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Cephalosporin C Biosynthesis : On The Mechanism Of Hydroxylation Of Deacetoxycephalosporin C To Deacetylcephalosporin C.

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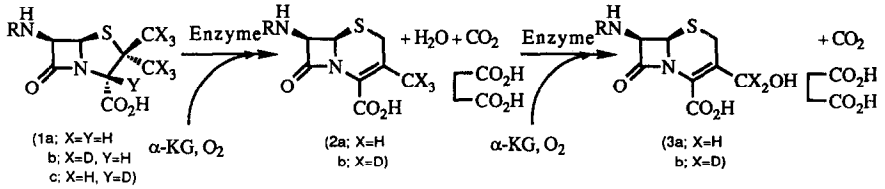
Abstract: Incubation of an equal mixture of deacetoxycephalosporin C and [3'-$^2H_3$]deacetoxy-cephalosporin C with partially purified deacetoxycephalosporin C / deacetylcephalosporin C synthase from Cephalosporium acremonium CO728 in a mixed competitive kinetic isotope effect experiment resulted in enzymic discrimination between labelled and unlabelled substrates.

The enzyme deacetoxycephalosporin C / deacetylcephalosporin C synthase (DAOC/DAC synthase)[1,2,3] from *Cephalosporium Sp.* is responsible for the ring expansion of penicillin N (**1**) to deacetoxycephalosporin C (DAOC, **2**) and for the subsequent hydroxylation of DAOC to deacetylcephalosporin C (**3**) (Scheme 1). This enzyme has been the subject of intensive research which has culminated in the cloning and expression of this protein in *E. coli* [4]. Studies have shown that DAOC/DAC synthase is a bifunctional protein of molecular weight *ca* 40,000 which requires α-ketoglutarate and molecular oxygen as co-substrates and ferrous ion as a cofactor. Optimal *in vitro* enzyme activity is obtained in the presence of ascorbate and dithiothreitol.

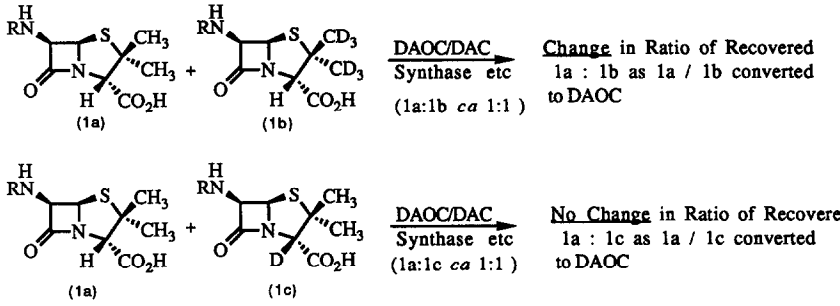


R = D-α-aminodipoyl
α-KG = α-ketoglutarate

Scheme 1

Until now, most investigations have concentrated on the ring expansion activity of this bifunctional enzyme *i.e.* the transformation of penicillin N (**1**) to DAOC (**2**). For example the use of mixed competitive kinetic isotope effect (Vmax/Km) experiments have enabled the order of hydrogen atom abstractions that occur in the ring expansion process, **1** to **2**, to be determined[5]. This

was achieved by incubation of 1:1 mixtures of either specifically deuterated penicillin Ns (**1b**) or (**1c**) with unlabelled penicillin N (**1a**) (Scheme 2).



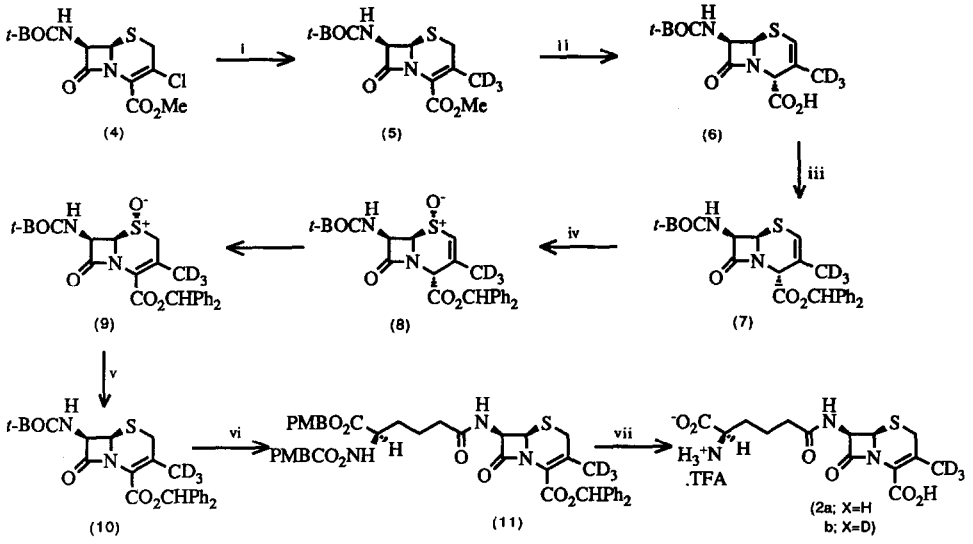
R = D-α-aminoadipoyl

Scheme 2

The observation that enzymic discrimination between labelled and unlabelled substrates occurred only in the case of the mixtures (**1a**) / (**1b**) was consistent with the first irreversible event in the ring expansion occurring by hydrogen abstraction at the β-methyl group of penicillin N.

