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# Incorporation of $^{18}O$-Labelled Water into Oxygenated Products Produced by the Enzyme Deacetoxy/deacetylcephalosporin C Synthase

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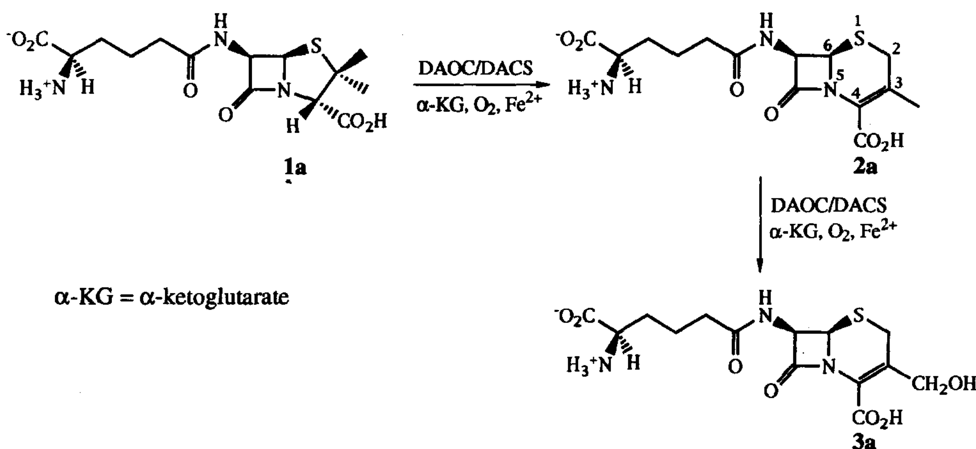
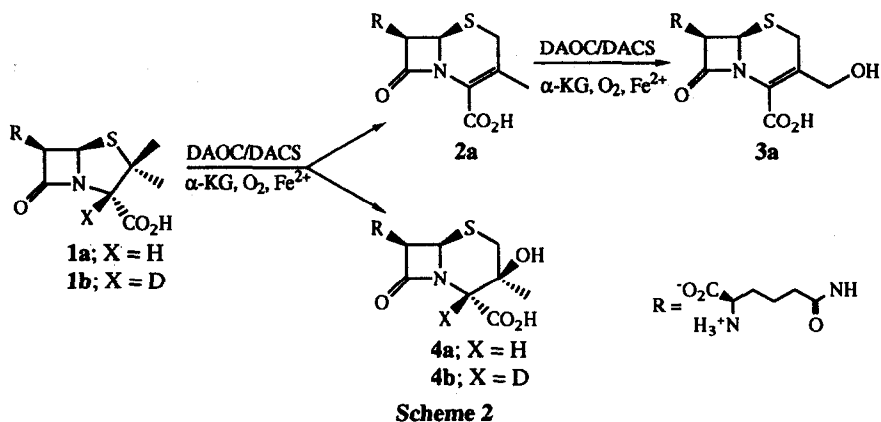
**Abstract** : $^{18}O$-labelling experiments have been conducted with the enzyme deacetoxy/deacetylcephalosporin C synthase. Incubations of [2-$^{13}C$,3-$^{2}H$]penicillin N and [4-$^{2}H$]exomethylene cephalosporin C were carried out with $^{18}O_2$ or $H_2{}^{18}O$, and $^{18}O$-incorporation from both label sources was observed into the oxygenated enzymic products. This is believed to be the first example of $H_2{}^{18}O$ incorporation into products of an $\alpha$-ketoglutarate dependent dioxygenase. An oxygen-exchange process at an enzyme-bound intermediate is proposed to account for this incorporation.

The enzyme deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) is an $\alpha$-ketoglutarate dependent dioxygenase from *Cephalosporium acremonium* responsible for the enzymic ring-expansion of penicillin N **1a** to the first of the cephalosporins, deacetoxycephalosporin C (DAOC) **2a**. The same enzyme also catalyses the hydroxylation of DAOC to deacetylcephalosporin C (DAC) **3a**. Both steps require $\alpha$-ketoglutarate and molecular oxygen as co-substrates and ferrous ion as a cofactor (Scheme 1).[1,2]



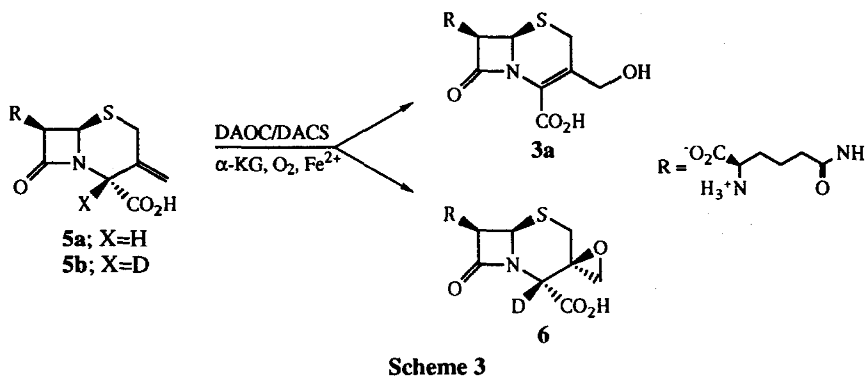

**Scheme 1**

During the course of our studies on this important enzyme we have observed an isotopically induced branching of the normal biosynthetic pathway during conversion of the specifically deuterated [3-$^{2}H$]penicillin N **1b**.[3,4] The result of this branching is a marked increase in the level of production of a shunt metabolite, the 3$\beta$-hydroxycepham **4b**, over the normal products DAOC **2a** and DAC **3a** (Scheme 2).



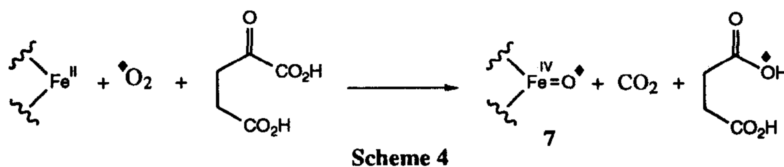
Scheme 2

Exomethylene cephalosporin C **5a** was the first unnatural substrate for DAOC/DACS to be recognised, and much effort was made to identify an intermediate between **5a** and its enzymic product, DAC **3a**.[5,6] However, the intermediacy of DAOC **2a** in this process was never observed. Recently, we have demonstrated a similar isotope induced branching of the enzymic reaction in the conversion of the specifically deuterated substrate, [4-$^{2}$H]exomethylene cephalosporin C **5b** (Scheme 3), which results in the formation of the novel spiro-epoxide cepham **6** in addition to the expected product, DAC **3a**.[7] Our results indicate that **6** is a shunt metabolite, as is the 3β-hydroxycepham **4a/b** described earlier.



Scheme 3

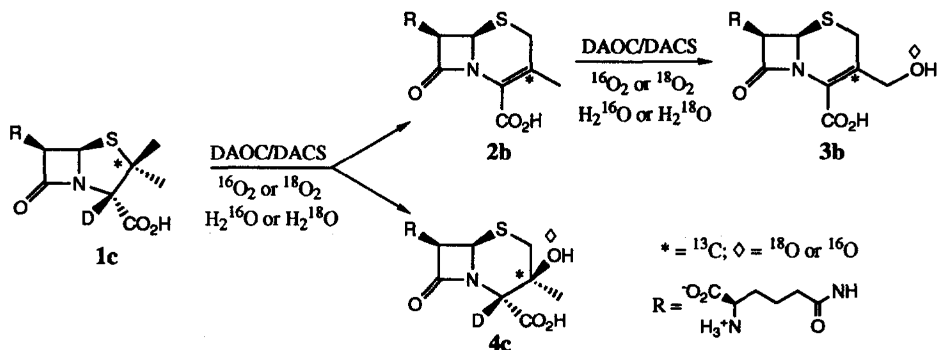
It is generally accepted that in α-ketoglutarate dioxygenases an iron-oxene intermediate [$Fe^{IV}$=O, **7**] is formed through the oxidative decarboxylation of α-ketoglutarate to succinate mediated by the iron and $O_2$ (Scheme 4). The ferryl species **7** is then responsible for reaction with the substrate, donating its oxygen atom in the case of an oxygenation reaction.



