Tetrahedron Vol. 44, No. 2, pp. 643 to 650, 1988
Printed in Great Britain.

0040-4020/88 $3.00+.00

THE ENZYMATIC CONVERSION OF EXOMETHYLENE CEPHALOSPORIN C INTO DEACETYL CEPHALOSPORIN C AND THE ROLE OF MOLECULAR OXYGEN IN CEPHALOSPORIN C BIOSYNTHESIS

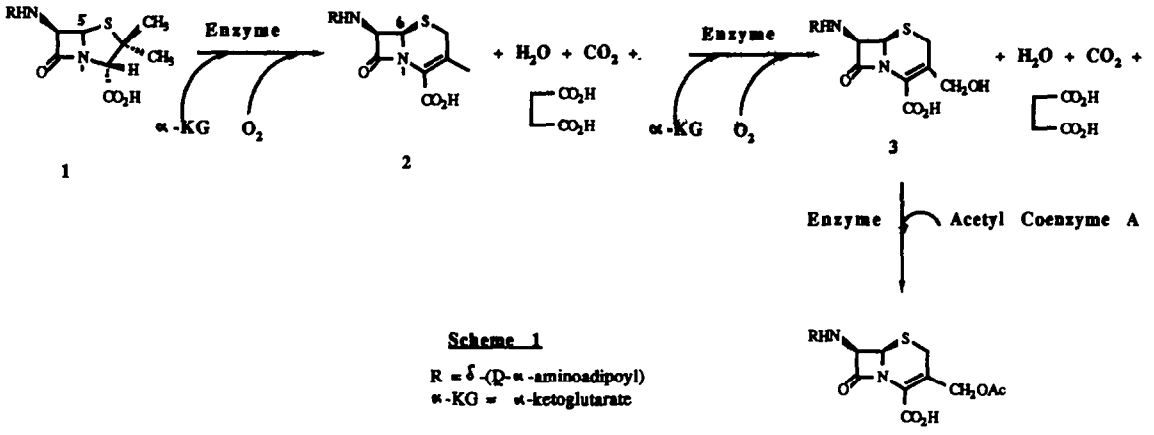
JACK E. BALDWIN,* ROBERT M. ADLINGTON, NICHOLAS P. CROUCH AND CHRISTOPHER J. SCHOFIELD,

The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.

(*Received in UK* 2 *November* 1987)

Abstract: Cell free extracts from Cephalosporium acremonium containing deacetoxy/-deacetyl cephalosporin C Synthetase activities have been shown to convert Exomethylene cephalosporin C directly to deacetyl cephalosporin C. Labelling experiments in the presence of $^{18}O_2$, indicated the source of the 3-methylene-oxygen of the latter was dioxygen.[1]

Until recently mechanistic investigations into the biosynthesis of cephalosporins have been hampered by the lack of reliable cell free extracts. Despite this it has been demonstrated that the immediate biosynthetic precursor of the cephalosporins is penicillin N (1).[2,3] Ring expansion of 1 to give deacetoxy cephalosporin C (DAOC)[2,3] (2) is followed by hydroxylation to give deacetyl cephalosporin C (DAC) (3)[4] which is then acetylated to give cephalosporin C (4) (Scheme 1).[5]



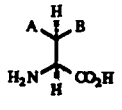
1 2 3

Scheme 1

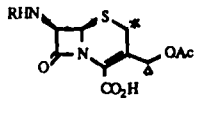
R = δ-(D-α-aminoadipoyl)
α-KG = α-ketoglutarate

4 = Cephalosporin C

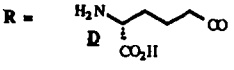
Intact cell experiments have demonstrated that 2S,3R,-^{13}C-valine (5a) gave 4-^{13}C-cephalosporin C (6a)[6] whilst 2S,3S-^{13}C-valine (5b) gave 3-methylene-^{13}C-Cephalosporin C (6b).[7]