Comparatively little is known about the mechanism of the hydroxylation reaction. It has been shown that in contrast to the loss of stereochemistry observed during the ring expansion process, the hydroxylation reaction proceeds with retention of configuration at the exocyclic methyl group and, in addition, displays a primary kinetic isotope effect[6]. We anticipated that observation of enzymic discrimination between labelled and unlabelled DAOC (**2a**) and (**2b**) in a mixed competitive kinetic isotope effect experiment would suggest that the first irreversible event in the hydroxylation reaction did indeed occur at the methyl group from which it would be possible to eliminate alternative pathways for the hydroxylation reaction.

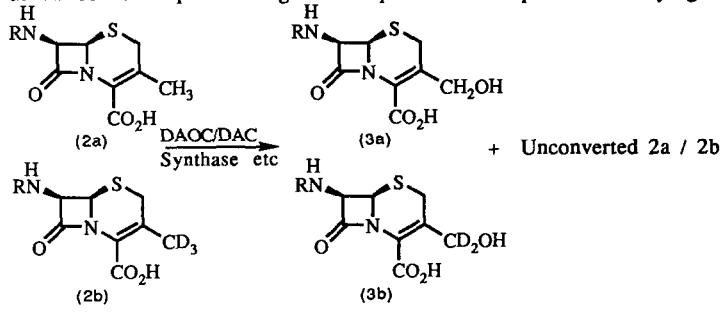
Investigation of the hydroxylation reaction *via* mixed competitive isotope effect experiments required the synthesis of the trideuterated DAOC (**2b**). Synthesis of this compound was achieved by application of previously reported cuprate chemistry on the 3-chlorocephem (**4**) to give *via* an addition/elimination mechanism, the C-3 trideuteromethyl cephem **5**[7](Scheme 3). Thus the cephem **4** was treated with an appropriately deuterated Lipschutz cuprate reagent to give **5** in 52% yield. Use of **4** as the methyl ester was found to be neccessary in order to obtain good yields of **5** - use of the diphenylmethyl ester in the same reaction gave low yields of **5** and some isomerised starting material presumably due to competing C-2 deprotonation. The resulting methyl ester **5** was then hydrolysed to the free acid **6** with concomitant isomerisation of the double bond, re-esterified to the diphenylmethyl ester **7** and then converted back to the Δ3 cephem isomer **10** by methods analogous to those previously reported[8] in an overall yield of 43%. After treatment with *p*-toluenesulphonic acid to remove the *t*-butyloxycarbonyl group, the amine was coupled to appropriately protected D-α-aminoadipic acid to give the protected target **11**. Subsequent treatment of **11** with trifluoroacetic acid / anisole at room temperature then liberated the trideutero-DAOC (**2b**) as its ammonium trifluoroacetate salt.



Scheme 3

Reagents: i) $(CD_3)_2Cu(CN)Li_2$, THF, -50°C; ii) 1eq NaOH, pyridine / H_2O (1:1 v/v); then H_3O^+; iii) Ph_2CN_2, MeCN; iv) MCPBA, DCM; v) MeCOCl, KI, DMF; vi) *p*-toluenesulphonic acid, PhMe then basic work-up followed by EEDQ, (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanoic acid, DCM; vii) trifluoroacetic acid, PhOMe, room temperature.

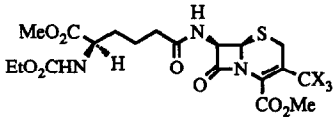
A one-to-one mixture of trideuterated DAOC (**2b**) and unlabelled DAOC (**2a**) was then prepared and incubated with DAOC/DAC synthase in a competitive isotope effect experiment (Scheme 4). Portions of the incubation mixture were removed and the enzymic reaction quenched by the addition of acetone at various time points to give a sequence of samples with varying degrees of conversion.



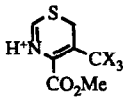
R = D-α-aminoadipoyl

Scheme 4

The DAOC mixture (**2a** and **2b**) was then isolated by chromatography (h.p.l.c, reverse phase) from each of these samples and derivatised to the N-ethoxycarbonyl, dimethyl esters **12** as previously described[9,10]. Analysis of the ratio of **2a** to **2b** was then performed by mass spectroscopy (NH_3, DCI) on each sample corresponding to the different % conversions.