Scheme 4

Previous studies with DAOC/DACS on the conversion of **1b** and **5a** using $^{18}O_2$ revealed there was incorporation of $^{18}O$-label into the oxygenated products formed, but the observed levels of incorporation were substantially lower than expected.[4,6] Further experiments revealed that in an incubation of DAOC **2a** with DAOC/DACS under $^{18}O_2$, the succinate formed retained over 90% $^{18}O$-label, whereas the product DAC **3a** exhibited an incorporation level of only 50%.[2] These results suggested that loss of label was occurring in a step subsequent to the formation of the ferryl **7**, and it was proposed that this process could result from oxygen-exchange between **7** and water from the medium. In order to investigate this process in more detail it was decided to carry out further labelling experiments with both $^{18}O_2$ and $H_2{}^{18}O$. We now report the results of this study with the substrates [2-$^{13}$C,3-$^{2}$H]penicillin N **1c** and [4-$^{2}$H]exomethylene cephalosporin C **5b**.[8]

Initially, we incubated [2-$^{13}$C,3-$^{2}$H]penicillin N† **1c** with DAOC/DACS under an atmosphere of $^{16}O_2$, and isolated the products [3-$^{13}$C]DAOC **2b**, [3-$^{13}$C]DAC **3b** and [3-$^{13}$C,4-$^{2}$H]-3β-hydroxycepham **4c** by HPLC (Scheme 5). The products were analysed by $^{1}$H-NMR and electrospray mass spectroscopy (ESMS) (see Table 1, Expt. 1), and a $^{13}$C-NMR spectrum of the [3-$^{13}$C,4-$^{2}$H]-3β-hydroxycepham **4c** also obtained which clearly showed an enhanced resonance at δ 65.46 ppm due to the $^{13}$C label at C3.




**Scheme 5**

The incubation was then repeated using exactly the same conditions except under an atmosphere of $^{18}O_2$ (98 atom % $^{18}$O, supplied by MSD Isotopes Ltd). The products were isolated by HPLC and analysed as described above. Electrospray mass spectroscopy revealed significant incorporation of labelled oxygen into the hydroxylated products [3-$^{13}$C]DAC **3b** and [3-$^{13}$C,4-$^{2}$H]-3β-hydroxycepham **4c** (see Table 1, Expt. 2a), and as expected no incorporation into [3-$^{13}$C]DAOC **2b**. Repetition of this incubation with a second sample of [2-$^{13}$C,3-$^{2}$H]penicillin N **1c** gave similar levels of incorporation (see Table 1, Expt. 2b) with both experiments comparing favourably with the levels of $^{18}O$-incorporation from $^{18}O_2$ previously determined from incubations of [3-$^{2}$H]penicillin N **1b** with DAOC/DACS.[3,4]

| Expt. | Conditions | | | | | | | | % Incorporation $^{18}O$[††] |
|---|---|---|---|---|---|---|---|---|---|
| **[3-$^{13}$C]DAOC 2b** | | | | | | | | | |
| | | *m/z* ($MH^+$) | *358* | *359* | *360* | *361* | *362* | | |
| | | Calcul.[a] (%) | – | 100 | 17 | 7 | 1 | | – |
| Expt. 1 | $^{16}O_2/H_2{}^{16}O$ | Found (%) | 14 | 100 | 20 | 9 | 4 | | – |
| Expt. 2a | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 12 | 100 | 20 | 9 | 3 | | – |
| | | *m/z* ($MNa^+$) | *380* | *381* | *382* | *383* | *384* | | |
| Expt. 2b | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 20 | 100 | 21 | 15 | 4 | | – |
| **[3-$^{13}$C,4-$^{2}$H]-3β-Hydroxycepham 4c** | | | | | | | | | |
| | | *m/z* ($MH^+$) | *377* | *378* | *379* | *380* | *381* | *382* | |
| | | Calcul.[a] (%) | – | 100 | 17 | 7 | 1 | – | |
| Expt. 1 | $^{16}O_2/H_2{}^{16}O$ | Found (%) | 15 | 100 | 26 | 10 | 2 | – | – |
| Expt. 2a | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 9 | 42 | 29 | 100 | 21 | 9 | 71 |
| | | *m/z* ($MNa^+$) | *399* | *400* | *401* | *402* | *403* | *404* | |
| Expt. 2b | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 11 | 47 | 31 | 100 | 20 | 9 | 69 |
| **[3-$^{13}$C]DAC 3b** | | | | | | | | | |
| | | *m/z* ($MH^+$) | *374* | *375* | *376* | *377* | *378* | *379* | |
| | | Calcul.[a] (%) | – | 100 | 17 | 7 | 1 | – | |
| Expt. 1 | $^{16}O_2/H_2{}^{16}O$ | Found (%) | 14 | 100 | 20 | 11 | 3 | – | – |
| Expt. 2a | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 5 | 90 | 34 | 100 | 25 | 13 | 52 |
| Expt. 2b | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 10 | 74 | 21 | 100 | 20 | 15 | 57 |

**Table 1**: $^{18}O$-oxygen incorporations[††] into the products **2b**, **4c** and **3b** from incubations of **1c** under $^{18}O_2$.
[a] calculated using oxygen at natural abundance.

In addition, incorporation of $^{18}O$ into the C3 of [3-$^{13}$C,4-$^{2}$H]-3β-hydroxycepham **4c** was confirmed by the observation of a $^{13}C$-$^{18}O$ shift[9] in the $^{13}C$-NMR spectrum of the isolated and purified cepham **4c** ($\Delta\delta$ = 0.03 ppm) and of the lactone **8**[10] ($\Delta\delta$ = 0.04 ppm) derived from it by decomposition, for which better $^{13}C$ spectroscopic data was obtained (see Figure 1). Observation of such a shift confirms both the position and to an approximate value, the level of isotopic incorporation.

We next incubated the unnatural substrate [4-$^{2}$H]exomethylene cephalosporin C **5b**[7] in an $^{16}O_2$ atmosphere. Again, the products DAC **3a** [isolated and analysed as the lactone **9**[6]] and the spiro-epoxide cepham **6** (Scheme 6) were isolated by HPLC and their $^{1}H$-NMR and electrospray mass spectra recorded (see Table 2, Expt. 1). This incubation was then performed under an atmosphere of $^{18}O_2$, the products isolated by HPLC and then examined by $^{1}H$-NMR and the level of $^{18}O$-incorporation determined by ESMS. Analysis of the mass spectra obtained indicated the level of isotope incorporation into the epoxide cepham **6** to be 94%, and that into DAC **3a** [isolated and analysed as the lactone **9**[†††]] 46% (see Table 2, Expt. 2a). This incubation was then repeated with a second sample of **5b** and the degree of incorporations determined to be in accord with the earlier result (see Table 2, Expt. 2b). Both experiments indicate that the epoxide oxygen in **6** is derived from molecular oxygen, as anticipated. The results reveal a consistently high incorporation of label in the case of **6**, but less so in the case of **9**.

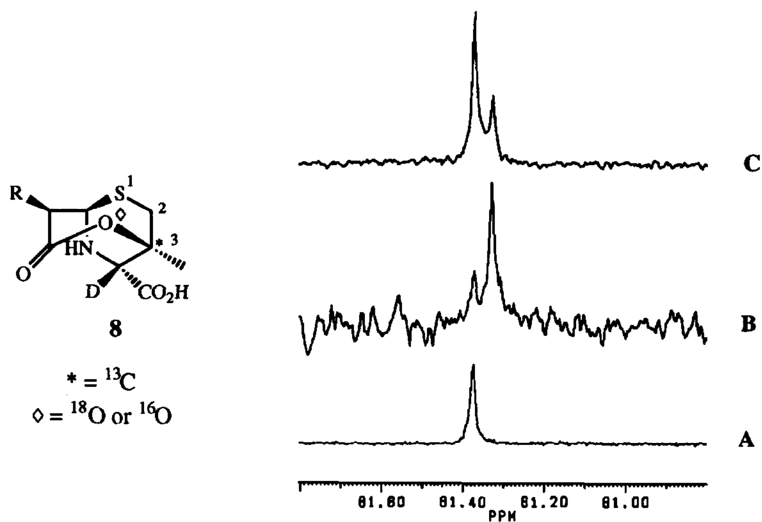
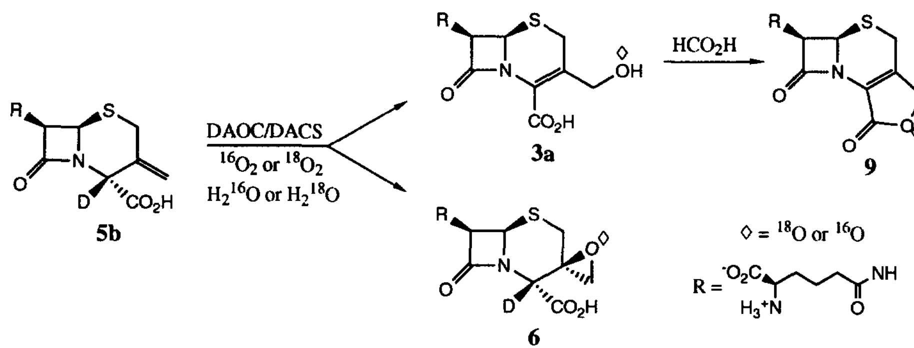



**Figure 1** : The C3-$^{13}$C resonance of **8** isolated from **A**) incubation of **1c** under $^{16}O_2$, **B**) incubation of **1c** under $^{18}O_2$ (98 atom % $^{18}$O), and **C**) B doped with A.

