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Evidence for Epoxidase Activity in Deacetoxy/deacetylcephalosporin C Synthase

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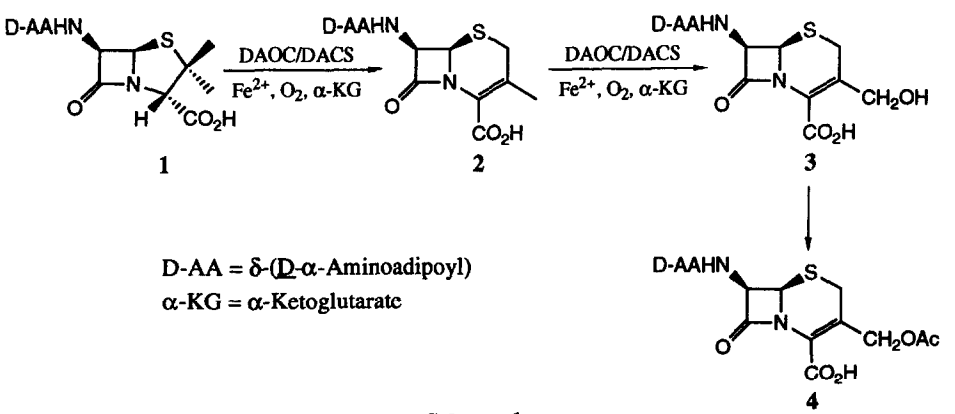
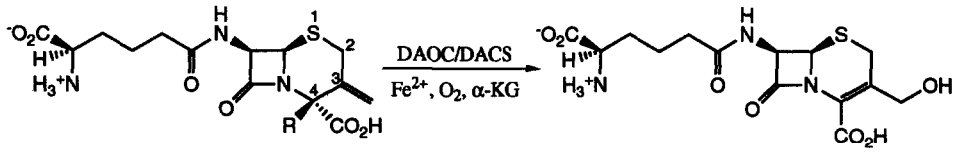
Abstract : The conversion of the unnatural substrate exomethylene cephalosporin C by deacetoxy/ deacetylcephalosporin C synthase (DAOC/DACS) has been studied by the use of competitive kinetic isotope experiments. These suggest that the first irreversible event in this conversion does not involve C4-hydrogen abstraction. A novel epoxide cepham metabolite has been isolated from the enzymic conversion of [4-^{2}H]exomethylene cephalosporin C. It is believed that increased formation of this shunt metabolite is caused by the operation of a deuterium kinetic isotope effect on an enzyme-bound intermediate. The epoxide cepham is also accepted as a substrate by DAOC/DACS, being oxidised to an aldehyde product.

Deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) isolated from *Cephalosporium acremonium*, is a crucial enzyme in the biosynthesis of cephalosporins. It catalyses the ring expansion of penicillin N **1** to deacetoxycephalosporin C (DAOC) **2** and the hydroxylation of this to deacetylcephalosporin C (DAC) **3**.[1] *In vivo*, DAC **3** is then acetylated by a different enzyme to give cephalosporin C **4** (Scheme 1).[2] DAOC/DACS is a non-heme ferrous dioxygenase which couples the oxidation of the substrate to the oxidative decarboxylation of α-ketoglutarate to succinate.[3] Both steps require Fe^{II} as a cofactor and α-ketoglutarate and dioxygen as cosubstrates. A partial amino-acid sequence has been obtained for this protein which permitted the cloning and over-expression of the corresponding gene in *E. coli* under the control of the λP_L promoter.[4] This system resulted in the expression of DAOC/DACS in an insoluble form, but recently use of a different promoter has enabled expression of soluble recombinant protein, which showed identical substrate specificity to the wild type enzyme.[5]



Scheme 1

DAOC/DACS has also been shown to convert the unnatural substrate exomethylene cephalosporin C **5a** directly to DAC **3** with no intermediates being observed (Scheme 2).[6,7]



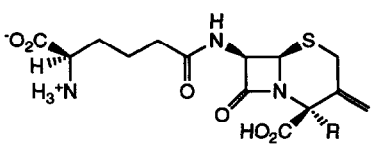
5a; R=H
5b; R=D

3

Scheme 2

The mechanisms previously proposed for this conversion consider abstraction of the C4-hydrogen† to be the first step in the reaction.[7] This proposal could be investigated by carrying out competitive kinetic isotope experiments (previously used to elucidate the order of events in the ring expansion of penicillin N **1** to DAOC **2**[8,9]), which look for enzymic discrimination between **5a** and the specifically deuterated [4-^{2}H]exomethylene cephalosporin C **5b**. We now report the results of these experiments and in addition, the isolation and characterisation of a new metabolite produced by DAOC/DACS in the conversion of **5b**.[10]

We synthesised **5a** by electrolysis of cephalosporin C **4**[11], and also isolated from this reaction the product of isomerisation, DAOC **2** and the C4 exomethylene cephalosporin C epimer **6a**. Formation of analogous exomethylene C4-epimers and of a cephem product was also reported on the electrolysis of cephalotin [7-(thiophene-2-acetamido)-cephalosporin C].[12]



6a; R=H
6b; R=D

Incubation of **5a** with DAOC/DACS gave DAC **3** as previously reported, with no other products or intermediates being observed. Incubation of the epimer **6a** resulted in no conversion indicating that the R-C4 stereochemistry of the exomethylene system is essential for enzymic conversion.

The [4-^{2}H]exomethylene cephalosporin C **5b** was synthesised by electrolysis of cephalosporin C **4** in a deuterated buffer. Analysis by mass spectrometry and ^{1}H-NMR showed that *ca.* 95 atom % deuterium incorporation had been achieved. The isomerised product DAOC **2** and **6b** were also obtained. When [4-^{2}H]exomethylene cephalosporin C **5b** was incubated with DAOC/DACS we observed formation of the expected product DAC **3** (by ^{1}H-NMR analysis), and of a second β-lactam product previously undetected in incubations of **5a**. Further incubations of **5b** with DAOC/DACS showed that the ratio of DAC **3** to the new β-

lactam compound varied with the overall degree of enzymic conversion (Table 1), with only DAC **3** being observed when conversion was complete.

