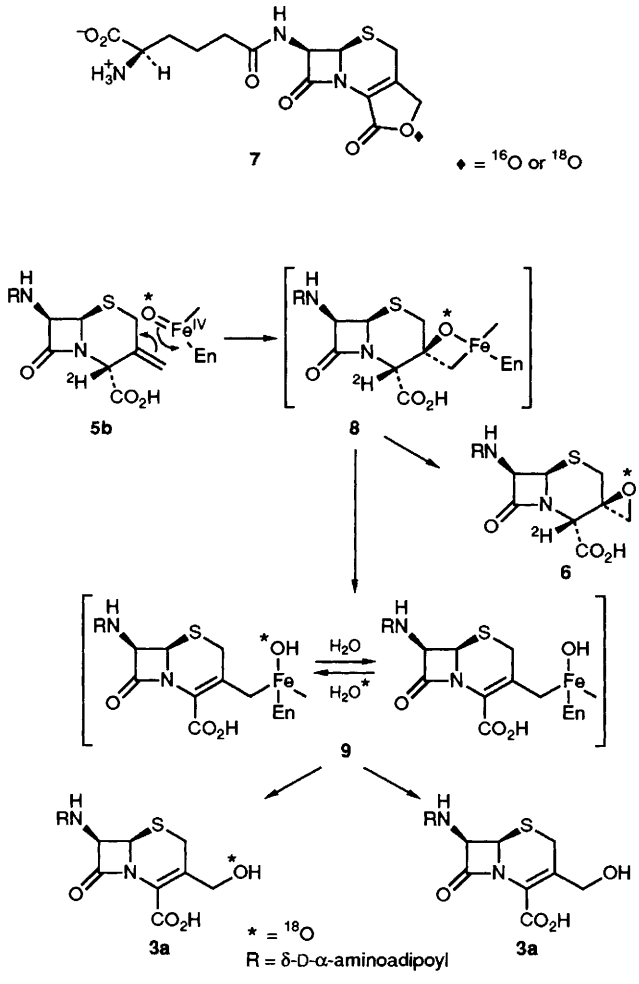
Scheme 2

In separate control experiments, no loss of labelled oxygen from the products **4c** and **3c** was observed when these compounds were individually incubated with denatured enzyme in $H_2{}^{16}O$ and in the presence of the usual cofactors. In the case of the epoxide **6**, a control experiment where unlabelled **6** was incubated with denatured enzyme in $H_2{}^{18}O$ and cofactors, showed no incorporation of oxygen from labelled water into **6**.

It is of particular interest to consider first the origin of the two products **3a** and **6** derived from [4-^{2}H]exomethylene cephalosporin C **5b** and the mechanism of their formation, in relation to the large differences observed in the level and source of oxygen incorporation between them. Both products

† Examination of the isolated [3-^{13}C, 4-^{2}H]-3β-hydroxycepham **4c** by ^{13}C NMR (125.77 MHz, D_2O) confirmed that the labelled water had been incorporated into the hydroxy group attached to the ^{13}C-label, due to a clearly visible ^{18}O-^{13}C isotope shift[18,19] ($\Delta\delta$ = 0.03 ppm) of the resonance at δ 65.457.

‡ We have previously shown that no exchange of the hydroxy oxygen occurs during lactonisation of **3** to **7**.[6]



Scheme 3

are formed from the same substrate *via* a deuterium isotope induced branching of the normal enzymic pathway. In addition, no V_{max}/K_m isotope effect[8] was observed in competitive incubations of exomethylene cephalosporin C **5a** and the specifically deuteriated [4-^{2}H]exomethylene cephalosporin C **5b**, a result strongly suggesting that the first irreversible event occurs by addition of an iron-oxene to the olefin.[8] The implication of these two experimental observations is that both products, DAC **3a** and the spiroepoxide **6** are formed from a common enzyme-bound intermediate **8** and that branching of the normal pathway to provide **6** arises owing to the operation of a primary deuterium isotope effect on the reactions from this intermediate (Scheme 3, illustrated for an iron-oxene generated from $*O_2$).