(12a; X=H
b; X=D)



(13a; X=H
b; X=D)

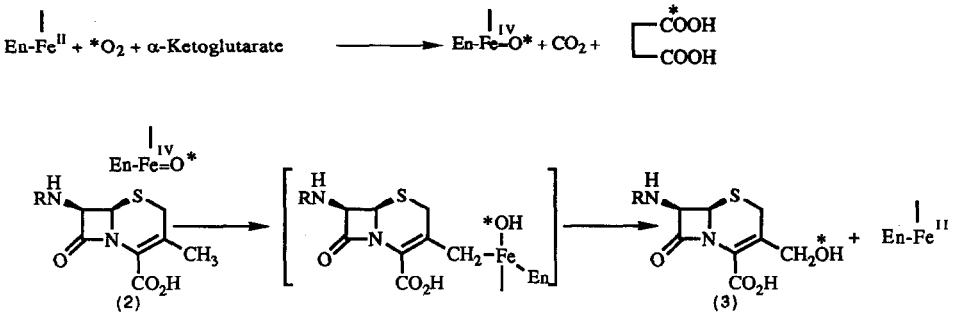
The results of these experiments are displayed below (Table 1). In a separate experiment a one to one mixture of (**2a**) and (**2b**) was incubated with denatured DAOC/DAC synthase (the enzyme being denatured by heating at 100°C for 5 minutes prior to use) under exactly the same conditions as the incubations with 'live' enzyme. No conversion of DAOC (**2a/b**) to DAC (**3a/b**) was observed as judged by ^{1}H-nmr (500MHz, D_2O). The DAOC was isolated by h.p.l.c. and then derivatised prior to examination by mass spectroscopy which indicated that, to within experimental error, no change in the ratio (**2a**) : (**2b**) had occurred.

Table 1. Enzymic Conversion of (**2a**) + (**2b**).

		Relative intensities of fragment ions (**13a/b**) (MH^+) from DAOC derivatives (**12a**) + (**12b**) m/z									
Expt.	% Conversions of DAOCs	171	172	173	174	175	176	177	178	179	Ratio (**2a**):(**2b**)
1	0	0	100	16	18	95	14	9	2	3	1.05 : 1.00
	55	0	74	21	37	100	25	22	9	9	0.74 : 1.00
2	0	0	89	13	0	100	13	10	2	2	0.89 : 1.00
	20	0	79	11	0	100	12	10	1	2	0.79 : 1.00
	35	0	73	10	0	100	13	10	1	2	0.73 : 1.00
	60	0	55	10	0	100	15	13	2	4	0.55 : 1.00
Denatured Control											
	0	0	99	14	0	100	13	7	1	1	0.99 : 1.00
	120 mins	8	100	27	0	97	13	9	0	2	1.02 : 1.00

		Relative intensities of molecular ions (MH^+) for DAOC derivatives (**12a**) + (**12b**) m/z									
Expt.	% Conversions of DAOCs	457	458	459	460	461	462	463	464	465	Ratio (**2a**): (**2b**)
1	0	0	100	26	22	98	25	17	4	1	1.02 : 1.00
	55	0	71	20	21	100	25	20	5	3	0.71 : 1.00
2	0	0	84	21	23	100	24	22	5	2	0.84 : 1.00
	20	0	77	19	21	100	25	23	5	2	0.77 : 1.00
	35	0	70	17	19	100	25	22	5	2	0.70 : 1.00
	60	0	52	13	22	100	26	34	8	3	0.52 : 1.00
Denatured Control											
	0	8	93	29	19	100	26	14	3	0	0.93 : 1.00
	120 mins	11	100	56	34	99	28	19	0	2	1.01 : 1.00

It is apparent from these results that a mixed competitive isotope effect is manifest in the hydroxylation of DAOC to DAC and hence that the data is consistent with a mechanism in which the first irreversible event takes place at the C-3' methyl group (Scheme 5). We thus propose that the hydroxylation reaction proceeds *via* a mechanism involving direct insertion of a ferryl [$\geq Fe^{(IV)}O$] species into a C-H bond of the exocyclic methyl group as an irreversible step. Reductive elimination then regenerates Enz-Fe^{II} and liberates free DAC in readiness for the next catalytic cycle.



R = δ-D-α-Aminoadipoyl

Scheme 5

Acknowledgements: We thank the S.E.R.C. (for a studentship to N.P.C.), Eli Lilly & Co., Indianapolis, U.S.A. for financial support, Mrs. W. Sobey and Mr. J.W. Keeping for the preparation of enzyme extracts, and Mr. R. Proctor for mass spectrometric analysis.

EXPERIMENTAL.

Standard experimental procedures as previously reported were used.[11] (Procedures for the conversion of (4) to (5) to (6) etc. were performed on unlabelled material prior to preparation of the deuterated compounds. All the compounds prepared for the unlabelled series gave correct spectroscopic data and acceptable elemental analyses where determined.