Approximate % conversion of **5b**	Ratio of DAC **3** to Unknown
25	1 : 0.4
50	1 : 0.25
75	1 : 0.17
92	1 : 0.1
100	1 : 0

Table 1

We next carried out competitive kinetic isotope experiments by incubating an approximately 1:1 mixture of **5a** and **5b** with DAOC/DACS. Portions of the incubation mixture were removed at various times in order to obtain a range of samples with varying degrees of enzymic conversion. The unconverted exomethylene cephalosporin C **5a/b** was re-isolated from these samples and the ratio of **5a** to **5b** determined by mass spectrometry (Table 2).

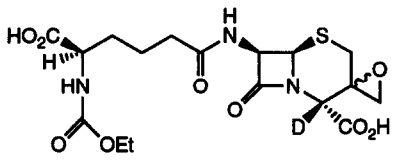
		Relative intensities of molecular ions (MH^+) for recovered **5a/b** *m/z*							
	% conversions of **5a/b** mixture								Ratio **5a : 5b**
Expt.		356	357	358	359	360	361	362	
1	0	1	8	92	100	18	7	1	1 : 0.92
	15	1	2	96	100	16	6	-	1 : 0.86
	35	1	4	93	100	18	9	2	1 : 0.90
	60	3	6	88	100	15	10	3	1 : 0.95
2	0	3	8	92	100	18	7	1	1 : 0.92
	10	2	2	97	100	24	6	1	1 : 0 85
	25	3	4	94	100	17	8	2	1 : 0.88
	40	1	3	94	100	14	5	1	1 : 0.88

Table 2

The results of the two experiments show that to within experimental error there was no isotopic enrichment of **5** during conversion, which indicates that the enzyme converts deuterated and protiated exomethylene cephalosporin C **5a/b** at the same rate. Competitive mixed-label experiments reflect differences in the parameter V_{max}/K_m[13], which expresses the enzymic events up to and including the first irreversible step[14]. Thus, the absence of discrimination between **5a** and **5b** enables us to conclude that the C4-H bond is not involved in steps up to and including the first irreversible one. These results, combined with the observation of a new metabolite from the incubation of **5b** which did not accumulate, strongly suggested that

an enzyme-free intermediate between **5b** and **3** was being produced in the conversion of **5b** by DAOC/DACS. To test this hypothesis it was necessary to isolate the new metabolite and re-incubate it with DAOC/DACS.

HPLC purification of the crude incubation mixture of DAOC/DACS with **5b** revealed that the new compound and DAC **3** co-eluted in all the systems that were tried. The first approach to solving this problem was to attempt HPLC separation of the two products after N-derivatisation of the mixture. Several N-protecting groups were tried, but this method was only partially successful when incorporating an N-ethoxycarbonyl group, which provided a derivative of the new metabolite in sufficiently pure form for its structure to be elucidated as the spiro-epoxide cepham **7**[5b]. However, the N-ethoxycarbonyl group could not be removed from **7** for re-incubation of the free metabolite with DAOC/DACS.



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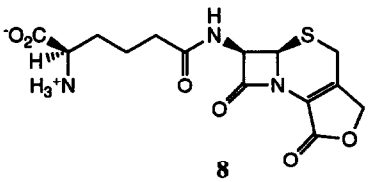


8

A procedure for isolating the free epoxide from its mixture with DAC **3** was found when it was discovered that acid treatment of this mixture resulted in lactonisation of DAC **3** to give the cephalosporin C lactone **8**, with only a little decomposition of the epoxide being observed. Thus, a mixture of the two compounds was treated with formic acid to give, after HPLC purification, the lactone **8** and the spiro-epoxide cepham which gave data consistent with structure **9**.

Assignment of the β-stereochemistry for C3 of the epoxide **9** was based on the following observations. Firstly, the ^{1}H-NMR spectrum of **9** (Figure 1) showed a splitting of the C2 hydrogen resonances which was very similar to that observed for the 3β-hydroxycepham **10**[9,15], where in both cases the β-C2 hydrogen is shifted upfield in comparison to other cephams. NOe studies on **9** (Table 3) were consistent with a computer model†† for the β-stereochemistry (Figure 2) and revealed similar spatial relationships to those observed for **10** (Table 4).

		nOe Effect				
		2β-H (2.44 ppm)	Epoxide H (3.28ppm)	Epoxide H (3.40 ppm)	2α-H (3.64 ppm)	6H (5.37 ppm)
Proton irradiated	2α-H (3.64 ppm)	12	–	–	–	3
	2β-H (2.44 ppm)	–	5	3	5	–
	Epoxide H (3.28ppm)	7	–	30	–	–
	Epoxide H (3.40 ppm)	3	28	–	–	–
	6H (5.37 ppm)	–	s	s	4	–

Table 3 : nOe results on **9** (in %; s = too small to quantify)

nOe Effect

H irradiated	Me (1.39 ppm)	2β-H (2.66 ppm)	2α-H (3.56 ppm)	6H (5.29 ppm)
2α-H (3.56 ppm)	2	10	–	4
2β-H (2.66 ppm)	6	–	19	–

Table 4 : nOe results on **10** (in %)