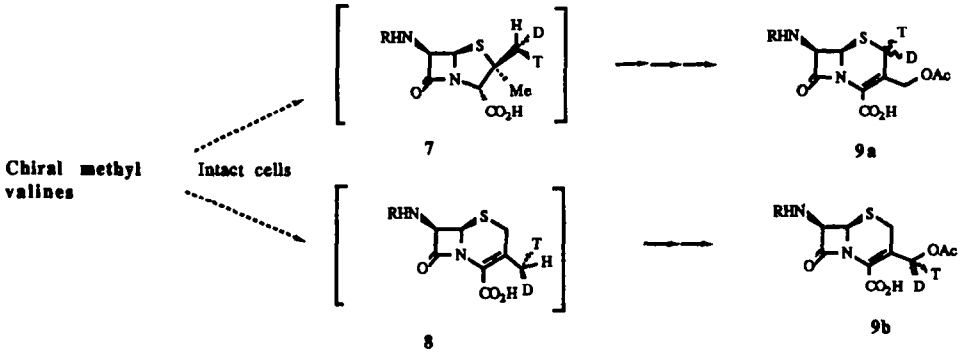
5 a) A = $^{13}CH_3$; B = $^{12}CH_3$
b) A = $^{13}CH_3$; B = $^{13}CH_3$



6 a) * = $^{13}CH_2$; Δ = $^{12}CH_2$
b) * = $^{12}CH_2$; Δ = $^{13}CH_2$



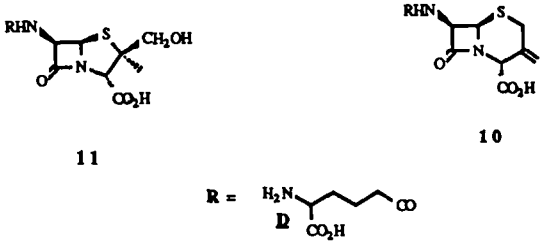
Townsend[8,9] and Abraham et al[10] have independently demonstrated by feeding chiral methyl valines to intact cell-systems, that the resultant cephalosporin C showed complete tritium scrambling at the C-2 position, to give 9a (presumably via 7) (a substantial primary isotope effect was noted)[8] and that the allylic hydroxylation occurred with complete retention of stereochemistry to give 9b[9] (presumably via 8) (Scheme 2). We have also investigated several putative intermediates for the ring expansion process,[11] including the 3β-methylene hydroxypenam (11). However incubation of 11 with a freshly prepared extract of DAOC/DAC Synthetase gave no cephalosporin products.



Scheme 2

R = 6-(D-α-aminoadipoyl)

In our laboratories we have demonstrated, in accord with the results of Scheidigger et al,[12] using purified extracts from C.acremonium CO 728, that the biosynthetic conversions 1 → 2 and 2 → 3 are catalysed by a single bifunctional non haem iron oxygenase (termed DAOC/DAC Synthetase).[11] The enzyme was shown to require oxygen and α-ketoglutarate as co-substrates and needed ascorbate for optimal activity. (In contrast using prokaryotic systems Wolfe et al have reported the separation of the DAOC Synthetase and DAC Synthetase activities[13]).



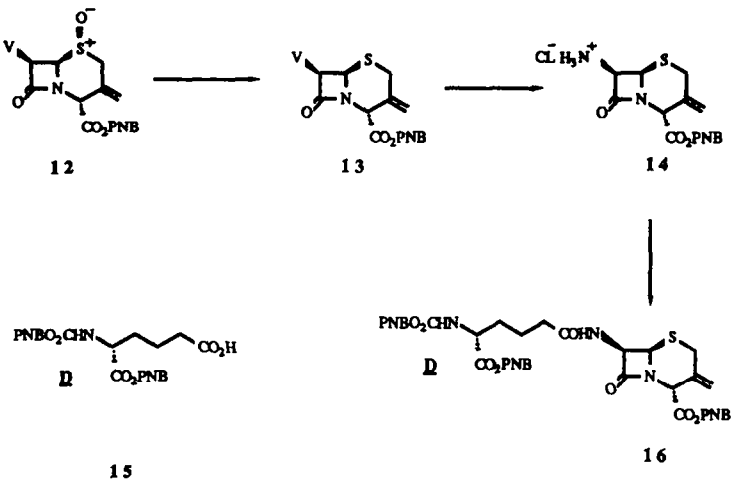
11 10

R = H_2N, D, CO_2H, CO

A mechanism involving the intermediacy of exomethylene cephalosporin C (10) may be considered for the ring expansion of 1 to 2. To investigate this possibility we have synthesised 10 and incubated it with purified extracts of DAOC/DAC Synthetase, from C.acremonium CO 728.