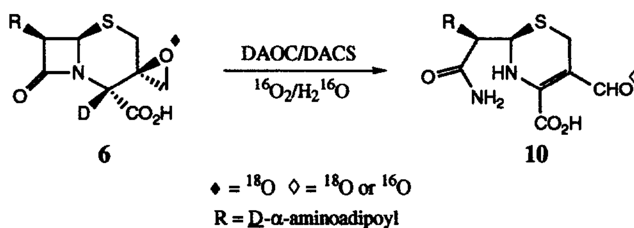



**Scheme 6**

| Expt. | Conditions | | | | | | | | % Incorporation $^{18}O$†† |
|---|---|---|---|---|---|---|---|---|---|
| DAC lactone **9** | | m/z (MH+) | 356 | 357 | 358 | 359 | 360 | 361 | |
| | | Calcul.[a] (%) | 100 | 18 | 7 | 1 | – | – | |
| Exp. 1 | $^{16}O_2/H_2{}^{16}O$ | Found (%) | 100 | 20 | 8 | 2 | – | – | – |
| Exp. 2a | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 100 | 24 | 88 | 22 | 7 | 2 | 46 |
| Exp. 2b | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 77 | 22 | 100 | 19 | 7 | 1 | 56 |
| Epoxide **6** | | m/z (MH+) | 375 | 376 | 377 | 378 | 379 | 380 | |
| | | Calcul.[a] (%) | 100 | 18 | 7 | 1 | – | – | |
| Exp. 1 | $^{16}O_2/H_2{}^{16}O$ | Found (%) | 100 | 20 | 8 | 2 | – | – | – |
| Exp. 2a | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 9 | 12 | 100 | 19 | 8 | 2 | 94 |
| Exp. 2b | $^{18}O_2/H_2{}^{16}O$ | Found (%) | 7 | 9 | 100 | 20 | 8 | 2 | 95 |

**Table 2**: $^{18}O$-oxygen incorporations†† into the products **9** and **6** from incubations of **5b** under $^{18}O_2$.
[a] calculated using oxygen at natural abundance.

It was previously found that the epoxide cepham **6** is also a substrate for DAOC/DACS, being oxidised to the aldehyde cephalosporoate **10** (Scheme 7).[7] Given the very high level of $^{18}O$-labelling of the epoxide **6** obtained in the $^{18}O_2$ incubations of **5b**, it was decided to re-incubate it with the enzyme in order to investigate whether the epoxide oxygen was retained in the product. Thus, the $^{18}O$-labelled epoxide cepham **6** (94% $^{18}O$) was incubated with DAOC/DACS under normal conditions. The product **10** was isolated by HPLC, and analysed by electrospray mass spectrometry which revealed it retained only about 14% $^{18}O$-label. However, the apparent loss of $^{18}O$ revealed in this experiment was not considered conclusive in terms of the enzymic mechanism because of the possibility that the exchange of oxygen might have occurred between the formyl group of the product and water, either before or after hydrolysis of the β-lactam ring took place.




**Scheme 7**

We next carried out incubations with $^{18}O$-labelled water. Thus, [2-$^{13}C$,3-$^{2}H$]penicillin N **1c** and co-factors were prepared in $H_2{}^{18}O$ (2 ml, 95 atom % $^{18}O$, supplied by Aldrich Chemical Co.) and then mixed with concentrated DAOC/DACS (0.5 ml in 50 mM TRIS.HCl buffer, pH 7.5) resulting in a final concentration of $H_2{}^{18}O$ of approximately 76%. The mixture was then incubated under standard conditions and the products isolated by HPLC. Analysis of the purified products [3-$^{13}C$]DAOC **2b**, [3-$^{13}C$]DAC **3b** and [3-$^{13}C$,4-$^{2}H$]-3β-hydroxycepham **4c** by ESMS revealed significant incorporation of labelled water into both hydroxylated products, but not into [3-$^{13}C$]DAOC **2b** (see Table 3).

| Conditions | | | | | | | | % Incorporation $^{18}O$†† |
|---|---|---|---|---|---|---|---|---|
| [3-$^{13}$C]DAOC **2b** | | | | | | | | |
| | *m/z* ($MH^+$) | *358* | *359* | *360* | *361* | *362* | | |
| | Calcul.[a] (%) | – | 100 | 17 | 7 | 1 | | |
| $^{16}O_2/H_2{}^{16}O$ | Found (%) | 14 | 100 | 20 | 9 | 4 | | – |
| $^{16}O_2/H_2{}^{18}O$ | Found (%) | 14 | 100 | 22 | 9 | 2 | | – |
| [3-$^{13}$C-4-$^{2}$H]-3β-Hydroxycepham **4c** | | | | | | | | |
| | *m/z* ($MH^+$) | *377* | *378* | *379* | *380* | *381* | *382* | |
| | Calcul.[a] (%) | – | 100 | 17 | 7 | 1 | – | |
| $^{16}O_2/H_2{}^{16}O$ | Found (%) | 15 | 100 | 26 | 10 | 2 | – | – |
| $^{16}O_2/H_2{}^{18}O$ | Found (%) | 18 | 100 | 23 | 26 | 7 | 3 | 21 |
| [3-$^{13}$C]DAC **3b** | | | | | | | | |
| | *m/z* ($MH^+$) | *374* | *375* | *376* | *377* | *378* | *379* | |
| | Calcul.[a] (%) | – | 100 | 17 | 7 | 1 | – | |
| $^{16}O_2/H_2{}^{16}O$ | Found (%) | 14 | 100 | 20 | 11 | 3 | – | – |
| $^{16}O_2/H_2{}^{18}O$ | Found (%) | 17 | 92 | 37 | 100 | 21 | 10 | 66 |

**Table 3** : $^{18}O$-oxygen incorporations†† into the products **2b**, **4c** and **3b** from an incubation of **1c** in $H_2{}^{18}O$ ($^{16}O_2/H_2{}^{16}O$ data from Table 1).

[a] calculated using oxygen at natural abundance.

Examination of the isolated [3-$^{13}$C,4-$^{2}$H]-3β-hydroxycepham **4c** by $^{13}$C-NMR revealed 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 ($\Delta\delta = 0.03$ ppm) for the resonance at δ 65.46 ppm (see Figure 2).

As a control experiment, the $^{18}O$-labelled products **3b** (16% $^{18}O$) and **4c** (50% $^{18}O$) were individually incubated with denatured DAOC/DACS under normal incubation conditions. Analysis by $^{1}$H-NMR showed both compounds were still intact, and these were recovered by HPLC purification from the incubation mixtures. Mass spectroscopic analysis revealed no loss of label had occurred from either product, to within experimental error.

Incubation with DAOC/DACS in $^{18}O$-enriched water was then repeated with [4-$^{2}$H]-exomethylene cephalosporin C **5b**, and the products DAC **3a** [isolated and analysed as the lactone **9**[6]] and the spiro-epoxide **6** were isolated by HPLC. Analysis by ESMS again revealed incorporation of labelled water into both enzymic products (see Table 4), although the level of incorporation apparent in the spiro-epoxide **6** was much lower than that observed with molecular oxygen as the label source.

As a control experiment, some unlabelled epoxide-cepham **6** (obtained from an incubation of **5b** in $^{16}O_2/H_2{}^{16}O$) was incubated with denatured DAOC/DACS in $H_2{}^{18}O$. No conversion of **6** was observed and this was recovered by HPLC. Analysis by electrospray mass spectrometry showed no incorporation of $^{18}O$-label into **6** had occurred.