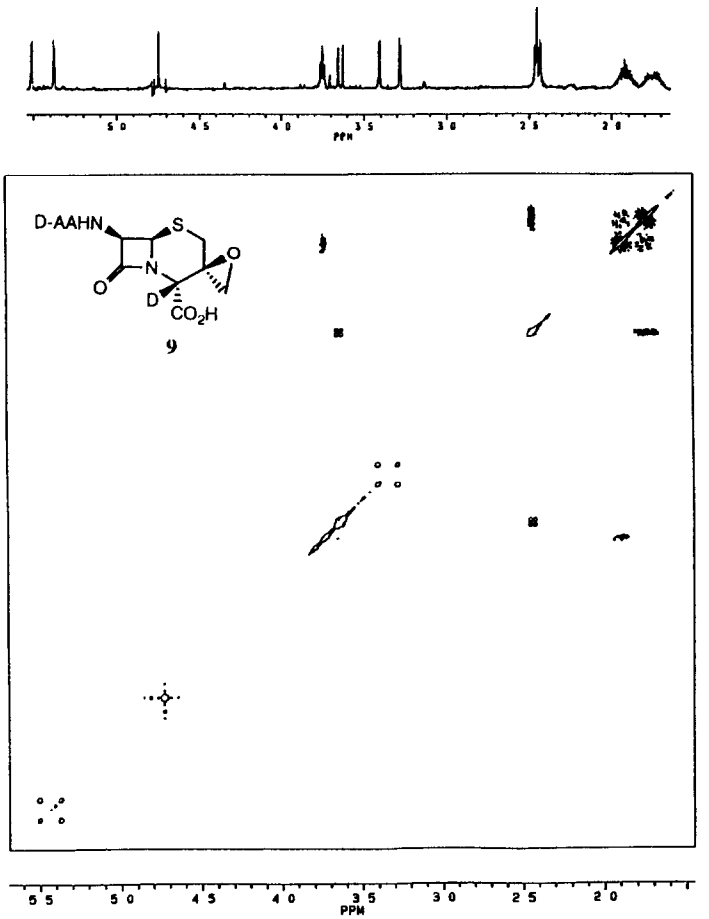


Figure 1 . ^{1}H-NMR and 2D-COSY spectra of **9**

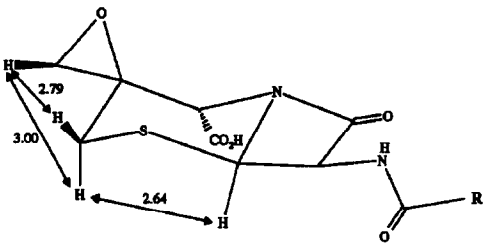
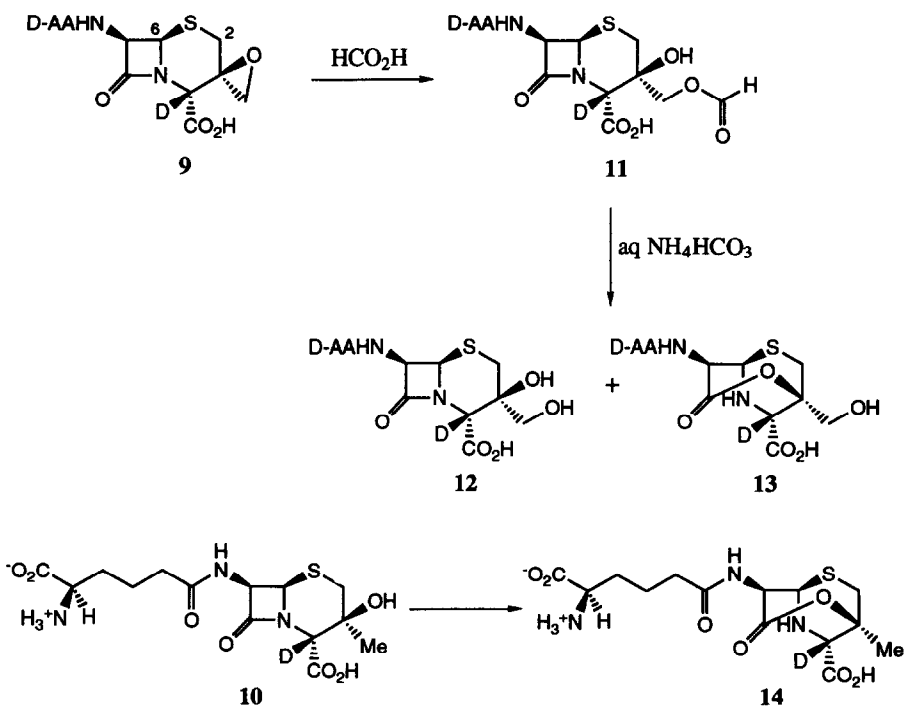


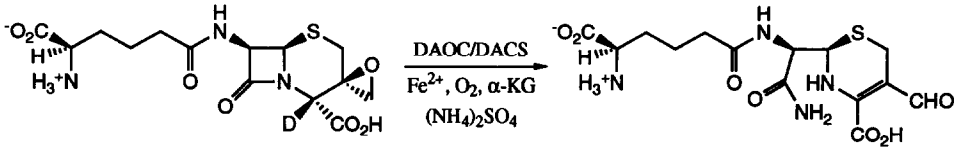
Figure 2 : Computer model†† for the ring-system of **9** (arrows represent distances between protons in angstroms)

In addition, a new compound was observed from treatment of the mixture of **3** and **9** with formic acid, which had a mass spectrum consistent with the hydroxyformate ester **11**. HPLC purification using ammonium hydrogen carbonate as solvent resulted in hydrolysis of **11**, and isolation of a mixture of two compounds with spectral data consistent with the diol **12** and the lactone **13**, which is analogous to lactone **14**[16] obtained from the 3β-hydroxycepham **10** by exposure to acid, base or heat (Scheme 3). Formation of these lactones is very facile provided a 3β-hydroxy group is present which attacks the β-lactam ring.



Scheme 3

We next re-incubated the epoxide cepham **9** with DAOC/DACS and cofactors. Unexpectedly, we observed conversion of **9** to the ring-opened deacetylcephalosporin C aldehyde **15** with only trace amounts of DAC being detected, even under conditions whereby some epoxide **9** remained unconverted (Scheme 4).