Thus the sulphoxide (12) (obtained from Eli Lilly & Co.) was deoxygenated (acetyl bromide/cyclohexene) to give the sulphide (13). Cleavage of the phenoxyacetyl side chain of 13 gave the ester (14), which was coupled with protected D-α-aminoadipic acid (15)[14] to yield the triprotected exomethylene cephem (16). Partial hydrogenation followed by purification (preparative electrophoresis at pH 3.5) gave 10.

When 10 was incubated with purified extracts of DAOC/DAC Synthetase, in the presence of the appropriate cofactors,[11] a single beta-lactam product was observed in the ^1H n.m.r. (500 MHz) of the crude incubation mixture (10-100% conversion of 10). This new product was identical (by ^1H n.m.r., h.p.l.c. retention time, and bioassay) after isolation, by reverse phase h.p.l.c., to an



12 13 14

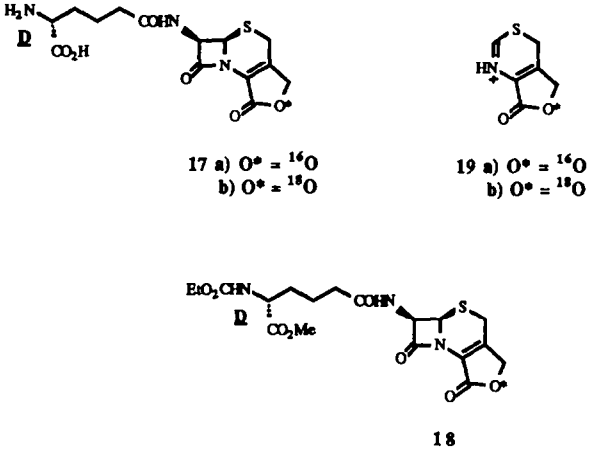
15 16

Scheme 3

PNB = 4-$O_2NC_6H_4CH_2$

V = $PhOCH_2CONH$

authentic sample of DAC (3). We did not observe any 2, either by ^1H n.m.r. or by h.p.l.c. analysis, thus 10 is not a free intermediate in the ring expansion process (1 → 2). Treatment of the biosynthetic (3) with formic acid gave the lactone (17), identical to an authentic sample (by ^1H n.m.r. and h.p.l.c. retention time), which was converted into the derivative (18)[15] (M^+ found 441.1206, $C_{18}H_{23}N_3O_6S$ requires 441.1206).

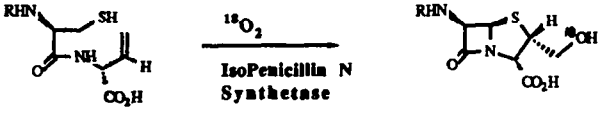


17 a) O* = ^{16}O
b) O* = ^{18}O

19 a) O* = ^{16}O
b) O* = ^{18}O

18

Recently, we have reported that the related enzyme isopenicillin N synthetase, is capable of producing hydroxylated β-lactam products from suitably unsaturated substrates[16,17] and shown that the source of the oxygen atom of the hydroxyl moiety was molecular oxygen[18] (for example Scheme 4). As we believed the conversion of 10 to 3, may arise from a similar mode of



Scheme 4

R = δ-(L-α-aminoadipoyl)

action by DAOC/DAC Synthetase, we have observed this conversion in an atmosphere of $^{18}O_2$. We also observed the cell free conversion of 2 to 3 in the presence of $^{18}O_2$. Stevens *et al*[19] have reported that the origin of the C3-methylene hydroxyl group of 3 from 2 was molecular oxygen, when an intact cell experiment in the presence of $^{18}O_2$ was performed. However, the level of incorporation was not reported.