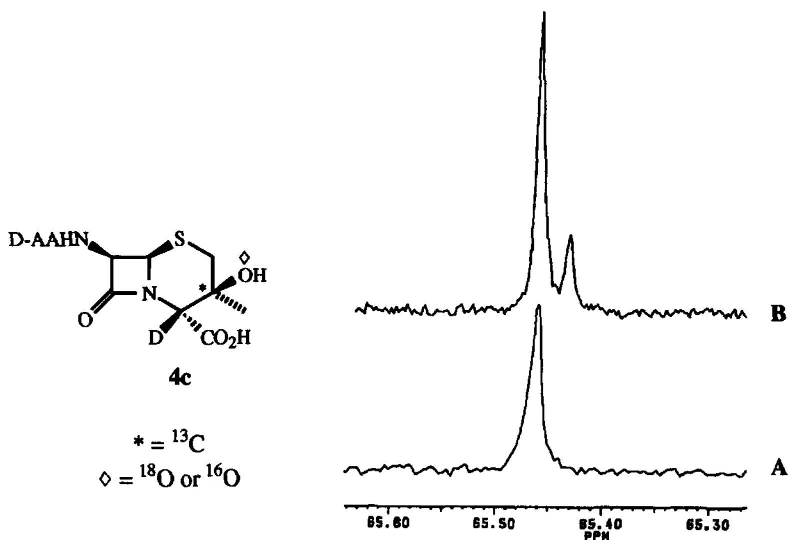



**Figure 2** : The C3-$^{13}$C resonance of **4c** isolated from A) incubation of **1c** in $H_2{}^{16}O$, B) incubation of **1c** in $H_2{}^{18}O$ (76 atom % $^{18}O$).

| Conditions | | | | | | | | % Incorporation $^{18}O$†† |
|---|---|---|---|---|---|---|---|---|
| DAC lactone **9** | | | | | | | | |
| | *m/z* ($MH^+$) | *356* | *357* | *358* | *359* | *360* | *361* | |
| | Calcul.[a] (%) | 100 | 18 | 7 | 1 | – | – | |
| $^{16}O_2/H_2{}^{16}O$ | Found (%) | 100 | 20 | 8 | 2 | – | – | – |
| $^{16}O_2/H_2{}^{18}O$ | Found (%) | 100 | 25 | 50 | 11 | 4 | 1 | 40 |
| Epoxide **6** | | | | | | | | |
| | *m/z* ($MH^+$) | *375* | *376* | *377* | *378* | *379* | *380* | |
| | Calcul.[a] (%) | 100 | 18 | 7 | 1 | – | – | |
| $^{16}O_2/H_2{}^{16}O$ | Found (%) | 100 | 20 | 8 | 2 | – | – | – |
| $^{16}O_2/H_2{}^{18}O$ | Found (%) | 100 | 20 | 22 | 5 | 2 | 1 | 17 |

**Table 4** : $^{18}O$-oxygen incorporations†† into the products **9** and **6** from an incubation of **5b** in $H_2{}^{18}O$ ($^{16}O_2/H_2{}^{16}O$ data from Table 2).

[a] calculated using oxygen at natural abundance.

## Conclusions

The results obtained clearly indicate that there is significant incorporation of oxygen from water into the oxygenated products formed by DAOC/DACS. It is believed that the incorporation values from $^{18}O_2$ or $H_2{}^{18}O$ into each individual product are complementary to each other, to within experimental error (± 10%)#. These observations give strong support to the proposed existence of a process involving exchange of oxygen with water from the medium. It is envisaged that such an exchange process could occur directly between the iron(IV)-oxene **7** and water, or between an intermediate subsequently formed and water.

Direct exchange between a metallo-oxene species and water has been reported in synthetic molybdenum complexes[11] and in iron[12] or chromium[13] containing porphyrins. Incorporation of $^{18}O$-oxygen into products formed in the presence of $H_2{}^{18}O$ has also been reported for some oxygenases where iron-oxenes are implicated; with hepatic cytochrome P-450 an 8.6% $^{18}O$-incorporation was observed in cyclohexanol derived from cyclohexane[14], and with toluene dioxygenase 68% $^{18}O$-incorporation was observed in the oxidation of indan to 1-indanol[15].

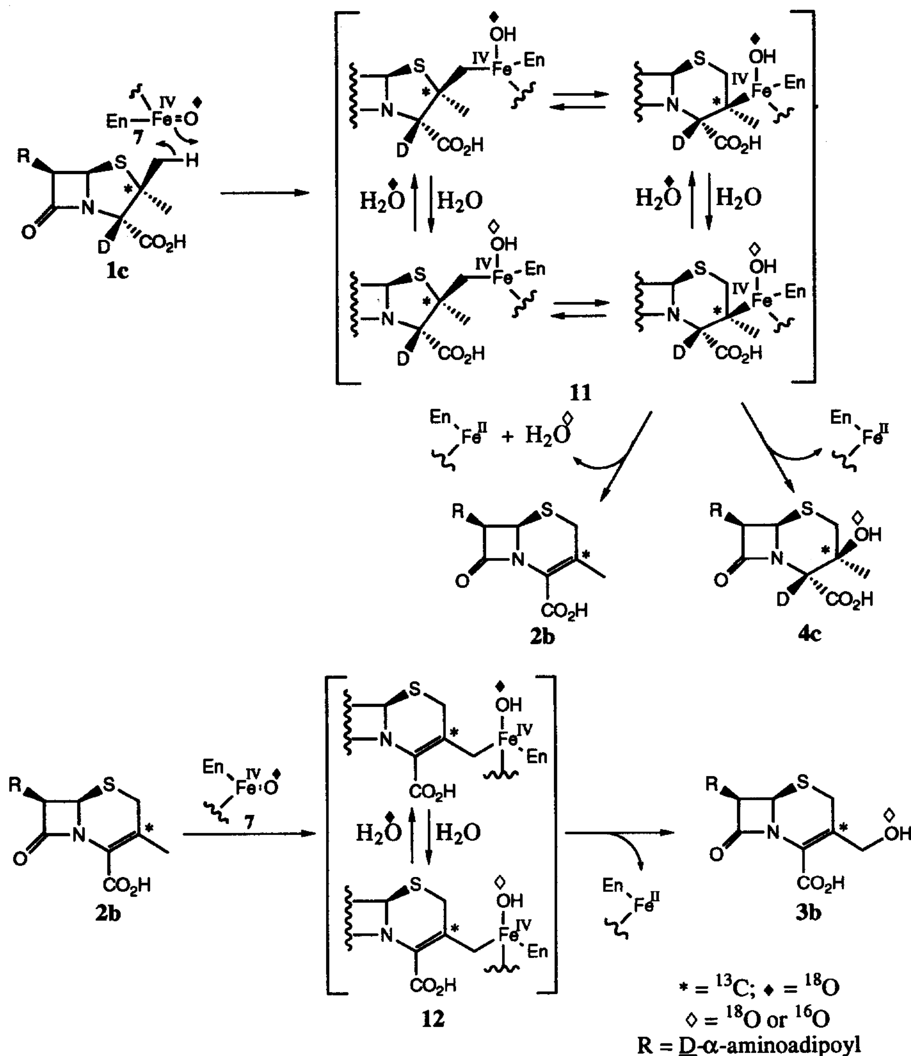
More detailed labelling studies with cytochrome P-450 revealed that oxygen incorporation from $H_2{}^{18}O$ into the product was complete if the iron-oxene was generated from iodosobenzene, but could not be observed if this species was generated from the physiological NADPH/$O_2$ system.[16] This result was interpreted as indicating that exchange between the iron-oxene and water could only occur if the iron-oxene was formed before binding of the substrate (as proposed to be the case with iodosobenzene), in which case it was free to exchange with water. In the physiological case it is known that the substrate binds before oxidation of the iron, and this was proposed to prevent exchange from occurring, possibly by preventing access of water to the active site.

Another pre-requisite for exchange in biological systems was proposed for horseradish peroxidase. Evidence suggested that exchange between the iron-oxene and water only occurred in this system in the presence of a source to protonate the ferryl-oxygen.[17] Exchange was observed only at acidic pH, when the iron-oxene was hydrogen-bonded to a protonated histidine residue of the active site.

In the present case of DAOC/DACS it is believed that the major exchange process does not occur directly between the ferryl species **7** and water, but between an enzyme-bound intermediate subsequently formed and water. This suggestion is based on the observation of the very large difference in label incorporation between the two products DAC **3a** and the epoxide cepham **6**, both derived from the conversion of the [4-$^2$H]exomethylene cephalosporin C **5b**. Previous studies on the conversion of **5b** gave strong indication that both products **3a** and **6** are formed from the same enzyme-bound intermediate (formed by addition of **7** to the double bond of **5b**) through isotope-induced branching of the normal pathway.[7] If the exchange process was occurring directly between **7** and water before reaction with substrate, then the same levels of $^{18}O$-incorporation should be observed in **3a** and **6**.