9 **15**

Scheme 4

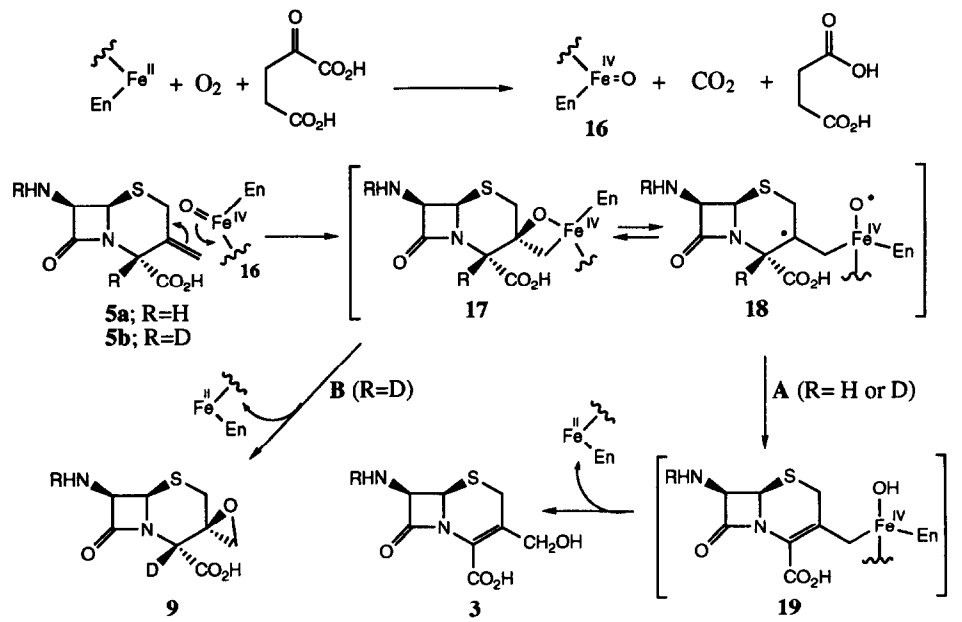
The aldehyde cephalosporoate **15** is also the product obtained on conversion of DAC by DAOC/DACS[17] [the β-lactam ring-opening was shown to be effected by several nucleophiles present in the incubation mixture, leading to the formation of different products; in the present case several aldehyde products were also observed, the major one isolated being **15**]. In a denatured enzyme control incubation with normal cofactors no conversion to aldehyde **15** or DAC **3** could be detected, and the epoxide **9** remained intact. The same was observed if the incubation was carried out with active enzyme but in the absence of α-ketoglutarate. These results indicated that the new metabolite **9** was not converted through to DAC **3** as predicted. A more thorough inspection of crude incubation mixtures of [4-^{2}H]exomethylene cephalosporin C **5b** with DAOC/DACS revealed the presence of previously undetected aldehyde products, in amounts that increased with the degree of conversion of **5b**.

From these results we conclude that the spiro-epoxide **9** is not an enzyme-free intermediate in the conversion of **5b** to **3** as first thought, but a shunt metabolite formed in the conversion of [4-^{2}H]exomethylene cephalosporin C **5b**, which can also serve as a substrate for DAOC/DACS. We believe this shunt metabolite is formed through the operation of a deuterium isotope effect on an enzyme-bound intermediate. The lack of a V_{max}/K_m isotope effect on the conversion of **5a/b** permits us to exclude involvement of the C4-H bond in the first irreversible step of this conversion. However, this bond must be broken in a subsequent step in order to allow formation of the major product DAC **3**. It is at this step that manifestation of the isotope effect on an enzyme-bound intermediate is observed, with the result that branching of the normal enzymic pathway occurs.

We propose that in the conversion of **5a/b** by DAOC/DACS (Scheme 5) the first irreversible step with respect to the substrate is the addition of the iron(IV)-oxene **16** to the double bond. The formation of the Fe^{IV}=O **16** (by oxidative decarboxylation of α-ketoglutarate) is not considered to be the first irreversible step (in terms of the β-lactam substrate), by analogy to the ring-expansion of penicillin N **1** and hydroxylation of DAOC **2** mediated by DAOC/DACS for both of which V_{max}/K_m isotope effects were observed[8,18], indicating that binding of both substrates **1** and **2** is still reversible after formation of **16**. In our proposed mechanism the first irreversible step leads to the formation of an enzyme-bound intermediate, which in the case of **5a** (R=H) is converted to intermediate **19** [pathway A, **18** to **19**], from which reductive elimination generates the product DAC **3** and returns En-Fe^{II} to the catalytic cycle. In the case of **5b** (R=D) a deuterium isotope effect leads to a decrease in the rate of hydrogen abstraction [pathway A], thus allowing reductive elimination and generation of

the epoxide **9** to compete [pathway B, **17** to **9**]. In the conversion of undeuterated exomethylene cephalosporin C **5a** both pathways are possible but only the more rapid pathway A is observed, with the result that no epoxide is formed. Branching of an enzymic pathway due to the operation of a deuterium isotope effect on an enzyme bound intermediate has previously been proposed to account for an increase in formation of the 3β-hydroxycepham **10** in the incubation of a specifically deuterated penicillin N.[9]

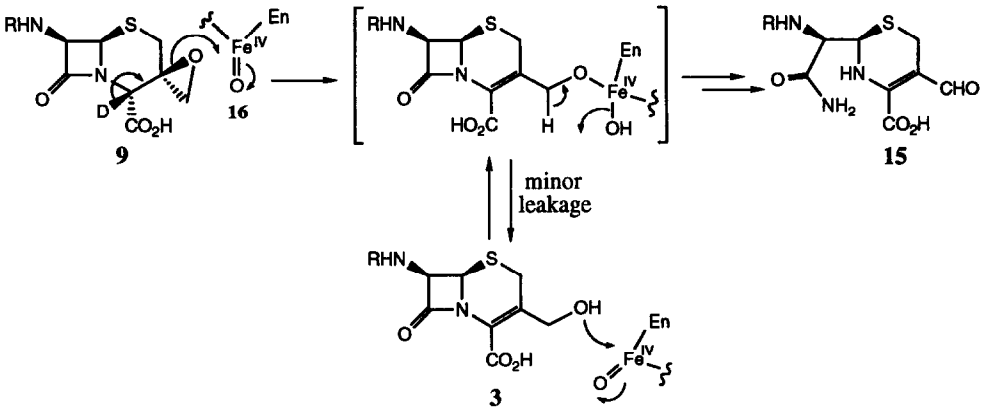
The observation that the ratio of spiro-epoxide cepham **9** to DAC **3** decreases with increased conversion level of [4-^{2}H]exomethylene cephalosporin C **5b** (Table 1) can be explained assuming that DAOC/DACS oxidises the epoxide **9** in preference to DAC **3**, *i.e.* that the epoxide **9** is a better substrate for oxidation than DAC **3** (presumably because of a lower K_m), and that this process competes with the conversion of **5b** at the enzyme active site. This was confirmed through comparison (by 500 MHz ^{1}H-NMR) of incubation mixtures at similar conversion levels, of protiated and deuterated exomethylene cephalosporin C **5a/b**. Incubation mixtures of deuterated exomethylene cephalosporin C **5b** showed evidence of aldehyde formation even at low levels of conversion (≥ 20%), whereas aldehyde present in incubations of protiated exomethylene cephalosporin C **5a** could only be detected when conversion of **5a** was pratically complete, and the enzyme switched to oxidising DAC **3**. It is not strange that the oxidation of DAC **3** should have a high K_m value, as this transformation is biologically undesirable leading to destruction of the β-lactam ring. A high K_m value also facilitates product release. It seems plausible that the *in vivo* acetylation of DAC **3** might have evolved to prevent the destruction of the bicyclic ring system.