Thus incubation of 2 and 10 independently with DAOC/DAC synthetase under an atmosphere of $^{18}O_2$ gas (99%) gave labelled (3a, b) which were purified and lactonised as before. Owing to the low levels of conversion (10 → 3, and 2 → 3), under the conditions of the labelling experiment, we were unable to obtain the lactone (17) cleanly as before. However, direct analysis of the underivatised lactone (17) was achieved by positive ion thermospray h.p.l.c. mass spectrometry, using a reverse phase octadecylsilane column. The results (Tables 1,2) indicate a significant (30-40%) incorporation of ^{18}O into the lactone (17), from both 10 and 3. The most probable explanation for the less than stochiometric levels of incorporation results from $^{18}O_2$ present in the enzyme preparation. We found that degassing of the enzyme solution led to loss of activity. However, we cannot eliminate the alternative explanation that the source of the hydroxylated oxygen atom was at least partly derived from water.

Experiment	Conditions	17 MH^{1+} ion/relative intensity (%)								Fragment 19 ion/relative intensity (%)							
1	$^{18}O_2$	m/e	354	355	356	357	358	359	360								
		Found	0	12	100	28	12	3	1								
		cal[d]	-	-	100	18	7	1	-								
2	$^{18}O_2$	m/e	354	355	356	357	358	359	360	361	m/e	154	155	156	157	158	159	1
		Found	4	1	100	23	58	13	8	2	Found	1	1	100	11	53	5	3
											Cal[d](a)	-	-	100	8	5	-	-

Table 1 (10 to 17, via 3)

a) for $C_4H_8N^{18}O_2S$

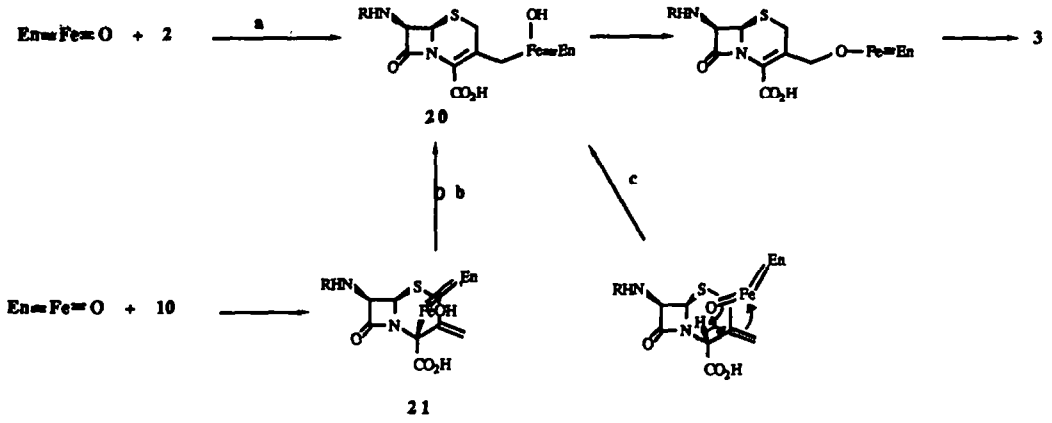
Experiment	Conditions	17 MH^{1+} ion/relative intensity (%)						Fragment 19 ion/relative intensity (%)						
1	$^{18}O_2$	m/e	355	356	357	358	359	360	m/e	155	156	157	158	159	160
		Found	2	100	30	14	7	4	Found	1	100	13	8	3	2
		Cal[d]	-	100	18	7	1	-	Cal[d]	-	100	8	5	-	-
2	$^{18}O_2$	m/e	355	356	357	358	359	360	m/e	155	156	157	158	159	160
		Found	4	100	25	72	27	22	Found	4	100	14	70	10	6

Table 2 (2 to 17, via 3)

We have proposed a mechanism for the enzymatic ring expansion of 1 involving an enzyme bound (Fe IV) ferryl oxo moiety,[20] in analogy with mechanisms proposed for the mode of action of other non haem oxygenases,[21] and believe the conversion of 10 to 3 may involve such a species. Iron, dioxygen and α-ketoglutarate can combine at the active site of DAOC/DAC synthetase to form the ferryl species, with the production of CO_2 and succinic acid. In the normal hydroxylation pathway from 2 this species can insert into a C-H bond of the 3-C-methyl group of 2 (Scheme 5, a) to give an enzyme bound intermediate (20), which can then collapse with retention[9] to give 3. In the case of 10 the enzyme bound ferryl species may insert into the 2-C-H bond to give 21, which may

rearrange (possibly via a radical process) to give 20 (Scheme 5, b). An alternative proposal is that an oxy-ene type reaction pathway (Scheme 5, c), such has been considered for cytochrome P-450[22], may give rise to the same intermediate.