To account for the large difference in ^{18}O incorporation between DAC **3a** and epoxide **6** we propose that exchange of label occurs between either the reactive ferryl species or an enzyme-bound intermediate such as **9** (Scheme 3) present at the active site, and the aqueous incubation mixture. Any exchange of label between the ferryl species and the incubation media should, however, be reflected in both oxygen containing products. As this is not the case we propose that the majority of exchange must occur between an enzyme-bound intermediate, formed after the first irreversible enzymic event, and the medium.

We believe these results represent the first example of exchange observed between water and the active ferryl species and/or enzyme-bound intermediates with an α-ketoglutarate, ferrous dependent dioxygenase. By comparison, exchange of this type was not observed during $^{18}O_2$ and $H_2{}^{18}O$ incorporation studies on the α-ketoglutarate, ferrous dependent dioxygenase prolyl-4-hydroxylase, despite incorporation of oxygen from $^{18}O_2$ into collagen hydroxyproline.[9,10] Of the ferrous dependent enzymic systems more closely related to DAOC/DAC synthase which have been shown to undergo analogous exchange, α-ketoisocaproate oxygenase[11] and *p*-hydroxyphenylpyruvate hydroxylase[12] are the best studied although the species undergoing exchange in these systems are not known.

Exchange at the proposed iron-oxene stage has been shown to occur with other biological systems, albeit to a much lower extent, such as with cytochrome P-450.[13] Such exchange, however, apparently requires two preconditions, firstly an accessible active site so that water can reach the ferryl centre and secondly a proton source sufficiently acidic to protonate the oxygen of the ferryl moiety. In certain P-450 systems, it is generally accepted that substrate binds prior to the binding of molecular oxygen and generation of the reactive ferryl species.[14] At this point, it is believed that the active site becomes inaccessible to water thus preventing exchange, as shown by incubations of purified liver microsomal P-450 in $H_2{}^{18}O$, which gave only 8.6% labelled oxygen incorporation.[13] Some attempts to implicate the protein surrounding the active site as the proton source have been made, for example the involvement of protonated histidine residues in catalysis of exchange as observed with horseradish peroxidase.[15] It is also noteworthy that other synthetic iron and chromium containing porphyrins do show marked levels of exchange.[16,17]

By comparing our results and those of others, we conclude that exchange at the ferryl oxidation state probably constitutes a minor pathway in the case of DAOC/DAC synthase, as shown by the low incorporation of oxygen from $H_2{}^{18}O$ into the spiroepoxide **6**, but propose that the majority of exchange occurs after insertion of the iron-oxene into the relevant carbon–hydrogen bond of the substrate. A possible explanation for these observations may be that binding of the substrate leads to a tightening or closing of the active site thereby hindering entry of water and reducing the possibility of exchange. Once the enzymic reaction is nearing completion, the active site must open in readiness for product release and at this point water could enter more readily and thus become more involved in exchange processes. In addition, exchange after reaction between the substrate and ferryl species would involve a hydroxy group coordinated to the iron and thus protonation would no longer be a prerequisite, unlike the case of exchange with the ferryl moeity. We thus propose the following mechanism for the formation of [4-^{2}H]-3β-hydroxycepham **4b** from penicillin N **1b** and for the hydroxylation of **2a** to **3a** (Scheme 4, illustrated for iron-oxene generated from $*O_2$), in which the intermediates **9** and **10** are capable of undergoing facile exchange. In the conversion **2a** to **3a** initial insertion of the iron-oxene into a C–H bond of the exocyclic methyl group results in formation of the same intermediate **9** we postulate in the formation of DAC **3a** from exomethylene cephalosporin C **5a** (Scheme 3). This view is supported by the observation of similar levels of oxygen incorporation from both $^{18}O_2$ and $H_2{}^{18}O$ into DAC **3a** from either **5b** or **2a**.§