R= D-α-aminoadipoyl

Scheme 5

We also propose that the conversion of the spiro-epoxide **9** to the ring-opened aldehyde **15** occurs by isomerisation of **9** to bound DAC **3** at the active site of DAOC/DACS (Scheme 6). This process does not lead to a change in oxidation level of either the substrate or the enzyme ferryl moiety, and thus permits the rapid oxidation of bound DAC **3** to an aldehyde, which is readily attacked on the β-lactam ring due to its increased reactivity. Leakage of isomerised epoxide from the active site would also account for the low levels of DAC **3** observed in the incubation mixtures of epoxide with DAOC/DACS.



R= D-α-aminoadipoyl

Scheme 6

In conclusion, we believe that the spiro-epoxide **9** is a shunt metabolite formed on conversion of [4-^{2}H]exomethylene cephalosporin C **5b** by DAOC/DACS, through the operation of a deuterium kinetic isotope effect on an enzyme-bound intermediate. The shunt product **9** is also a substrate for DAOC/DACS being converted to the aldehyde **15**. This is the first example of epoxidase activity so far detected with DAOC/DACS, and further demonstrates its versatility in terms of reactions catalysed. This multi-reactivity is a characteristic apparently shared with other α-ketoglutarate dependent dioxygenases (*e.g.* thymine 7-hydroxylase[19] and hyoscyamine 6β-hydroxylase[20]), and possibly results from the high reactivity and consequently low selectivity of the proposed iron(IV)-oxene intermediate.

Acknowledgements : We thank Dr. J. D. Sutherland, Mr. R. J. Heath, Mr. J. W. Keeping, and Mrs. W. J. Sobey for the supply of purified recombinant DAOC/DACS and Dr. R. T. Aplin and Mr. R. Proctor for mass spectral analysis. In addition we thank Eli Lilly & Co., Indianapolis, Indiana 46285, USA for financial and scientific assistance, the SERC, and the Junta Nacional de Investigação Científica e Tecnológica/Programa Ciência (Portugal) for a scholarship (to I.A.C.P.).

Footnotes

† In the text the classical numbering system for the β-lactam compounds has been used, but in the experimental section the IUPAC numbering is given to fully describe the structures.

†† The ring structure of **9** was built in SYBYL S.41, and energy-minimised using a standard force field.

Experimental

General

Standard chemical procedures as previously reported[7] were used. ^{1}H-NMR spectra were recorded at 500 MHz on a Brucker AM 500 spectrometer, and are internally referenced to 3-trimethylsilyl tetradeuteriopropionate (TSP) (δ_{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 125.77 MHz on a Brucker AM 500 spectrometer, and are internally referenced to 1,4-dioxan (δ_{ref} = 67.30 ppm). ^{13}C spectra were run using DEPT editing.

Fast atom bombardment mass spectra (FAB) were run on a VG Micromass ZAB 1F. 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 ^{1}H-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, HOD suppressed). Varying degrees of conversion were obtained by varying the amount of substrate or the incubation time.

Exomethylene cephalosporin C ; (2R,6R,7R)-1-Aza-3-methylene-7-[(5R)-5-amino-5-carboxy pentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid; 5a

A solution of cephalosporin C **4** (0.25 g, 0.602 mmol) in a sodium acetate buffer (0.1 M, pH 4) was electrolysed[11] at 15V using a mercury pool cathode and a platinum sheet anode separated by a Nafion membrane, and the reaction was followed by ^{1}H-NMR. After 5h there was no evidence of starting material, and the reaction mixture was lyophilized. The crude mixture was purified by chromatography (HPLC, Gilson system, 0.75% MeCN in 10mM aqueous NH_4HCO_3, 4 ml/min) to give 3-exomethylene cephalosporin C **5a** (retention time 4.5 min, *ca.* 48 mg by NMR calibration, 24%), DAOC **2** (retention time 6.5 min,*ca.* 23 mg by NMR calibration, 11%), and the 3-exomethylene cephalosporin C epimer **6a** (retention time 8 min, *ca.* 24 mg by NMR calibration, 12%).

Data for **5a** : δ_H (500 MHz, D_2O, HOD suppressed) : 1.64-1.92 (4H, m, $CHCH_2CH_2$), 2.40 (2H, t, J 7 Hz, CH_2CO), 3.38 and 3.61 (2H, ABq, J 14 Hz, CH_2S), 3.71 (1H, t, J 6Hz, $H_3N^+CHCO_2^-$), 4.97 (1H, s, $CHC=CH_2$), 5.24 and 5.28 (2H, 2 × s, $C=CH_2$), 5.40 and 5.41 (2H, ABq, J 4 Hz, HNCHCHS); δ_C (125.8 MHz, D_2O) : 22.60 (t, $CHCH_2CH_2$), 30.34 (t, CH_2S), 33.16 (t, $CHCH_2$), 35.87 (t, CH_2CO), 57.25, 57.55, 59.74 and 60.61 (4 × d, NHCHCHS, $H_3N^+CHCO_2^-$ and $CHC=CH_2$), 116.46 (t, $C=CH_2$), 137.97 (s, $C=CH_2$), 166.73, 174.26, 177.69 and 182.00 (4 × s, 4 × C=O); *m/z* (ESMS) : 380 ([MNa$^+$], 100%), 381 (19%), 382 (8%)

Data for **2** : δ_H (500 MHz, D_2O, HOD suppressed) : 1.63-1.95 (4H, m, $CHCH_2CH_2$), 1.93 (3H, s, CH_3), 2.42 (2H, t, J 7 Hz, CH_2CO), 3.26 and 3.60 (2H, ABq, J 18 Hz, CH_2S), 3.72-3.76 (1H, m, $H_3N^+CHCO_2^-$), 5.09 and 5.57 (2H, 2 × d, J 4 Hz, HNCHCHS); δ_C (125.8 MHz, D_2O) : 19.15 (q, CH_3), 22.65 (t, $CHCH_2CH_2$), 29.26 (t, CH_2S) 33.14 (t, $CHCH_2$), 35.92 (t, CH_2CO), 57.50, 57.67 and 59.50 (3 × d, NHCHCHS and $H_3N^+CHCO_2^-$), 123.35 and 127.66 (2 × s, C=C), 165.30, 170.54, 178.03 and 182.02 (4 × s, 4 × C=O); *m/z* (ESMS) : 358 ([MH$^+$], 100%), 359 (19%), 360 (8%). Identical to an authentic sample.