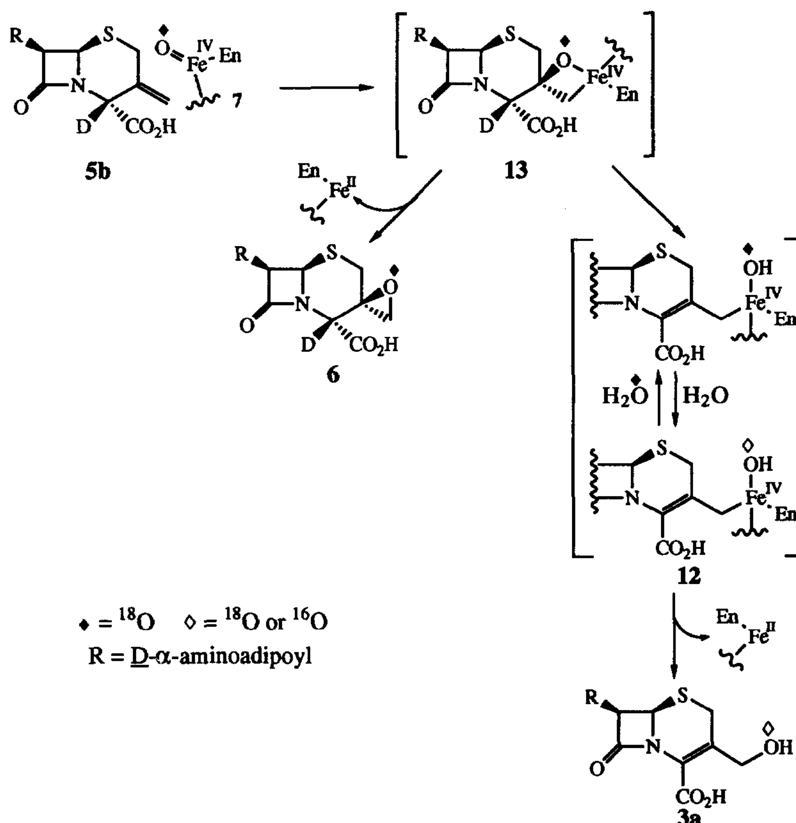
We propose that in DAOC/DACS the majority of exchange occurs after abstraction of a hydrogen atom from the substrate by the ferryl species **7**. At this point the intermediate formed involves a hydroxy group coordinated to the iron, a species which possibly undergoes exchange more readily than the iron-oxene **7**, no longer requiring the presence of a proton source for oxygen exchange. Such a hydroxyl intermediate is not thought to be involved in the formation of the epoxide cepham **6**, thus explaining the very high level of $^{18}O$-incorporation from $^{18}O_2$ observed into this product.

In terms of mechanism, it is proposed that the conversion of [2-$^{13}$C,3-$^{2}$H]penicillin N **1c** proceeds through the intermediate **11** which is capable of undergoing oxygen-exchange with water (Scheme 8, illustrated for an iron-oxene generated from $^{18}O_2$). The product [3-$^{13}$C]DAOC **2b** is further converted by DAOC/DACS to [3-$^{13}$C]DAC **3b**, through intermediate **12** which is also thought to be involved in a similar exchange process.[7] The different levels of labelling into the products **4c** and **3b** probably reflect different rates of conversion of the respective intermediates **11** and **12**, allowing for more or less exchange to occur.




**Scheme 8**

In the conversion of [4-$^{2}$H]exomethylene cephalosporin C **5b** (Scheme 9, illustrated for an iron-oxene generated from $^{18}O_2$) it is proposed that the intermediate **13** formed in the first irreversible step does not exchange oxygen with water, thus leading to a high retention of oxygen from $^{18}O_2$ into the epoxide **6** formed directly from it. The intermediate **13** is also converted further to intermediate **12** which then undergoes significant oxygen-exchange with water before reductive elimination of $Fe^{II}$ to generate the product DAC **3a**. The fact that similar $^{18}$O-incorporation values are observed in DAC (**3b** or **3a**) formed from either **2b** or **5b** supports the idea that the same intermediate **12** is responsible for oxygen-exchange in both cases. Direct exchange between the iron-oxene **7** and water is thought to occur only as a minor pathway, being responsible for the low $^{18}$O-incorporation from $H_2{}^{18}O$ into the epoxide cepham **6**.




**Scheme 9**

It is believed this study represents the first evidence for water incorporation into oxygenated products formed by an α-ketoglutarate dependent dioxygenase, and it has further refined our understanding of the mechanistic action of DAOC/DACS. In contrast, early studies into the hydroxylation of proline (for which the α-ketoglutarate dioxygenase prolyl 4-hydroxylase is responsible) using intact cell systems revealed there was incorporation of oxygen from $^{18}O_2$ into hydroxyproline, but not from $H_2{}^{18}O$.[18] However, in two closely related α-ketoacid dependent oxygenases, *p*-hydroxyphenylpyruvate oxygenase[19] and α-ketoisocaproate

oxygenase[20] significant $^{18}O$-incorporation values from $H_2{}^{18}O$ were also reported. Although different exchange processes were proposed for these systems, it is possible that a similar exchange mechanism to that proposed here could also be operating for these two enzymes.

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**Footnotes**

† Prepared by a method previously described.[4]

†† The incorporation values take into account the level of $^{18}O$-enrichment in the label source (98 atom % in $^{18}O_2$ and 76 atom % in $H_2{}^{18}O$).

††† We have previously shown that no exchange of the hydroxy oxygen occurs during lactonisation of **3** to **9**.[2]

# This large error is due to the technical difficulties involved in exactly reproducing the incubation conditions (e.g. enzyme activity and concentration) from one experiment to another.

## Experimental

### General

Standard chemical procedures as previously reported[4] were used. $^1H$-NMR spectra were recorded at 500 MHz on a Brucker AM 500 spectrometer, and are internally referenced to 3-trimethylsilyl tetradeuteriopropionate (TSP) ($\delta_{ref}$ = 0.00 ppm). Chemical shifts are reported in parts per million (δ p.p.m.) and coupling constants (J) are quoted to the nearest 0.5 Hz. $^{13}C$-NMR spectra were recorded at 125.8 MHz on a Brucker AM 500 spectrometer, and are internally referenced to 1,4-dioxan ($\delta_{ref}$ = 67.30 ppm). $^{13}C$ spectra were run using DEPT editing.

Electrospray mass spectra (ESMS) were run on a V.G. BIO-Q spectrometer. Peaks are quoted with percentage relative intensities in brackets.

High performance liquid chromatography (HPLC) was performed with either i) two Gilson 303 pumps, a Rheodyne 7125 injector, a Gilson holochrome variable wavelength detector and a column packed with Hypersil ODS (250 × 10 mm diameter) or ii) a Waters 600E Multisolvent Delivery System, a Rheodyne 7125 injector, a Waters 991 Photodiode Array Detector, and a column packed with Hypersil ODS (250 × 7 mm diameter).

### NMR calibration of aqueous samples

The sample to be calibrated was dissolved in $D_2O$ (0.5 - 1 ml) containing TSP (0.29 mM). The $^1H$-NMR spectrum (500 MHz, HOD suppressed) was recorded over at least 40 transients and the resonances due to TSP

and the β-lactam protons integrated. The concentration of the β-lactam compound was then calculated from the equation :

$$\text{β-lactam compound concentration} = \frac{1/2 \times \int \text{β-lactam protons}}{1/9 \times \int \text{TSP protons}} \times 0.29\ \text{mM}$$

**General procedure for DAOC/DACS incubations**

A cofactor solution was prepared with iron (II) sulphate (1.5 mg, 1 mM), α-ketoglutarate (21 mg, 14.4 mM), L-ascorbate (17.6 mg, 10 mM), dithiothreitol (30.8 mg, 20 mM) and ammonium sulphate (0.33 g, 0.25 M) in distilled water (10 ml), and pH adjusted to 7.5 with 1M NaOH. A solution of DAOC/DACS (2 ml, *ca.* 0.14 International Units) in TRIS-HCl buffer (pH 7.4, 50 mM) was pre-incubated with cofactor solution (200 μl) for 5 min at 27° C and 250 rpm. The substrate (1 mg) in water (800 μl) was added, and the resulting solution was incubated at 27°C and 250 rpm for 2h, after which time the protein was precipitated by the addition of acetone to 70% (v/v). After centrifugation (15 Krpm, 5 min, 0°C) the supernatant was evaporated to dryness, and the residue dissolved in $D_2O$ (0.5 ml) for examination by $^1$H-NMR (500 MHz, H̲OD suppressed).

**Incubation of [2-$^{13}$C,3-$^2$H]penicillin N 1c with DAOC/DACS under $^{16}O_2$**

[2-$^{13}$C,3-$^2$H]Penicillin N **1c**[4] (4 mg) was incubated with DAOC/DACS (4 ml, 0.74 IU) and cofactor solution (400 μl) according to the general procedure. Examination of the crude incubation mixture by $^1$H-NMR showed *ca.* 85% conversion to [3-$^{13}$C]DAOC **2b**, [3-$^{13}$C,4-$^2$H]-3β-hydroxycepham **4c** and [3-$^{13}$C]DAC **3b**. Integration of the β-lactam region showed the ratio of the products to be **2b**:**4c**:**3b** = 11:46:43. Purification of the crude incubation mixture by HPLC (Waters system, 10 mM aqueous $NH_4HCO_3$, 2 ml/min) gave **3b** (retention time 4.5 min, *ca.* 550 μg by NMR calibration), **4c** (retention time 5.8 min, *ca.* 765 μg by NMR calibration) and **2b** (retention time 10.4 min, *ca.* 174 μg by NMR calibration). The [3-$^{13}$C,4-$^2$H]-3β-hydroxycepham **4c** obtained from this experiment was analysed by $^{13}$C-NMR (overnight acquisition, 20,395 transients) and two peaks were observed, one for the C3 of **4c** (65.46 ppm, $^{13}$C̲Me) and one for the C3 of the corresponding lactone **8** (81.37, $^{13}$C̲Me).

