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COMPETING PATHWAYS IN THE OXIDATION OF Z-3-ETHYLIDINE CEPHALOSPORIN C BY THE ENZYME DEACETOXYCEPHALOSPORIN C SYNTHASE (DAOCS)

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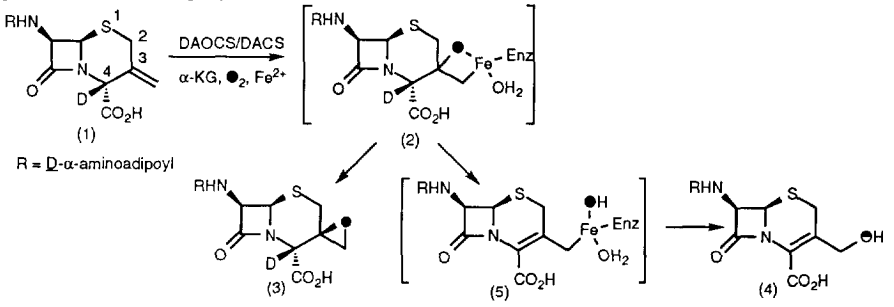
Abstract : DAOCS converts Z-3-ethylidene cephalosporin C to two diastereomeric alcohols in the ratio 7:2 with nearly complete incorporation of ^{18}O from $^{18}O_2$.

Recently,[1] it has been shown that DAOC/DACS (the bifunctional α-ketoglutarate dependent dioxygenase responsible for the conversion of penicillin N to deacetoxycephalosporin C and the hydroxylation of the latter to deacetylcephalosporin C) displayed epoxidase activity when 4-deuterio-3-exomethylene cephalosporin C (1) was employed as a substrate (Scheme 1).



Scheme 1

It was proposed that the spiro epoxide (3) was a shunt metabolite, formed as a consequence of the operation of a deuterium kinetic isotope effect, and that (3) and the other product i.e. deacetylcephalosporin C (DAC) (4) were formed from a common ferrocycle intermediate (2). Using $^{18}O_2$, the epoxide oxygen was shown to be derived largely (> 90 %) from molecular dioxygen, whereas the oxygen of the hydroxyl group of (4) was derived almost equally from both water and oxygen* . The oxygen exchange was proposed to occur *via* the iron-carbon species (5).

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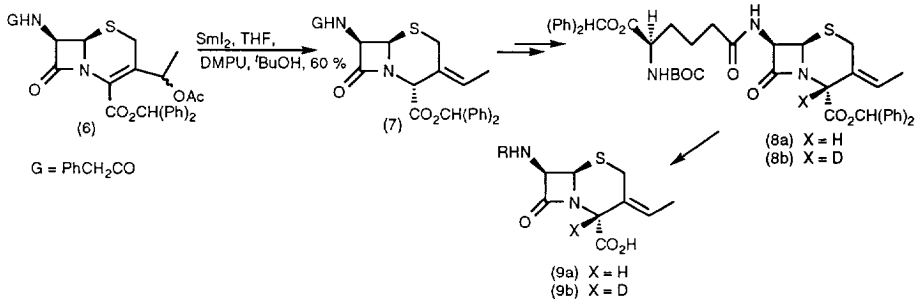
* In earlier work,[2] Baldwin *et al.* had found that incubation of the 4-deuterio-3-exomethylene cepham (1) with DAOCS/DACS under $^{18}O_2$ led to incorporation of 51 % (average) ^{18}O into deacetylcephalosporin C (DAC) (4) and 95 % (average) ^{18}O into the co-formed epoxide (3), while an incubation in $H_2{}^{18}O$ gave a 40 % ^{18}O incorporation into DAC (4) and a 17 % ^{18}O incorporation into the epoxide (3).

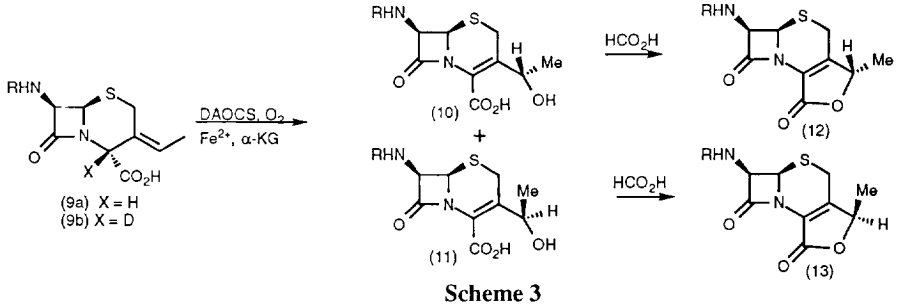
In order to assess the generality of such epoxidase activity, we prepared an alkylidene cephalosporin as a substrate for this class of enzyme. Thus Z-3-ethylidene cephalosporin C (8a) was synthesised, by samarium diiodide mediated formation of the Z-ethylidene cepham (7) from a mixture of the diastereomeric acetates[3] (6) as the key step (Scheme 2).



Scheme 2

Incorporation of deuterium into (8a) to give (8b) was accomplished by stirring (8a) in a buffer[4] at 35°C for 24 hours followed by deprotection using standard procedures[5] to give (9a) and (9b) respectively. Mass spectral analysis** showed the incorporation of at least 85 % deuterium into (8b).

Due to a lack of sufficiently active DAOC/DACS, incubations of (9a) and (9b) were performed with readily available DAOCS, the bacterial enzyme which ring expands penicillin N to DAOC, but which has also been shown to display epoxidase activity with 4-deuterioexomethylene cepham (1).[6] In this case the enzyme converted both (9a) and the deuteriated form (9b) into a mixture of two diastereomeric alcohols (10) and (11), in the ratio of 7:2 (Scheme 3). Thus no epoxide was obtained and no isotope effect on the product ratio was observed.