Data for **6a** : δ_H (500 MHz, D_2O, HOD suppressed) : 1.64-1.97 (4H, m, $CHCH_2CH_2$), 2.45 (2H, t, J 7 Hz, CH_2CO), 3.46 and 3.67 (2H, ABq, J 12 Hz, CH_2S), 3.72 (1H, t, J 6Hz, $H_3N^+CHCO_2^-$), 4.68 (1H, s, $CHC=CH_2$), 5.11 and 5.23 (2H, ABq, J 4 Hz, HNCHCHS), 5.33 and 5.37 (2H, 2 × s, $C=CH_2$); δ_C (125.8 MHz, D_2O) : 22.06 (t, $CHCH_2CH_2$), 29.04 (t, CH_2S), 32.09 (t, $CHCH_2$), 35.93 (t, CH_2CO), 55.83, 55.92, 60.07 and 64.81 (4 × d, NHCHCHS, $H_3N^+CHCO_2^-$ and $CHC=CH_2$), 118.28 (t, $C=CH_2$), 137.76 (s, $C=CH_2$), 165.78, 173.07, 177.47 and 182.26 (4 × s, 4 × C=O); *m/z* (ESMS) : 358 ([MH$^+$], 100%), 359 (20%), 360 (7%), 361 (2%)

Incubation of 5a and 6a with DAOC/DACS

Exomethylene cephalosporin C **5a** was incubated with DAOC/DACS according to the general procedure. Examination of the crude incubation mixture by ^{1}H-NMR revealed conversion to a single β-lactam product. Purification by HPLC (Waters system, 10 mM aqueous NH_4HCO_3, 1 ml/min) gave DAC **3** (retention time 9 min), identical to an authentic sample by ^{1}H-NMR (500 MHz, D_2O, HOD suppressed) : 1.65-1.97 (4H, m, $CHCH_2CH_2$), 2.43 (2H, t, J 7 Hz, CH_2CO), 3.47 and 3.67 (2H, ABq, J 18 Hz, SCH_2), 3.74 (1H, t, J 6 Hz, $H_3N^+CHCO_2^-$), 4.26 and 4.30 (2H, ABq, J 13 Hz, CH_2OH), 5.13 and 5.63 (2H, 2 × d, J 4.5 Hz, HNCHCHS).

The exomethylene cephalosporin C epimer **6a** was also incubated with DAOC/DACS according to the general procedure. Analysis of the crude incubation mixture by ^{1}H-NMR revealed that **6a** was still intact and no products could be observed. The incubation mixture was bioassayed against *E. coli* X580 and *Staphilococcus aureus* NCTC 6571 but showed no bioactivity, further indicating that no cephem product had been formed.

[4-^{2}H]Exomethylene cephalosporin C; (2R,6R,7R)-1-Aza-(2-^{2}H)-3-methylene-7-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid; 5b

A solution of cephalosporin C **4** (0.50 g, 1.2 mmol) in a sodium acetate buffer in deuterium oxide (0.1 M, pH 4) was electrolysed[11] for 7h. The same purification procedure as for **5a** gave **5b** (retention time 5 min, *ca.* 140 mg by NMR calibration, 32%), DAOC **2** (retention time 6.5 min,*ca.* 28 mg by NMR calibration, 7%), and **6b** (retention time 7.5 min, *ca.* 24 mg by NMR calibration, 6%).
Data for **5b** : ^{1}H-NMR identical to **5a** except for the absence of the C4-H resonance at 4.97 ppm.
m/z (ESMS) : 380 (6%), 381 ([MNa$^+$], 100%), 382 (18%), 383 (8%).
Data for **6b** : ^{1}H-NMR identical to **6a** except for the absence of the C4-H resonance at 4.68 ppm.
m/z (ESMS) : 358 (5%), 359 ([MH$^+$], 100%), 360 (18%), 361 (8%), 362 (2%).

Incubation of 5b with DAOC/DACS

[4-^{2}H]Exomethylene cephalosporin C **5b** (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 **3** and a second β-lactam product (β-lactam resonances at δ 5.37 and 5.50 ppm). On purification of the crude incubation mixture by HPLC (Gilson system, 10mM aqueous NH_4HCO_3, 2 ml/min) the two products eluted as one single peak (retention time 10 min) (*ca.* 3 mg DAC by NMR calibration; ratio DAC : new compound = 4 : 1).

Competitive kinetic isotope experiments

An approximately 1:1 mixture of **5a** and **5b** was prepared (*ca.* 2 mg) and a small sample removed representing a time-zero reference point. This mixture was incubated with DAOC/DACS (2 ml, *ca.* 0.1 IU) and cofactor solution (200 µl) according to the general procedure. Portions of the incubation mixture were removed at varying times to give samples with varying degrees of enzymic conversion. The protein was precipitated in each of these samples and centrifuged off. The supernatant was evaporated to dryness, and re-dissolved in D_2O for examination by ^{1}H-NMR (500 MHz, HOD suppressed). The β-lactam region (δ 6.0 to 4.5 ppm) was integrated and the percentage conversion of the starting material calculated by comparison of the integrals of the β-lactam resonances of **5a/b** and DAC **3**. The unconverted **5a/b** present in this samples was then isolated by HPLC [Gilson system, 10 mM aqueous NH_4HCO_3, 2 ml/min], and then analysed by mass spectrometry (FAB). This experiment was repeated a second time.