If a ferryl-oxo (Fe=O) complex is indeed the enzymatic oxidant, then it is possible that the isotopic exchange of oxygen occurs at the active site. Groves et al have reported evidence for exchange processes of this type in oxidations utilising an oxoporphinate chromium (V) complex[23] or the complex resulting from the reaction of chloro-5,10,15,20-tetramesitylporphinatoiron (III) with m-chloroperoxybenzoic acid.[24,25]. There is, however, no precedent for an exchange of this type in a non-haem oxygenase and in the case of the decarboxylation coupled oxygenase, L-prolyl hydroxylase, Fujimoto et al[26] and Prockop et al[27] have independently demonstrated that $^{18}O_2$, but not H_2O^{18} was incorporated into collagen hydroxyproline.



Scheme 5

En = DAOC/DAC Synthetase

Acknowledgements: We thank the S.E.R.C. and Eli Lilly & Co. Indianapolis U.S.A. for financial support and Mr. G.C. Knight, Mr. J.W. Keeping and Dr. H-H. Ting for the preparation of the enzyme extracts and Mr. R. Procter and Dr. R. Aplin for mass spectrometric analysis.

GENERAL EXPERIMENTAL

Standard chemical procedures as previously reported were used.[11] Melting points were recorded on a Büchi 510 apparatus and are uncorrected. Infra red spectra were recorded on a Perkin Elmer 681 spectrophotometer. ^1H n.m.r. were recorded at 300 MHz on a Bruker WH300 spectrometer or at 500 MHz on a Bruker AM500 spectrometer. ^{13}C n.m.r. were recorded at 62.85 MHz on a Bruker AM250 spectrometer. Mass spectra in the electron impact or chemical ionisation modes were recorded on a VG Micromass 16F spectrometer. Samples requiring desorption chemical ionisation or fast atom bombardment were run on VG Micromass 30F or ZAB 1F spectrometers. The h.p.l.c./mass spectrometry was carried out using a VG Micromass 2250 spectrometer coupled to VG Thermospray interface Rheodyne 7R5 injector, two Water 510 pumps and a Phase Sep S5 ODS2 column (150 x 4.6 mm internal diameter).

H.p.l.c. of crude incubation mixtures was carried out using a Waters M-6000 A pump, Rheodyne 7R5 injector, PYE Unicam LC3 UV detector or Waters 441 Absorbance Detector and columns packed with Hypersil ODS (250 x 4.6 mm internal diameter).

Paper electrophoresis (analytical and preparative) was carried out at pH3.5 on Whatman No.1 filter paper at 70 volts cm^{-1} using a Locarte power pack. The buffer consisted of water, acetic acid and pyridine (135:10:1 v/v). Cadmium/ninhydrin reagent was used to locate amino acids.[29]

(2R,6R,7R)-1-Aza-3-methylene-7-[5R-5-p-nitrobenzyloxycarbonylamino-5-p-nitrobenzyloxycarbonylpentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid p-nitrobenzyl ester (16)