Data for [3-$^{13}$C]DAOC **2b** : $\delta_H$ (500 MHz, $D_2O$, H̲OD suppressed) : 1.67-1.95 (4H, 2 × m, $CH̲_2CH̲_2CH_2CO$), 1.94 (3H, d, $J_{13C\text{-}H}$ 7 Hz, $^{13}CH̲_3$), 2.41 (2H, t, J 7 Hz, $CH̲_2CO$), 3.25 and 3.59 (2H, AB part of ABX system (X=$^{13}$C), $J_{AB}$ 18 Hz, $J_{AX}$ 7 Hz, $J_{BX}$ 5 Hz, $^{13}CCH̲_2S$), 3.75 (1H, t, J 6 Hz, $H_3N^+CH̲CO_2^-$), 5.08 and 5.56 (2H, 2 × d, J 4 Hz, NHCH̲CH̲S); Partial $\delta_C$ (125.8 MHz, $D_2O$) : 122.80 (s, C=$^{13}$C̲); *m/z* (ESMS) : 358 (14%), 359 ([MH$^+$], 100%), 360 (20%), 361 (9%), 362 (4%)

Data for [3-$^{13}$C,4-$^2$H]-3β-hydroxycepham **4c** : $\delta_H$ (500 MHz, $D_2O$, H̲OD suppressed) : 1.38 (3H, d, $J_{13C\text{-}H}$ 4 Hz, $^{13}CH̲_3$), 1.70-1.97 (4H, 2 × m, $CH̲_2CH̲_2CH_2CO$), 2.43 (2H, t, J 7 Hz, $CH̲_2CO$), 2.64 (1H, dd, $J_{H\text{-}H}$ 14 Hz, $J_{H\text{-}13C}$ 5 Hz, $^{13}$CCH̲HS), 3.55 (1H, d, $J_{H\text{-}H}$ 14 Hz, $^{13}$CCHH̲S), 3.73-3.77 (1H, m, $H_3N^+CH̲CO_2^-$), 5.28 and 5.44 (2H, 2 × d, J 4 Hz, NHCH̲CH̲S); Partial $\delta_C$ (125.8 MHz, $D_2O$) : 65.46 (s, $^{13}C̲H_3$); *m/z* (ESMS) : 377 (15%), 378 ([MH$^+$], 100%), 379 (26%), 380 (10%), 381 (2%)

Data for [3-$^{13}$C]DAC **3b** : $\delta_H$ (500 MHz, $D_2O$, H̲OD suppressed) : 1.70-1.96 (4H, 2 × m, $CH̲_2CH̲_2CH_2CO$), 2.43 (2H, t, J 7 Hz, $CH̲_2CO$), 3.47 and 3.66 (2H, AB part of ABX system (X=$^{13}$C), $J_{AB}$ 18 Hz, $J_{AX}$ 7 Hz, $J_{BX}$ 6 Hz, $^{13}CCH̲_2S$), 3.74 (1H, t, J 6 Hz, $H_3N^+CH̲CO_2^-$), 4.26 and 4.30 (2H, AB part of ABX system (X=$^{13}$C),

$J_{AB}$ 13 Hz, $J_{AX}$ 4 Hz, $J_{BX}$ 3 Hz, $^{13}CC\underline{H}_2OH$) 5.13 and 5.63 (2H, 2 × d, J 5 Hz, NHC$\underline{H}$C$\underline{H}$S); Partial $\delta_C$ (125.8 MHz, $D_2O$) : 121.81 (s, C=$^{13}\underline{C}$);
*m/z* (ESMS) : 374 (14%), 375 ([$MH^+$], 100%), 376 (20%), 377 (11%), 378 (3%)

**Incubation of [2-$^{13}$C,3-$^2$H]penicillin N 1c with DAOC/DACS under $^{18}O_2$**

The head space of an intact glass vial of $^{18}O_2$ (MSD Isotopes, 98 atom % $^{18}$O, 100 ml) was repeatedly evacuated and flushed with argon, and then septum sealed. A solution of [2-$^{13}$C,3-$^2$H]-penicillin N **1c** (1.75 mg) in water (1.65 ml) was degassed and flushed with argon, after addition of the standard cofactor solution (350 µl). DAOC/DACS (5 ml, 0.02 IU) was also briefly degassed and flushed with argon. After breaking the glass neck seal of the septum sealed $^{18}O_2$ vial, the enzyme was introduced *via* syringe, followed by the solution of the substrate and cofactors. The sealed vial was incubated for 2h at 27°C and 250 rpm. Acetone was added *via* syringe to a final concentration of 70% (v/v), the vial opened to air, and the incubation worked up according to the general procedure. Examination by $^1$H-NMR showed *ca.* 87% conversion to [3-$^{13}$C]DAOC **2b**, [3-$^{13}$C,4-$^2$H]-3β-hydroxycepham **4c** and [3-$^{13}$C]DAC **3b**. Integration of the β-lactam region revealed the ratio of products to be **2b**:**4c**:**3b** = 20:53:27. Purification of the crude incubation mixture as before gave **3b** (*ca.* 128 µg by NMR calibration), **4c** (*ca.* 185 µg by NMR calibration) and **2b** (*ca.* 143 µg by NMR calibration), identified by $^1$H-NMR and HPLC retention times. Mass spectral analysis of the products indicated no $^{18}$O incorporation into **2b**, 71% incorporation of $^{18}$O into **4c** and 52% incorporation into **3b**[††] (see below).

This experiment was repeated with [2-$^{13}$C,3-$^2$H]penicillin N **1c** (2.3 mg), cofactor solution (300 µl) and DAOC/DACS (3.7 ml, 0.07 IU). $^1$H-NMR analysis of the crude incubation mixture showed *ca.* 75% conversion to **2b**, **4c** and **3b** [ratio **2b**:**4c**:**3b** = 26:52:22]. Purification by HPLC as before gave **3b** (*ca.* 170 µg by NMR calibration), **4c** (*ca.* 420 µg by NMR calibration) and **2b** (*ca.* 213 µg by NMR calibration), identified by $^1$H-NMR and HPLC retention times. Mass spectral analysis of the products showed no $^{18}$O incorporation into **2b**, 69% incorporation of $^{18}$O into **4c** and 57% incorporation into **3b**[††] (see below). The [3-$^{13}$C,4-$^2$H]-3β-hydroxycepham **4c** obtained on this second experiment was analysed by $^{13}$C-NMR (overnight acquisition, 31,925 transients), and an $^{18}$O-$^{13}$C shift was observed for both **4c** and the lactone **8**. The $^{16}$O-$^{13}$C peak for both **4c** and **8** had an intensity of about 30% of that of the $^{18}$O-$^{13}$C peak, confirming the mass spectral results for $^{18}$O incorporation (see Figure 1).

Mass spectrum of [3-$^{13}$C]DAOC **2b** (ESMS) :

| | | | | | | |
|---|---|---|---|---|---|---|
| | *m/z* ($MH^+$) | *358* | *359* | *360* | *361* | *362* |
| Experiment 1a | Found (%) | 12 | 100 | 20 | 9 | 3 |
| | *m/z* ($MNa^+$) | *380* | *381* | *382* | *383* | *384* |
| Experiment 1b | Found (%) | 20 | 100 | 21 | 15 | 4 |

Mass spectrum of [3-$^{13}$C,4-$^2$H]-3-β-Hydroxycepham **4c** (ESMS) :

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | *m/z* ($MH^+$) | *377* | *378* | *379* | *380* | *381* | *382* |
| Experiment 1a | Found (%) | 9 | 42 | 29 | 100 | 21 | 9 |
| | *m/z* ($MNa^+$) | *399* | *400* | *401* | *402* | *403* | *404* |
| Experiment 1b | Found (%) | 11 | 47 | 31 | 100 | 20 | 9 |

Mass spectrum of [3-$^{13}$C]DAC **3b** (ESMS) :

| | *m/z* ($MH^+$) | *374* | *375* | *376* | *377* | *378* | *379* |
|---|---|---|---|---|---|---|---|
| **Experiment 1a** | **Found (%)** | 5 | 90 | 34 | 100 | 25 | 13 |
| **Experiment 1b** | **Found (%)** | 10 | 74 | 21 | 100 | 20 | 15 |

$^{13}$C-NMR analysis (125.8 MHz, $D_2O$) of [3-$^{13}$C-4-$^{2}$H]-3β-hydroxycepham **4c** and lactone **8** :

| **4c** | $^{13}\underline{C}$-$^{16}$OH | $^{13}\underline{C}$-$^{18}$OH | Δδ |
|---|---|---|---|
| δ(ppm) | 65.45(7) | 65.42(7) | 0.03(0) |

| **8** | $^{13}\underline{C}$-$^{16}$OH | $^{13}\underline{C}$-$^{18}$OH | Δδ |
|---|---|---|---|
| δ(ppm) | 81.37(3) | 81.41(6) | 0.04(3) |