Mass spectroscopic results for the **5a/b** mixture in samples taken from the incubations with DAOC/DACS :

Expt.1	Time (min)	% Conversion	Relative Intensities of the Molecular Ions (MH^+) of **5a/b** (*m/z*)						
			356	357	358	359	360	361	362
	0	0	1	8	92	100	18	7	1
	10	15	1	2	96	100	16	6	–
	30	35	1	4	93	100	18	9	2
	120	60	3	6	88	100	15	10	3

Expt.2	Time (min)	% Conversion	Relative Intensities of the Molecular Ions (MH^+) of **5a/b** (*m/z*)						
			356	357	358	359	360	361	362
	0	0	3	8	92	100	18	7	1
	15	10	2	2	97	100	24	6	1
	60	25	3	4	94	100	17	8	2
	120	40	1	3	94	100	14	5	1

N-protection of a DAC/unknown mixture - Isolation of (2R,6R,7R)-1-Aza-2-[^{2}H]-3-spiroepoxy-7-[(5R)-5-N-ethoxycarbonylamino-5-carboxypentanamido]-8-oxo-5-thiabicyclo [4.2.0]octane-2-carboxylic acid; 7

A mixture of DAC **3** (*ca.* 4.5 mg) and the new metabolite (ratio 4:1) was dissolved in distilled water (2 ml). The pH was adjusted to 8 with a sat. $NaHCO_3$ solution (100 µl) and diethylpyrocarbonate (13 µl, 0.09 mmol, *ca.* 6 eq.) was added. The reaction mixture was stirred at room temperature for 20 min, washed twice with ether and lyophilized. Analysis by ^{1}H-NMR showed reaction was quantitative and the ratio of the two products was still the same. Purification of the crude mixture by HPLC (Waters system, 8% MeOH in aqueous 10 mM NH_4HCO_3, 1 ml/min) revealed two partially coeluting peaks which were collected as two fractions (fr.1:15-17 min; fr.2:17-19 min). Fr.1 consisted of N-ethoxycarbonyl-DAC (*ca.* 2.1 mg by NMR calibration). Fr.2 had the two products in a ratio of 1.3:1 (*ca.* 490 µg of N-ethoxycarbonyl-DAC by NMR calibration) and was again purified by HPLC using the same procedure as before. The second fraction of this run contained the N-ethoxycarbonyl derivatives of DAC and the unknown in a ratio of 1:2, and the latter could be identified as the spiro-epoxide **7**.

Data for N-ethoxycarbonyl DAC : δ_H (500 MHz, D_2O, HOD suppressed) : 1.24 (3H, t, J 7 Hz, CH_2CH_3), 1.64-1.88 (4H, m, $CHCH_2CH_2$), 2.38-2.45 (2H, m, CH_2CO), 3.46 and 3.66 (2H, ABq, J 18 Hz, CH_2S), 3.92-3.98 (1H, m, $HNCHCO_2H$), 4.08-4.14 (2H, m, CH_2CH_3), 4.26 and 4.30 (2H, ABq, J 13 Hz, CH_2OH), 5.12 and 5.62 (2H, 2 × d, J 5 Hz, $HNCHCHS$); δ_C (125.8 MHz, D_2O) : 14.60 (q, CH_3), 22.65 (t, $CHCH_2CH_2$), 26.19 (t, CH_2S), 32.20 and 35.65 (2 × t, $CHCH_2$ and CH_2CO), 56.93, 58.09 and 59.75 (3 × d, $NHCHCHS$ and $HNCHCO_2H$), 61.82 (t, CH_2OH), 62.57 (t, CH_2CH_3), 122.26 and 130.47 (2 × s, C=C), 158.97, 165.67, 169.65, 177.93 and 180.19 (5 × s, 5 × C=O); *m/z* (ESMS) : 468 ([MNa^+], 100%), 469 (30%), 470 (13%), 471 (3%)

Data for **7** : δ_H (500 MHz, D_2O, HOD suppressed) : 1.24 (3H, t, J 7 Hz, CH_2CH_3), 1.65-1.86 (4H, m, $CHCH_2CH_2$), 2.34-2.45 (2H, m, CH_2CO), 2.43 and 3.63 (2H, ABq, J 15 Hz, CH_2S), 3.40 and 3.63 (2H, 2 × d, J 3.5 Hz, epoxide H's), 3.92-3.98 (1H, m, $HNCHCO_2H$), 4.07-4.15 (2H, m, CH_2CH_3), 5.36 and 5.49 (2H, 2 ×

d, J 4 Hz, HNCHCHS) (the couplings were confirmed by a COSY experiment); *m/z* (ESMS) : 469 ([MNa$^+$], 100%), 470 (21%), 471 (11%), 472 (3%), 447 ([MH$^+$], 20%)

Isolation of the spiro-epoxide cepham, (2R,3R,6R,7R)-1-Aza-2-[^{2}H]-3-spiroepoxy-7-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid; 9

A mixture of DAC **3** (*ca.* 7.6 mg) and the epoxide metabolite (ratio 4 : 1) was dissolved in distilled formic acid (9 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 to the lactone **8**, and the presence of a third β-lactam product in trace amounts indicating that some decomposition had occured. The crude mixture was purified by HPLC (Waters system, 4.5% MeCN/H_2O, 1 ml/min) to give a mixture of the epoxide **9** and the third compound coeluting as one peak (retention time 4.5 min), and the lactone **8** (retention time 15 min). A mass spectrum (ESMS) on the fraction containing the epoxide and the third compound revealed peaks at 375 ([MH$^+$], epoxide) and 421 ([MH$^+$], hydroxyformate ester **11**(?)). This fraction was further purified by HPLC (Waters system, 10 mM aqueous NH_4HCO_3, 1 ml/min) to give the pure spiro-epoxide cepham **9** (*ca.* 960 μg by NMR calibration, retention time 8.5 min), and the diol **12** (*ca.* 38 μg by NMR calibration) and lactone **13** (*ca.* 100 μg by NMR calibration) which coeluted as one peak (retention time 13.2 min). The epoxide **9** showed no bioactivity against *E. coli* X580.