(6R,7R)-1-Aza-3-chloro-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid methyl ester (4)

To (6R,7R)-1-aza-3-chloro-7-amino-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (2.0 g, 9.0 mmol) dissolved/suspended in saturated aqueous $NaHCO_3$ (15 ml) and dioxan (15 ml) was added di-*t*-butylpyrocarbonate (2.9 g, 13.5 mmol) in one portion and the resulting suspension stirred overnight at room temperature. After washing with DCM (3 x 50 ml) the solution was acidified to *ca* pH 2 by the addition of 2N HCl and then rapidly extracted with EtOAc (3 x 50 ml). The EtOAc layers were combined, dried (anhydrous Na_2SO_4), filtered and evaporated to dryness to give a N-*t*-BOC free acid (2.1 g, 6.2 mmol, 69% yield) which was redissolved in EtOAc (20 ml) at 0°C and treated with excess diazomethane. Chromatography [flash silica, DCM / EtOAc (3:2, v/v)] gave 4 (1.74 g , 5.0 mmol, 55% yield); t.l.c.,[DCM/ EtOAc (3:2 v/v)] R_f 0.7; m.p. 151-152°C (from DCM/petrol); υ_{max}($CHCl_3$) 3440 (w), 1793 (s), 1723 (s), 1506 (s), 1370 (m); δ_H (500MHz, $CDCl_3$) 1.45 (9H, s, $C(CH_3)_3$), 3.51 and 3.82 (2 x 1H, 2 x d, *J* 18Hz, CH_2S), 3.89 (3H, s, CO_2CH_3), 5.02 (1H, d , *J* 4.5Hz, CHS), 5.21 (1H, d, *J* 9Hz, NH), 5.61 (1H, dd, *J* 9, 4.5Hz, NHCHCH); *m/z* (DCI, NH_3) 366 (MNH_4^+ ^{35}Cl, 100%), 367 (23), 368 (47), 369 (9), 370 (3).

$^2H_3$-Methyllithium[12]

Lithium metal containing 1% sodium (2.3 g, 83.0 mmol, 2.4 eq, as a 50% dispersion in oil) was placed in a two necked flask equipped with a stirrer bar, reflux condenser and dropping funnel.

The lithium was washed with dry diethyl ether (2 x 10 ml) and then re-suspended in diethyl ether (10ml). $^2H_3$-Iodomethane (5.0 g, 34.5 mmol, 99.5 atom % D) was added at such a rate as to maintain gentle reflux. The resulting solution was stirred overnight at room temperature and then filtered *via* cannula into a dry flask to give $^2H_3$-methyllithium (16.0 ml, 0.91 molar [as determined by calibration against diphenylacetone tosylhydrazone][13], 14.5 mmol, 42% yield).

(6R,7R)-1-Aza-3-[($^2H_3$)-methyl]-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid methyl ester (5)

Copper cyanide (630 mg, 7.0 mmol) was azeotroped with toluene (3 x 10 ml) on a high-vacuum line, each time equilibrating under an atmosphere of argon. The cyanide was suspended in THF (20 ml) and cooled with stirring to -78°C for 5 minutes after which time $^2H_3$-methyllithium (15.4 ml, 0.91 M, 14.0 mmol) was added dropwise with concomitant formation of a heavy white precipitate, presumed to be lithium iodide. This mixture was stirred at 5°C for 20 minutes and then cooled back to -78°C, and a solution of the chlorocephem (4) (810 mg, 2.3 mmol) in degassed THF (20 ml) added dropwise. The suspension was warmed to -50°C for 30 minutes and then quenched by the addition of saturated aqueous NH_4Cl (20 ml) and EtOAc (150 ml) and finally filtered. The organic layer was separated, washed with water (3 x 30 ml), brine (30 ml), dried (anhydrous Na_2SO_4), filtered and then evaporated *in vacuo* to dryness. Chromatography [flash silica, EtOAc / DCM (1:4 v/v)] gave **5** (397 mg, 1.2 mmol, 52% yield); t.l.c.,[EtOAc / DCM (35 : 65 v/v)] R_f 0.65; m.p. 109-112°C (from DCM/petrol); υ_{max}($CHCl_3$) 1780 (m), 1720 (m), and 1560 (m); δ_H (500MHz, $CDCl_3$) 1.46 (9H, s, $C(CH_3)_3$), 3.22 and 3.51 (2 x 1H, 2 x d, *J* 18Hz, CH_2S), 3.84 (3H, s, CO_2CH_3), 4.95 (1H, d, *J* 4.5Hz, CHS), 5.21 (1H, d, *J* 13Hz, NH), 5.56 (1H, dd, *J* 13 and 4.5Hz, NHCHCH); *m/z* (DCI, NH_3) 332 (MH^+, 39%), 333 (6), 334 (4), 335 (1).