**Incubation of [4-$^2$H]exomethylene cephalosporin C 5b with DAOC/DACS under $^{16}O_2$**

[4-$^2$H]Exomethylene cephalosporin C **5b**[7] (5 mg) was incubated with DAOC/DACS (10 ml, 0.7 IU) and cofactor solution (1 ml) according to the general procedure. Examination of the crude incubation mixture by $^1$H-NMR showed *ca.* 65% conversion to DAC **3a** and **6**[7] (ratio **3a**:**6** = 4:1). On purification of the crude incubation mixture by HPLC (Gilson system, 10 mM aqueous $NH_4HCO_3$, 2 ml/min) the two products eluted as one single peak (retention time 10 min). The mixture of the two products was dissolved in distilled formic acid (5 ml) and left to stand at room temperature for 45 min. The formic acid was evaporated under *vacuum* with no heating. $^1$H-NMR analysis showed complete lactonisation of DAC **3a** to the lactone **9**[6], and only minor decomposition of the epoxide cepham **6**. The reaction mixture was purified by HPLC (Waters system, 4.5% MeCN/$H_2O$, 1 ml/min) to give a mixture of the epoxide **6** with some impurities (retention time 4.5 min), and the lactone **9** (retention time 15 min). The fraction containing the epoxide **6** was further purified by HPLC (Waters system, 10 mM aqueous $NH_4HCO_3$, 1 ml/min) to give the pure epoxide cepham **6** (retention time 8.5 min).

Data for **9** : $\delta_H$ (500 MHz, $D_2O$, $\underline{H}$OD suppressed) : 1.68-1.96 (4H, m, CHC$\underline{H}_2$C$\underline{H}_2$), 2.44 (2H, t, J 7 Hz, C$\underline{H}_2$CO), 3.74 and 3.91 (2H, ABq, J 18 Hz, C$\underline{H}_2$S), 3.74 (1H, t, J 6Hz, $H_3N^+$C$\underline{H}$$CO_2^-$), 5.10 and 5.15 (2H, ABq, J 18Hz, C$\underline{H}_2$OCO), 5.24 and 5.80 (2H, 2 × d, J 5 Hz, HNC$\underline{H}$C$\underline{H}$S);

$\delta_C$ (125.8 MHz, $D_2O$) : 21.62 (t, CHCH$_2\underline{C}$H$_2$), 23.41 (t, $\underline{C}$H$_2$S) 30.61 (t, CH$\underline{C}$H$_2$), 35.32 (t, $\underline{C}$H$_2$CO), 55.31, 57.96 and 60.57 (3 × d, NH$\underline{C}$H$\underline{C}$HS and $H_3N^+\underline{C}$H$CO_2^-$), 73.49 (t, $\underline{C}$H$_2$OCO) 123.75 and 144.59 (2 × s, $\underline{C}$=$\underline{C}$), 165.69, 170.19, 174.91 and 177.15 (4 × s, 4 × $\underline{C}$=O); *m/z* (ESMS) : 356 ([$MH^+$], 100%), 357 (20%), 358 (8%), 359 (2%)

Data for **6** : $\delta_H$ (500 MHz, $D_2O$, $\underline{H}$OD suppressed) : 1.66-1.95 (4H, m, CHC$\underline{H}_2$C$\underline{H}_2$), 2.44 (2H, t, J 7 Hz, C$\underline{H}_2$CO), 2.44 and 3.64 (2H, ABq, J 15 Hz, C$\underline{H}_2$S), 3.28 and 3.40 (2H, 2 × d, J 4 Hz, epoxide H's), 3.74 (1H, *ca.* t, J 6 Hz, $H_3N^+$C$\underline{H}$$CO_2^-$), 5.37 and 5.50 (2H, 2 × d, J 4 Hz, HNC$\underline{H}$C$\underline{H}$S) (the couplings were confirmed by a COSY experiment); *m/z* (ESMS) : 375 ([$MH^+$], 100%), 376 (20%), 377 (8%), 378 (2%), 397 ([$MNa^+$], 44%).

**Incubation of [4-$^{2}$H]exomethylene cephalosporin C 5b with DAOC/DACS under $^{18}O_2$**

DAOC/DACS (2 ml, 1.3 IU) in TRIS-HCl buffer (50 mM, pH 7.4) was exchanged into ammonium hydrogen carbonate buffer (7 ml, 10 mM, pH 7.8, 2 mM DTT) on a pre-equilibrated Sephadex column (PD-10), and this solution was rapidly evacuated and flushed with argon. The head space of an intact glass vial of $^{18}O_2$ (MSD Isotopes, 98 atom % $^{18}$O, 100 ml) was repeatedly evacuated and flushed with argon, and then septum sealed. To a solution of [4-$^{2}$H]exomethylene cephalosporin C **5b** (8 mg) in water (1 ml) was added the cofactor solution (800 µl), and the resulting solution was also repeatedly evacuated and flushed with argon. After breaking the glass neck seal of the septum sealed $^{18}O_2$ vial, the enzyme was introduced *via* syringe, followed by the solution of the substrate and cofactors. The sealed vial was incubated for 2h at 27°C and 250 rpm. Acetone was added *via* syringe to a final concentration of 70% (v/v), the vial opened to air and the incubation worked up according to the general procedure. Examination by $^{1}$H-NMR showed *ca.* 4% conversion to DAC **3a** and the epoxide **6**. Integration of the β-lactam region revealed the ratio of products to be **3a**:**6** = 2.5:1. Purification of the crude incubation mixture by HPLC (Gilson system, 10 mM aqueous $NH_4HCO_3$, 2 ml/min) gave a mixture of DAC **3a** (*ca.* 210 µg by NMR calibration) and epoxide **6** (*ca.* 80 µg by NMR calibration) coeluting as one peak (retention time 9.7 min). This mixture was treated with formic acid as before to give after HPLC purification the DAC lactone **9** (*ca.* 105 µg by $^{1}$H-NMR analysis) and the epoxide **6** (*ca.* 60 µg by NMR calibration). Mass spectral analysis of the two products showed 46% incorporation of $^{18}$O into the DAC lactone **9** and 94% incorporation into the epoxide **6**†† (see below).

This experiment was repeated with [4-$^{2}$H]exomethylene cephalosporin C **5b** (8 mg), cofactor solution (700 µl), and DAOC/DACS (5 ml, 1.2 IU) (the enzyme was used in the TRIS-HCl buffer, without exchange as before). $^{1}$H-NMR analysis of the crude incubation mixture showed *ca.* 10% conversion to **3a** and **6** [ratio **3a**:**6** = 2:1]. Purification by HPLC as before gave a mixture of **3a** (*ca.* 442 µg by NMR calibration) and **6** (*ca.* 244 µg by NMR calibration) coeluting as one peak. This mixture was treated with formic acid as before, to give after HPLC purification the DAC lactone **9** (*ca.* 228 µg by $^{1}$H-NMR calibration) and the epoxide **6** (*ca.* 134 µg by $^{1}$H-NMR calibration). Mass spectral analysis of the two products showed 56% incorporation of $^{18}$O into the lactone **9** and 95% into the epoxide **6**†† (see below).

Mass spectrum of DAC-lactone **9** (ESMS) :

| | *m/z* (MH$^{+}$) | *356* | *357* | *358* | *359* | *360* | *361* |
|---|---|---|---|---|---|---|---|
| Experiment 1a | Found (%) | 100 | 24 | 88 | 22 | 7 | 2 |
| Experiment 1b | Found (%) | 77 | 22 | 100 | 19 | 7 | 1 |

Mass spectrum of the epoxide **6** (ESMS) :

| | *m/z* (MH$^{+}$) | *375* | *376* | *377* | *378* | *379* | *380* |
|---|---|---|---|---|---|---|---|
| Experiment 1a | Found (%) | 9 | 12 | 100 | 19 | 8 | 2 |
| Experiment 1b | Found (%) | 7 | 9 | 100 | 20 | 8 | 2 |

**Incubation of $^{18}$O-labelled epoxide cepham 6 with DAOC/DACS**

The $^{18}$O-labelled epoxide **6** (*ca.* 200 µg, 94% $^{18}$O label) obtained from the incubations of [4-$^{2}$H]exomethylene cephalosporin C **5b** under $^{18}O_2$ was incubated with DAOC/DACS (0.5 ml) according to

the general procedure. $^1$H-NMR analysis showed complete conversion to the aldehyde cephalosporoate **10**. Purification by HPLC (Waters system, 0.1% $HCO_2H$ in water, 2 ml/min, monitoring at λ 300 nm) gave the aldehyde **10** (retention time 6.6 min). Mass spectral analysis showed **10** retained only about 14% $^{18}$O-label.