Data for **8** : δ_H (500 MHz, D_2O, HOD suppressed) : 1.68-1.96 (4H, m, CHCH_2CH_2), 2.44 (2H, t, J 7 Hz, CH_2CO), 3.74 and 3.91 (2H, ABq, J 18 Hz, CH_2S), 3.74 (1H, t, J 6Hz, H_3N^+CHCO_2^-), 5.10 and 5.15 (2H, ABq, J 18Hz, CH_2OCO), 5.24 and 5.80 (2H, 2 × d, J 5 Hz, HNCHCHS);

δ_C (125.8 MHz, D_2O) : 21.62 (t, CHCH$_2$CH$_2$), 23.41 (t, CH$_2$S) 30.61 (t, CHCH$_2$), 35.32 (t, CH$_2$CO), 55.31, 57.96 and 60.57 (3 × d, NHCHCHS and H_3N^+CHCO_2^-), 73.49 (t, CH$_2$OCO) 123.75 and 144.59 (2 × s, C=C), 165.69, 170.19, 174.91 and 177.15 (4 × s, 4 × C=O); *m/z* (ESMS) : 356 ([MH$^+$], 100%), 357 (20%), 358 (8%), 359 (2%)

Data for **9** : δ_H (500 MHz, D_2O, HOD suppressed) : 1.66-1.95 (4H, m, CHCH_2CH_2), 2.44 (2H, t, J 7 Hz, CH_2CO), 2.44 and 3.64 (2H, ABq, J 15 Hz, CH_2S), 3.28 and 3.40 (2H, 2 × d, J 4 Hz, epoxide H's), 3.74 (1H, *ca.* t, J 6 Hz, H_3N^+CHCO_2^-), 5.37 and 5.50 (2H, 2 × d, J 4 Hz, HNCHCHS) (the couplings were confirmed by a COSY experiment - see Figure 1); *m/z* (ESMS) : 375 ([MH$^+$], 100%), 376 (20%), 377 (8%), 378 (2%), 397 ([MNa$^+$], 44%). The ^{1}H-NMR of **9** was further elucidated by a series of nOe experiments. Irradiation of one of the C4 hydrogens (δ 3.64) caused an enhancement of the other C4-H (δ 2.44, 12%) and of one of the β-lactam protons (δ 5.37, 3%), and so could be assigned to the C4α-H. Irradiation of the C4β-H (δ2.44) caused an enhancement of the C4α-H resonance (δ 3.64, 5%), and of both epoxide hydrogens (δ 3.28 and 3.40, 5% and 3%). Irradiation of the epoxide hydrogen (δ 3.28) caused an enhancement of the C4β-H (δ 2.44, 7%), and a very large enhancement of the other epoxide resonance (δ 3.40, 30%) thus confirming their closeness in space. Irradiation of the epoxide hydrogen at δ 3.40 caused a smaller enhancement of the C4β-H (δ 2.44, 3%), and again a very large enhancement of the other epoxide hydrogen (δ 3.28, 28%). Irradiation of the β-lactam hydrogen at δ 5.37 caused an enhancement of the other β-lactam (δ 5.50, 9%), of the C4α-H (δ 3.64, 4%) and a very small one of both the epoxide hydrogens (δ 3.28 and 3.40, *ca.* 1%). The unexpected relationship between

the C4β-H and the epoxide methylene hydrogens in **9** was confirmed through nOe studies on the 3β-hydroxycepham **10**. Thus irradiation of the C4-H (δ 3.56) caused an enhancement of the other C4-H (δ 2.66, 10%), of the C3α-methyl group (δ 1.39, 2%) and of one of the β-lactam hydrogens (δ 5.29, 4%), thus showing it to be the C4α-H. Irradiation of the C4β-H (δ 2.66) caused an enhancement of the C4α-H (δ 3.56, 19%), and of the C3α-methyl group (δ 1.39, 6%) thus confirming the closeness in space of the C3α-methyl group and the C4β-H in **10**, which is analogous to the position of the epoxide methylene and C4β hydrogen in **9**.

Data for **12** and **13** : δ_H (500 MHz, D_2O, HOD suppressed) : 1.68-1.95 (4H, m, $CHCH_2CH_2$ of **12** and **13**), 2.38-2.48 (2H, m, CH_2CO of **12** and **13**), 2.77 and 3.37 (2H, ABq, J 14 Hz, CH_2S of **12**), 3.11 and 3.15 (2H, ABq, J 13 Hz, CH_2S of **13**), 3.53 and 3.87 (2H, ABq, J 13 Hz, CH_2OH of **13**), 3.57 and 3.78 (2H, ABq, J 12 Hz, CH_2OH of **12**) 3.75 (1H, *ca.* t, J 6 Hz, $H_3N^+CHCO_2^-$ of **12** and **13**), 4.35 and 5.43 (2H, 2 × s, HNCHCHS of **13**), 5.32 and 5.46 (2H, 2 × d, J 4 Hz, HNCHCHS of **12**) (assignments based on relative intensities of the peaks and a COSY experiment). The diol **12** and lactone **13** are analogous to the 3β-hydroxycepham **10**[9,15] and known lactone **14**[16] respectively, by NMR comparison; *m/z* (ESMS): 393 ([MH$^+$], 100%), 394 (19%), 395 (8%), 396 (2%).

Incubation of the epoxide 9 with DAOC/DACS

The epoxide **9** (*ca.* 0.5 mg) was incubated with DAOC/DACS (1 ml) and cofactor solution (100 µl). Examination of the crude incubation mixture by ^{1}H-NMR showed complete conversion of **9** to a mixture of aldehydic products and a trace of DAC **3**. Purification of the mixture by HPLC (Waters system, 0.1% HCO_2H/H_2O, 2 ml/min, monitoring at 300 nm) enabled isolation of the aldehyde cephalosporoate **15** (retention time 8 min) which gave data identical to literature reference.[17] Data for **15** : δ_H (500 MHz, D_2O, HOD suppressed) : 1.60-1.90 (4H, m, $CHCH_2CH_2$), 2.43 (2H, t, J 7 Hz, CH_2CO), 3.46 and 3.62 (2H, ABq, J 16 Hz, SCH_2), 3.73-3.80 (1H, m, $H_3N^+CHCO_2^-$), (6H resonance obscured by HOD peak), 4.95 (1H, d, J 7.5 Hz, 7H), 9.19 (1H, s, CHO); *m/z* (ESMS) : 389 ([MH$^+$], 100%)

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