(2R,6R,7R)-1-Aza-3-[($^2H_3$)-methyl]-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic acid (6)

Procedure as described by Mobashery and Johnson[14] was followed. Thus to a stirred solution of **5** (202 mg, 0.61 mmol) in water / pyridine (1:1 v/v, 10ml) at 0°C was added 1N NaOH (630 µl, 0.63 mmol). The resulting solution was stirred at 0°C for a further 4 hours and then washed with DCM (2 x 20 ml), acidified to *ca* pH 2 with 2N HCl and rapidly extracted with EtOAc (3 x 30 ml). The combined EtOAc layers were dried (anhydrous Na_2SO_4), filtered, and evaporated to dryness to give **6** (160 mg, 0.53 mmol, 86% yield) as a white solid which was used without further purification; m.p. 168-171°C dec. (from Et_2O); υ_{max}($CHCl_3$) 3020 (s), 1760 (w), 1710 (s), and 1360 (m); δ_H(200MHz, $^2H_6$-acetone) 1.36 (9H, s , $C(CH_3)_3$), 4.67 (1H, s, $CHCO_2H$), 5.21 (1H, d, *J* 3Hz, CHS), 5.41 (1H, br.s, NHCHCH), 6.11 (1H, s, SCH-C), 6.72 (1H, br. s, NH); *m/z* (DCI, NH_3) 335 (MNH_4^+, 100%), 336 (19), 337 (11), 338 (2).

(2R,6R,7R)-1-Aza-3-[($^2H_3$)-methyl]-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-3-ene-2-carboxylic acid benzhydryl ester (7)

The free acid **6** (150 mg, 0.49 mmol) was dissolved in MeCN (10 ml) and the resulting solution stirred at room temperature. Diphenyldiazomethane (95 mg, 0.5 mmol) was added and the resulting solution was stirred until the esterification was complete as judged by the persistence of a faint pink colouration. The solvent was removed *in vacuo* to give **7** (236 mg, 0.48 mmol, 99%

yield); m.p 124-6°C (from DCM/petrol); (Found C 64.58, H 5.90 and N 5.79. $C_{26}H_{25}D_3N_2O_5S$ requires C 64.42, H 6.29, N 5.76%); υ_{max}(CHCl3) 3021 (m), 1776 (s), 1720 (s), and 1507 (s); δ_H (200MHz, $CDCl_3$) 1.46 (9H, s, C(C<u>H</u>3)3), 4.86 (1H, d, *J* 1.5Hz, C<u>H</u>CO2H), 5.22 (1H, d, *J* 3.5Hz, CHS), 5.38-5.45 (1H, br. m, NHC<u>H</u>CH), 5.95 (1H, d, *J* 1.5Hz, SCH=C), 6.91 (1H, s, C<u>H</u>Ar2), 7.34 (10H, s, Ar<u>H</u>); δ_C (50.3MHz, $CDCl_3$) 28.08 (C(<u>C</u>H3)3), 53.04 and 53.12 (2 x d, <u>C</u>HC<u>C</u>HS), 61.74 (d, <u>C</u>HCCD3), 78.86 (d, <u>C</u>HPh2), 81.30 (s, <u>C</u>(CH3)3), 114. 31 (d, <u>C</u>H=CCD3), 120.19 (s, CH=<u>C</u>CD3), 126.88-139.22 (8 x ArC), 154.90, 165.54 and 166.77 (3 x s, 3 x C=O); *m/z* (DCI, NH_3) 501 (MNH_4^+, 100%), 502 (31), 503 (11), 504 (3).

(2R,5S,6R,7R)-1-Aza-3-[(²H3)-methyl]-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid-S-oxide, benzhydryl ester (9)[8]

To a stirred solution of the Δ2-cephem (7) (243 mg, 0.50 mmol) in DCM (6 ml) at 0°C, was added dropwise MCPBA (147 mg, 0.85 mmol) in DCM (4 ml). The resulting solution was stirred at 0°C for 3 hours and then transfered to a separating funnel and washed with aqueous sodium thiosulphate (20 ml, 10% w/w), saturated aqueous $NaHCO_3$ (20 ml), brine (20 ml) and then dried (anhydrous Na_2SO_4). The solution was filtered and the solvent evaporated *in vacuo* to give **9** (270 mg) which was used without further purification; t.l.c.[EtOAc/DCM (1:4 v/v)] R_f 0.5; m.p 78-80°C (from Et_2O/petrol); υ_{max}(CHCl3) 3420 (w), 3020 (s), 1800 (s), 1706 (s), 1654 (w), 1456 (m), and 1230 (s); δ_H (200 MHz, $CDCl_3$) 1.46 (9H, s, C(C<u>H</u>3)3), 3.24 and 3.61 (2H, ABq, *J* 19Hz, SCH_2), 4.45 (1H, d, *J* 3Hz, CHC<u>H</u>SO), 5.75-5.80 (1H, br. m, NHC<u>H</u>CHS), 6.94 (1H, s, C<u>H</u>Ph2), 7.24-7.51 (10H, m, ArH); *m/z* (DCI, NH_3) 517 (MNH_4^+, 100%), 518 (33), 519 (15), 520 (1).