Mass spectrum of aldehyde **10** (ESMS) :

| | *m/z* ($MH^+$) | *388* | *389* | *390* | *391* | *392* |
|---|---|---|---|---|---|---|
| $^{16}O_2/H_2{}^{16}O$ | Calcul. (%) | - | 100 | 19 | 8 | 1 |
| $^{16}O_2/H_2{}^{16}O$ | Found (%) | 9 | 100 | 19 | 24 | 11 |

**Incubation of [2-$^{13}$C,3-$^2$H]penicillin N 1c with DAOC/DACS in $H_2{}^{18}O$**

DAOC/DACS (2 ml) was concentrated down to 0.5 ml on a centrifuge, using a Centricon 10 microconcentrator (6 Krpm, 25 min, 0°C), and 0.5 ml of $H_2{}^{18}O$ (Aldrich Chemical Co., 95 atom % $^{18}$O) was added. A cofactor solution (300 μl) that had previously been lyophilised was dissolved in 0.5 ml $H_2{}^{18}O$ and added to the enzyme solution, which was then pre-incubated for 5 min at 27°C and 250 rpm, after which [2-$^{13}$C,3-$^2$H]penicillin N **1c** (2 mg) dissolved in 1 ml $H_2{}^{18}O$ and added. The resulting solution (final volume 2.5 ml) was incubated for 2h at 27°C and 250 rpm, after which time the protein was precipitated by the addition of acetone to 70% (v/v), and the incubation worked up as in the general procedure. Analysis by $^1$H-NMR showed *ca.* 59% conversion to **2b**, **4c** and **3b** [ratio **2b**:**4c**:**3b** = 21:44:35]. Purification by HPLC (Gilson system, 10 mM aqueous $NH_4HCO_3$, 4 ml/min) gave [3-$^{13}$C]DAC **3b** (retention time 5 min, *ca.* 174 μg by NMR calibration), [3-$^{13}$C,4-$^2$H]-3β-hydroxycepham **4c** (retention time 6.3 min, *ca.* 402 μg by NMR calibration) and [3-$^{13}$C]DAOC **2b** (retention time 13 min, *ca.* 354 μg by NMR calibration). Mass spectral analysis of the products indicated no $^{18}$O incorporation into **2b**, about 16% incorporation of $^{18}$O into **4c** and 50% incorporation into **3b** (see below). Considering the $^{18}$O enrichment of the incubation was 76%, the corrected $^{18}$O incorporation into **4c** is 21%, and into **3b** 66%. Analysis of **4c** by $^{13}$C-NMR (overnight acquisition, 29,437 transients) showed a peak for the $^{18}$O-$^{13}\underline{C}$ resonance with an intensity of about 17% of the peak for the $^{16}$O-$^{13}\underline{C}$ resonance, in accordance with mass spectral results (see Figure 2).

Mass spectrum of [3-$^{13}$C]DAOC **2b** (ESMS) :

| *m/z* ($MH^+$) | *358* | *359* | *360* | *361* | *362* |
|---|---|---|---|---|---|
| Found (%) | 14 | 100 | 22 | 9 | 2 |

Mass spectrum of [3-$^{13}$C,4-$^2$H]-3β-Hydroxycepham **4c** (ESMS) :

| *m/z* ($MH^+$) | *377* | *378* | *379* | *380* | *381* | *382* |
|---|---|---|---|---|---|---|
| Found (%) | 18 | 100 | 23 | 26 | 7 | 3 |

Mass spectrum of [3-$^{13}$C]-DAC **3b** (ESMS) :

| *m/z* ($MH^+$) | *374* | *375* | *376* | *377* | *378* | *379* |
|---|---|---|---|---|---|---|
| Found (%) | 17 | 92 | 37 | 100 | 21 | 10 |

$^{13}$C-NMR analysis (125.8 MHz, $D_2O$) of [3-$^{13}$C,4-$^2$H]-3-β-Hydroxycepham **4c** :

| **4c** | $^{13}\underline{C}$-$^{16}$OH | $^{13}\underline{C}$-$^{18}$OH | Δδ |
|---|---|---|---|
| δ(ppm) | 65.46(1) | 65.43(4) | 0.03 |

**Control incubations of $^{18}O$-labelled 3b and 4c with DAOC/DACS**

The $^{18}O$-labelled products **3b** (*ca.* 100 μg, 16% $^{18}O$) and **4c** (*ca.* 400 μg, 50% $^{18}O$) obtained on the incubation of [2-$^{13}$C,3-$^{2}$H]penicillin N **1c** in $H_2{}^{18}O$ were independently incubated with denatured DAOC/DACS (1 ml) (pre-inactivated by heat) and cofactors according to the general procedure. $^{1}$H-NMR analysis of the crude incubation mixtures showed the two products were still intact, and these were isolated using the usual HPLC system. Analysis by electrospray mass spectrometry of the recovered **3b** and **4c** showed no loss of label had occurred (to within experimental error).

Mass spectrum of [3-$^{13}$C,4-$^{2}$H]-3β-Hydroxycepham **4c** (ESMS) :

| | *m/z* ($MH^+$) | *377* | *378* | *379* | *380* | *381* | *382* |
|---|---|---|---|---|---|---|---|
| Before control inc. | Found (%) | 18 | 100 | 23 | 26 | 7 | 3 |
| After control inc. | Found (%) | 21 | 100 | 31 | 34 | 8 | 2 |

Mass spectrum of [3-$^{13}$C]DAC **3b** (ESMS) :

| | *m/z* ($MH^+$) | *374* | *375* | *376* | *377* | *378* | *379* |
|---|---|---|---|---|---|---|---|
| Before control inc. | Found (%) | 17 | 92 | 37 | 100 | 21 | 10 |
| After control inc. | Found (%) | 22 | 80 | 33 | 100 | 17 | 15 |

**Incubation of [4-$^{2}$H]exomethylene cephalosporin C 5b with DAOC/DACS in $H_2{}^{18}O$**

DAOC/DACS (1.5 ml, 0.3 IU) was concentrated down to 0.5 ml as described for the incubation of [2-$^{13}$C,3-$^{2}$H]penicillin N **1c** in $H_2{}^{18}O$. Exactly the same procedure was followed using [4-$^{2}$H]exomethylene cephalosporin C **5b** (2.7 mg) and lyophilised cofactor solution (400 μl) (final volume 2.5 ml). Analysis by $^{1}$H-NMR showed 18% conversion to DAC **3a** and the epoxide **6** [ratio **3a**:**6** = 3:1]. Isolation of the products as described before gave DAC lactone **9** (*ca.* 96 μg by $^{1}$H-NMR calibration) and the epoxide **6** (*ca.* 25 μg by $^{1}$H-NMR calibration). Mass spectral analysis of the products showed 30% $^{18}O$ incorporation into **9** and 13% incorporation into the epoxide **6**. Considering the $^{18}O$ enrichment of the incubation solution was 76%, the corrected $^{18}O$-incorporation into **9** is 40%, and into **6** 17%.

Mass spectrum of DAC-lactone **9** (ESMS) :

| *m/z* ($MH^+$) | *356* | *357* | *358* | *359* | *360* | *361* |
|---|---|---|---|---|---|---|
| Found (%) | 100 | 25 | 50 | 11 | 4 | 1 |

Mass spectrum of epoxide **6** (ESMS) :

| *m/z* ($MH^+$) | *375* | *376* | *377* | *378* | *379* | *380* |
|---|---|---|---|---|---|---|
| Found (%) | 100 | 20 | 22 | 5 | 2 | 1 |

**Control incubation of 6 with DAOC/DACS**

The unlabelled epoxide cepham **6** [*ca.* 200 μg; previously obtained from an incubation of [4-$^{2}$H]exomethylene cephalosporin C **5b** under normal conditions] was dissolved in $H_2{}^{18}O$ (0.5 ml), and a previously lyophilised cofactor solution (200 μl) was also dissolved in $H_2{}^{18}O$ (0.5 ml). These two solutions were added to denatured DAOC/DACS (250 μl) and the incubation performed as usual. $^{1}$H-NMR analysis of the crude incubation mixture showed no conversion of the epoxide **6** which was recovered by HPLC purification (Waters system, 10 mM aqueous $NH_4HCO_3$, 1 ml/min). Analysis by mass spectrometry showed no incorporation of $^{18}$O-label into **6** had occurred.

Mass spectrum of **6** (ESMS) :

| | *m/z* ($MH^+$) | *375* | *376* | *377* | *378* | *379* | *380* |
|---|---|---|---|---|---|---|---|
| Before control inc. | Found (%) | 100 | 20 | 8 | 2 | – | – |
| After control inc. | Found (%) | 100 | 16 | 11 | 3 | 1 | – |

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