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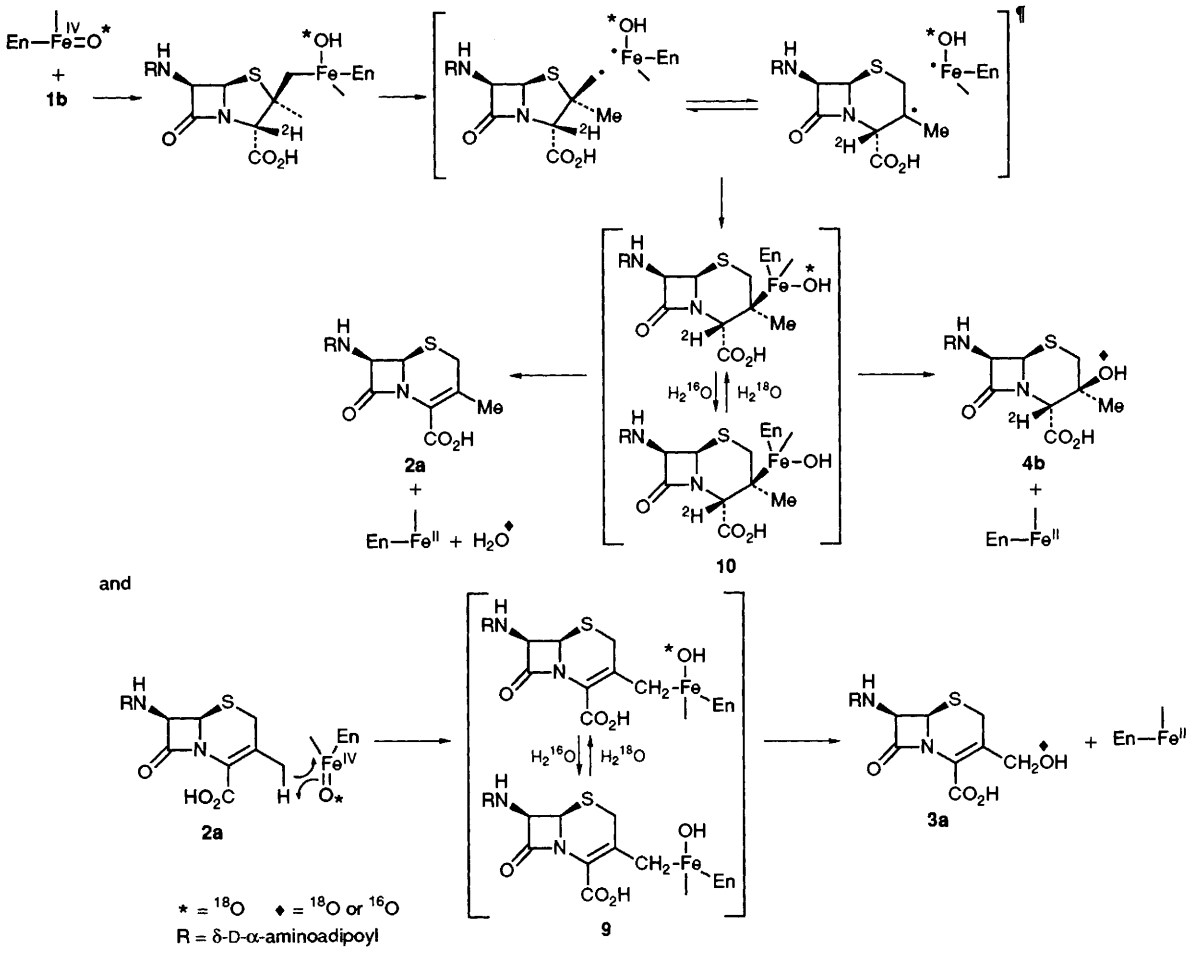
§ We believe the levels of $^{18}O_2$ incorporation and $H_2{}^{18}O$ incorporation into the individual oxygenated products described in this communication are complementary to each other to within experimental error.



Scheme 4

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¶ We favour the view that this intermediate has radical as opposed to cationic character.