(6R,7R)-1-Aza-3-[(²H3)-methyl]-7-amino-(N-*t*-butyloxycarbonyl)-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid benzhydryl ester (10)

To a stirred solution of the sulphoxide **9** (250 mg, 0.51 mmol) in dry DMF (4 ml) at 0°C was added potassium iodide (1.2 g) and acetyl chloride (0.2 ml) (freshly distilled from quinoline)[15]. The resulting solution was stirred at 5°C for 1 hour and then saturated aqueous sodium metabisulphite (8 ml) was added. This mixture was extracted with EtOAc (3 x 10 ml) and the combined organic layers washed with water (20 ml), saturated aqueous $NaHCO_3$ (20 ml), brine (20 ml) and dried (anhydrous Na_2SO_4). After filtering, the solvent was evaporated *in vacuo* to give **10** (120 mg, 0.25 mmol, 50% from **7**); t.l.c.[EtOAc/DCM (2:3 v/v)] R_f 0.8; m.p. 90-92°C (from DCM/petrol); υ_{max}(CHCl3) 3435 (w), 3020 (w), 1785 (s), 1720 (s), 1505 (s), and 1371 (m); δ_H (500 MHz, $CDCl_3$) 1.46 (9H, s, C(C<u>H</u>3)3), 3.21 and 3.48 (2H, ABq, *J* 18Hz, SCH_2), 4.95 (1H, d, *J* 4Hz, CHS), 5.30 (1H, d, *J* 9Hz, N<u>H</u>CH), 5.59 (1H, dd, *J* 4 and 9Hz, NHC<u>H</u>CHS), 6.92 (1H, s, C<u>H</u>Ph2), 7.24-7.46 (10H, m, CH<u>Ph</u>2); δ_C (125.77 MHz, $CDCl_3$) 18.8-19.5 (br. m, CD_3), 28.04 (q, C(<u>C</u>H3)3), 55.47 (t, S<u>C</u>H2), 60.44 (d, CH<u>C</u>H), 67.01 (d, NH<u>C</u>H), 79.46 (d, <u>C</u>HAr2), 80.84 (s, <u>C</u>(CH3)3), 122.31 and 125.82 (2 x s, C=<u>C</u>CD3 and <u>C</u>=CCD3), 127-139 (Ar<u>C</u>), 154.55, 160.28 and 164.88 (3 x s, <u>C</u>=O); *m/z* (DCI, NH_3) 501 (MNH_4^+, 100%), 502 (31), 503 (12), 504 (3).

(6R,7R)-1-Aza-3-[(²H3)-methyl]-7-amino-[(5R)-5-N-*p*-methoxybenzyl-oxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid benzhydryl ester (11)

The *t*-BOC-cephem **10** (100 mg, 0.21 mmol) was dissolved in EtOH/Et_2O [1:1, v/v (2 ml)] and

p-toluenesulphonic acid (40 mg, 0.21 mmol) added. The resulting solution was stirred at room temperature for 48 hours and the solvent evaporated to give **10** as the ammonium tosylate salt (115 mg, 0.21 mmol, 100% yield). The crude tosylate salt was dissolved in saturated aqueous $NaHCO_3$ (10 ml) and the resulting solution then extracted with EtOAc (3 x 15 ml). The combined organic layers were dried (anhydrous Na_2SO_4), filtered and the solvent evaporated *in vacuo* to give a free amine (79 mg, 0.21 mmol). The amine was then coupled with (5R)-5-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanoic acid by previously reported methodologies[16] (92 mg, 0.21 mmol) to give after chromatography [flash silica, EtOAc/petrol (1:1 v/v)] compound **11** (135 mg, 0.17 mmol, 81% yield); t.l.c.[EtOAc] R_f 0.2; m.p 68-9°C (from DCM/Et_2O/petrol); δ_H (200MHz, $CDCl_3$) 1.68 (4H, m, $CH_2CH_2CH_2CO$), 2.25 (2H, m, CH_2CO), 3.09 and 3.60 (2H, ABq, *J* 19Hz, SCH_2), 3.79 (6H, s, $ArOCH_3$), 4.32-4.44 (1H, m, CHNHCO), 4.91 (1H, d, *J* 4Hz, CHS), 5.02 and 5.11 (2 x 2H, 2 x s, 2 x CH_2Ar), 5.62 (1H, d, *J* 8Hz, NHCH), 5.78 (1H, dd, *J* 4 and 8Hz, NHCHCH), 6.81- 6.97 and 7.25-7.48 (18H, 2 x m, ArH); δ_C (50.31MHz, $CDCl_3$) 21.28 (t, $CH_2CH_2CH_2CO$), 30.12 (t, $CH_2CH_2CH_2CO$), 31.81 (t, CH_2CO), 34.88 (t, SCH_2), 53.44, 55.19, and 57. 40 (3 x d, NHCH, CHCHS and $NHCHCH_2$), 58.97 (q, CH_3OAr), 66.85 and 67.05 (2 x t, CH_2Ar), 79.02 (d, $CHPh_2$), 114.03 and 114.11 (2 x d, ArC), 122.73 (s, $C{=}CCD_3$), 127.06-130.31 (7 x s, ArC), 133.93 (s, $C{=}CCD_3$), 139.72, 139.87 (2 x s, ArC), 154.53, 156.61, 159.79 and 159.99 (4 x s, MeOC of ArOMe and ArO_2CNH), 161.67, 165.32, 172.46, and 173.26 (4 x s, C=O); *m/z* (FAB, MCA) 811 (MH^+, 100%), 812 (39), 813 (17), 814 (7).