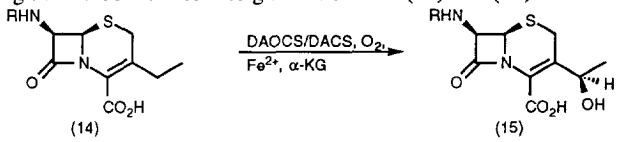


Scheme 3

The stereochemistry of (10) was determined by conversion (formic acid) to lactone (12), on which n.O.e. studies were performed. Insufficient quantities of the minor alcohol (11) were available for n.O.e. studies, however doping experiments (^{1}H NMR and HPLC) with a cephem alcohol (15), isolated by Wood[7] in an incubation of 3-ethyl cephalosporin C (14) with DAOCS/DACS, confirmed the identity of the "Wood alcohol" (15) with the minor alcohol (11) (Scheme 4). N.O.e. studies on the lactone (13) derived from the

** Mass spectral data for (9a): m/e (ESMS) 374 (6%), 373 (18%), 372 (MH^+, 100%); Mass spectral data for (9b): 375 (6%), 374 (14%), 373 (MH^+, 100%), 372 (33%).

"Wood alcohol" (15) were complementary to those found for the lactone (12) derived from the major alcohol (10) (Table 1) permitting the stereochemical assignment of both (10) and (11).



Scheme 4

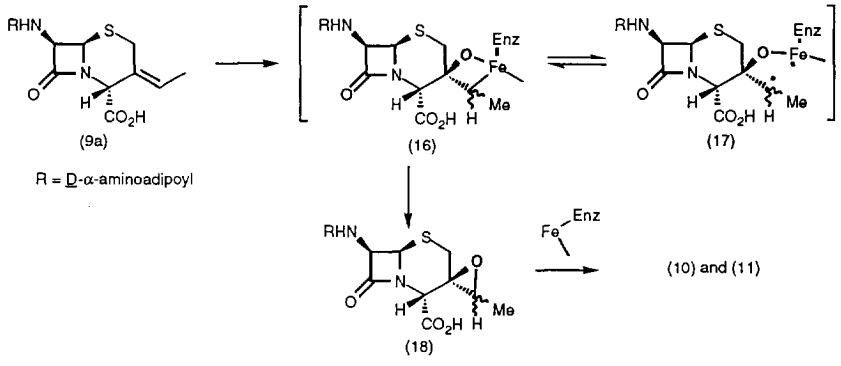
N.O.e data for (12)

Irradiation	n.O.e.
lactone-Me	12 % to lactone H
lactone-Me	5 % to CHCHS
2α-$C\underline{H}_2S$	4 % to CHCHS
CHC$\underline{H}$S	7 % to C$\underline{H}$CHS

N.O.e data for (13)

Irradiation	n.O.e.
lactone-Me	21 % to lactone H
lactone-Me	9 % to 2β-$C\underline{H}_2S$
2α-$C\underline{H}_2S$	9 % to 2β-$C\underline{H}_2S$
C$\underline{H}$CHS	10 % to CHC$\underline{H}$S
2β-$C\underline{H}_2S$	26 % to 2α–$C\underline{H}_2S$
2β-$C\underline{H}_2S$	5 % to lactone-Me

Table 1



Scheme 5

In view of the large differences in oxygen isotope incorporation into (3) and (4), *vide supra*, oxygen-18 studies were undertaken. In contrast, in this case, using $^{18}O_2$, both alcohols (10) and (11) were essentially fully labelled (98% and 99% respectively), whereas with $H_2{}^{18}O$ incorporations of 9% and 12% ^{18}O were observed†. These results strongly suggest that both (10) and (11) are derived from the "epoxide" pathway, e.g. (2) to (3) in the previous study*. We propose that as in the previous case a ferrocycle (16) is initially

† This seemingly large combined incorporation of approximately 110 % from $^{18}O_2$ and $H_2{}^{18}O$ may result from exchange and isotopic biasing of an intermediate e.g. ferryl (IV) oxene with water, prior to cycloaddition; for analogous exchange with a related alpha-keto acid dependant ferrous oxygenase see Sabourin P.J. and Bieber L.L., *J. Biol. Chem.*, **1982**, *257*, 7468.

formed which on reversible ring opening to the diradical form (17) with bond rotation inverts the methyl stereochemistry. Reductive elimination, as previously proposed, would give the isomeric epoxides (18) (Scheme 5). Ring opening with loss of the C-4 proton, or deuteron, occasioned by the extra methyl group in this series then provides the epimeric alcohols with the observed high level of ^{18}O incorporation. This process is probably mediated by the iron centre acting as a Lewis acid. Thus the combined stereochemical and oxygen isotope studies presented here further reveal the delicate balance between competing pathways in these dioxygenases.

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3. Kang, H-Y.; Seo Cho, Y.; Yeong Koh, H.; Ho Chang, M. *Syn. Commun.*, **1993**, *23*, 2977.

4. K_2HPO_4 / K_3PO_4, 14:1, w/w in D_2O, the volume of THF and D_2O being the same.

5. Trifluoroacetic acid/Anisole, 4:1, v/v, in toluene under ambient conditions.

6. DAOCS is the effectively monofunctional enzyme responsible for the ring expansion of penicillin N to DAOC. DAOCS was prepared from *S. clavuligerus*, cloned and overexpressed in *E. coli* BL21 (DE3)/pML1.

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