(6R,7R)-1-Aza-3-[($^2H_3$)-methyl]-7-amino-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (2b), [(3'-$^2H_3$-methyl)-DAOC]

To protected **11** (120 mg, 0.15 mmol) was added a solution of anisole and TFA [1:4 v/v (2 ml)] in toluene (4 ml) and the resulting mixture stirred at room temperature for 30 minutes. The solvent was then evaporated and the residue partitioned between water (5 ml) and EtOAc (5 ml). The aqueous phase was extracted with EtOAc (3 x 5 ml) and freeze-dried to give **2b** as its ammonium trifluoroacetate salt (58.5 mg, 0.13 mmmol, 88% yield) which was used without further purification. δ_H(500 MHz, D_2O, HOD suppressed); 1.67-2.03 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.44 (2H, *ca* t, CH_2CO), 3.38 and 3.61 (2 x 1H, 2 x d, *J* 18Hz, SCH_2), 4.02 (1H, *ca* t, $CHNH^+_3$), 5.14 and 5.58 (2H, ABq, *J* 4Hz, CHCHS); *m/z* (FAB, MCA) 360 (4%), 361 (MH^+, 100), 362 (18), 363 (13), 364 (2). [Identical to authentic DAOC (**2a**) by ^{1}H-n.m.r. except for the absence of the methyl group at δ1.94 ppm].

Mixed Competitive Isotope Effect Experiments with (2a) and (2b)

An approximately 1 : 1 mixture of **2a** and **2b** (*ca* 2mg) was prepared and a small sample taken representing a time zero reference point. DAOC/ DAC synthase (2 ml, *ca* 0.1 International Units) in TRIS-HCl buffer (pH 7.4, 50 mM) was pre-incubated for 5 minutes at 27°C and 250 rpm with 200 µl of cofactor solution, prepared from α-ketoglutarate (14.6 mg), L-ascorbate (17.6 mg), dithiothreitol (30.8 mg), iron (II) sulphate (1.4 mg) and ammonium sulphate (1.32 g) in distilled water (10 ml). The remaining substrate (*ca* 1.8 mg) in TRIS-HCl (1.8 ml, 50 mM, pH 7.4) was added and the pH adjusted to pH 7.4 by the addition of 1N NaOH solution. The resulting solution was incubated at 27°C and 250 rpm and portions removed at varying times to give samples with varying degrees of

enzymic conversion. The protein was precipitated in each of the samples by the addition of acetone to 70% v/v and the samples then centrifuged. The supernatant was evaporated to dryness and re-dissolved in D_2O, prior to examination by ^{1}H-nmr (500MHz, HOD suppressed). The region δ 6.0 to 4.5ppm was integrated and the percentage conversion of the starting material calculated by comparison of the integrals of the β-lactam resonances of DAOC (**2**) and DAC (**3**). Unconverted DAOC (**2a/b**) present in the incubation samples was then isolated by chromatography [reverse phase h.p.l.c, Gilson system with a C_{18} stationary phase eluting with 10mM NH_4HCO_3] and the DAOC (**2a/b**) samples converted to the N-ethoxycarbonyl, dimethyl ester derivatives [9,10] **12a/b** *via* standard methodologies prior to analysis by mass spectrometry.

FAB Mass Spectroscopic Results for DAOC derivatives (12a/12b)

Expt.1 Time (minutes)	% Conversion	Relative Intensities of the Molecular Ions (**12a/12b**)(*m/z*)								
		457	458	459	460	461	462	463	464	465
0	0	0	100	26	22	98	25	17	4	1
33	55	0	71	20	21	100	25	20	5	3

Expt.1 Time (minutes)	% Conversion	Relative Intensities of the Fragment Ions (**13a/13b**) (*m/z*)								
		171	172	173	174	175	176	177	178	179
0	0	0	100	16	18	95	14	9	2	3
33	55	0	74	21	37	100	25	22	9	9

Expt.2 Time (minutes)	% Conversion	Relative Intensities of the Molecular Ions (**12a/12b**)(*m/z*)								
		457	458	459	460	461	462	463	464	465
0	0	0	84	21	23	100	24	22	5	2
15	20	0	77	19	21	100	25	23	5	2
35	35	0	70	17	19	100	25	22	5	2
45	60	0	52	13	22	100	26	34	8	3

Expt.2 Time (minutes)	% Conversion	Relative Intensities of the Fragment Ions (**13a/13b**) (*m/z*)								
		171	172	173	174	175	176	177	178	179
0	0	0	89	13	0	100	13	10	2	2
15	20	0	79	11	0	100	12	10	1	2
35	35	0	73	10	0	100	13	10	1	2
45	60	0	55	10	0	100	15	13	2	4

Incubation of (2a) and (2b) in a Denatured Control Experiment.

An approximately 1 : 1 mixture of **2a** and **2b** (*ca* 2mg) was prepared and a sample (*ca* 1mg) taken thus representing a time zero reference point. DAOC/DAC synthase (0.05 I.U. in 50mM Tris-HCl buffer (2ml) at pH 7.4) was heated in a heating block previously equilibrated at 100°C for 5 minutes and then allowed to cool to room temperature. Cofactor solution (200 µl) (prepared as described above) was added and the pH adjusted to pH 7.4 by the addition of 1N NaOH solution. The resulting

solution was then pre-incubated at 27°C at 250 rpm for 5 minutes. and the remaining substrate (*c a* 1mg) in water (1.8mls) added. This mixture was then incubated for a further 2 hrs after which time acetone was added to 70% v/v (5mls). The precipitated protein was removed by centrifugation and the supernatant solution evaporated to dryness on a dry-ice rotavapour. After re-dissolution in D_2O, the sample was examined by ^{1}H-nmr (500MHz, HOD suppressed) in the region δ 6.0 to 4.5ppm. No conversion of DAOC (**2a/b**) to DAC (**3a/b**) was observed. The DAOC (**2a/b**) was isolated by chromatography [reverse phase h.p.l.c, Gilson system eluting with 10mM NH_4HCO_3] and the recovered (**2a/b**) then converted to the N-ethoxycarbonyl, dimethyl ester[9,10] (**12a/b**) derivative *via* the standard method prior to analysis by mass spectrometry.

Time (minutes)	% Conversion	Relative Intensities of the Molecular Ions (**12a/12b**) (*m/z*)								
		457	458	459	460	461	462	463	464	465
0	0	8	93	29	19	100	26	14	3	0
120	0	11	100	56	34	99	28	19	0	2

Time (minutes)	% Conversion	Relative Intensities of the Fragment Ions (**13a/13b**) (*m/z*)								
		171	172	173	174	175	176	177	178	179
0	0	0	99	14	0	100	13	7	1	1
120	0	8	100	27	0	97	13	9	0	2

References.

1 Scheidegger, A.; Kuenzi, M. T.; and Nuesch, J.; J. Antibiotics., 1984, 37, 522.
2 Baldwin, J. E.; Adlington, R. M.; Coates, J. B.; Crabbe, M. J. C.; Crouch, N. P.; Keeping, J. W.; Knight, G. C.; Schofield, C. J.; Ting, H.-H.; Vallejo, C. A.; Thorniley, M.; and Abraham, E. P.; Biochem. J., 1987, 245, 831.
3 Baldwin, J. E.; Adlington, R. M.; Crouch, N. P.; Coates, J. B.; Keeping, J. W.; Schofield, C. J.; Shuttleworth, W. A.; and Sutherland, J. D.; J. Antibiotics., 1988, 41, 1694.
4 Samson, S. M.; Dotzlaf, J. E.; Slisz, M. L.; Becker, G. W.; VanFrank, R. M.; Veal, L. E.; Yeh, N.-K; Miller, J. R.; Queener, S. W.; and Ingolia, T. D.; Biotechnology, 1987, 5, 1207.
5 Baldwin, J. E.; Adlington, R. M.; Aplin, R. T.; Crouch, N. P.; Schofield, C. J.; and Ting, H.-H.; J. Chem. Soc., Chem. Commun., 1987, 1654.
6 Townsend, C. A.; and Barrabee, E. B.; J. Chem. Soc., Chem. Commun., 1984, 1586.
7 Spry, D. O.; and Bhala, A. R.; Heterocycles, 1985, 23, 1901.
8 Kaiser, G. V.; Cooper, R. D. G.;Kochler, R. E. ; Murphy, C. F.; Webber, J. A.; and Van Heyningen, E. M.; J. Org. Chem., 1970, 35, 2430.
9 Loder, P. B.; and Abraham, E. P.; Biochem. J., 1971, 123, 471.
10 Baldwin, J. E.; Adlington, R. M.; Aplin, R. T.; Crouch, N. P.; Schofield, C. J.; and Ting, H.-H.; J. Chem. Soc., Chem. Commun., 1987, 1654.
11 Baldwin, J. E.; Adlington, R. M.; Crouch, N. P.; and Schofield, C. J.; Tetrahedron, 1988, 44, 643.
12 Schollkopf, U.;Paust, J.; and Patsch, M. R.; Org. Synth. Coll. Vol. V, p. 860, Note 4.
13 Lipton, M. F.; Sorensen, C. M.; Sadler, A. C.; and Shapiro, R. H.; J. Organometal. Chem., 1980, 186, 155.
14 Mobashery, S.; and Johnston, M.; J. Org. Chem., 1986, 51, 4723.
15 Perrin, D. D.; Armarego, W. L. F.; and Perrin, D. R.; in 'Purification of Laboratory Chemicals', p. 83, 2nd Ed. 1980, Pergamon Press.
16 Prepared by methods analogous to those previously used. See Baldwin, J. E.; Herchen, S. R.; Johnson, B. L.; Jung, M.; Usher, J. J.; and Wan, T.; J. Chem. Soc., Perkin Trans.1., 1981, 2253., and Baldwin, J. E.; Adlington, R. M.; Bradley, M.; Norris, W. J.; and Turner, N. T.;Tetrahedron 1991, 47 (3), 457.