A solution of 14[28] (73 mg, 0.21 mmol), 5R-5-p-nitrobenzyloxycarbonylamino-5-p-nitrobenzyloxycarbonylpentanoic acid (15)[14] (100 mg, 0.21 mmol) and 1-ethoxycarbonyl-2-ethoxy-dihydroquinoline (52 mg, 0.21 mmol) in dichloromethane (6 ml) was stirred at room temperature for 15 hours. The solvent was removed *in vacuo*, the residue dissolved in ethyl acetate (25 ml), washed with 2N hydrochloric acid (25 ml), aqueous sodium bicarbonate (25 ml) and brine (25 ml), dried (magnesium sulphate) and evaporated to dryness. Chromatography [preparative layer chromatography (dichloromethane/ethyl acetate, 7:3) Rf 0.25] gave 16 (75 mg, 45%). δ_H (300 MHz, $CDCl_3$) 1.67-2.00(4H, m, $CH_2CH_2CH_2CO$), 2.24-2.41(2H, m, CH_2CO), 3.21, 3.63(2H, ABq, J 14 Hz, 4-H), 4.39-4.49(1H, m, $NHCHCO_2$), 5.18-5.34(8H, m, 3 x CH_2Ar, $C{=}CH_2$), 5.41(1H, d, J 4.5 Hz, 6-H), 5.54(1H, d, J 8 Hz, NH), 5.68(1H, dd, J 10, 4.5 Hz, 7-H), 6.31(1H, d, J 10 Hz, NH), 7.38-7.58(6H, m, ArH), 8.19-8.30(6H, m, ArH). ν_{max} ($CHCl_3$) 3400 m, 1770 s, 1740 s, 1710 s, 1680 s, 1525 m, 1350 s cm^{-1}. m/e (Field desorption) 806 (M^+); Found C, 54.06; H, 4.31; N, 27.63%. $C_{38}H_{34}N_6O_{14}S$ requires C, 54.00; H, 4.24; N, 27.78%.

Exomethylene Cephalosporin C-(2R,6R,7R)-1-Aza-3-methylene-7-[5R-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid (10)

The protected cepham (16) (50 mg, 6.2×10^{-2} mmol) was hydrogenated over 10% Pd on charcoal (100 mg) in the presence of sodium bicarbonate (6 mg, 7.1×10^{-2} mmol) in tetrahydrofuran/water (15 ml, 1:1) at 20°C and atmospheric pressure (hydrogen uptake = 8 ml). The solution was then filtered (celite), washed with water (10 ml) and evaporated to dryness. The residue was then partitioned between water (25 ml) and ethyl acetate (25 ml), the aqueous layer was separated, washed [2 x ethyl acetate (25 ml)] and evaporated to dryness. The residue was then purified by electrophoresis (pH 3.5, 4 KV, 1 hour) and the ninhydrin active band 15-20 cm from the origin extracted to give 10 (18 mg, 81%). δ_H (300 MHz, D_2O) 1.35-1.80(4H, m, $CH_2CH_2CH_2CO$), 2.32-2.52(2H, m, CH_2CO), 3.18, 3.68(2H, ABq, J 13 Hz, 2 x 4-H), 3.70-3.73(1H, m, $HNCHCO_2$), 4.81(1H, s, 2-H), 5.08, 5.19(2H, 2 x s, $C{=}CH_2$), 5.39, 5.47(2H, 2 x d, J 4.5 Hz, 6-H, 7-H). m/e (positive argon fast atom bombardment) 358 (MH^+). Alternative purification was obtained by h.p.l.c. (25 mM ammonium bicarbonate, retention time ca 11 min.).

Incubation of 10 with DAC/DOAC Synthetase

Partially purified DAC/DAOC Synthetase (2 ml, ca 1.0 International Unit) in TRIS-HCl buffer (pH 7.4, 50mM)[11] was preincubated for 2 minutes at 27°C and 250 r.p.m. with 200 µl of pH 7.4 co-factor solution prepared from α-ketoglutarate (14.6 mg), L-ascorbate (17.6 mg), dithiothreitol (30.8 mg), ferrous sulphate (1.4 mg) and ammonium sulphate (1.32 g) in water (10 ml). 10 (1 mg) in TRIS-HCl (pH 7.4, 1.8 ml, 50 mM) was added and the pH adjusted to 7.4 (c. NaOH). The resulting solution was incubated at 27°C and 250 r.p.m. for 2 hours, after which protein was precipitated by the addition of acetone to 70% v/v. After centrifugation (10 K r.p.m., 2 minutes at 0°C) the supernatent was evaporated to dryness. The resultant solid was suspended in D_2O (1.0 ml), re-evaporated and re-suspended in D_2O (ca 0.6 ml), filtered (ACRO LC13 disposable filter assembly, Product No. 4450, Gelman Sciences) and examined by ^1H n.m.r. (500 MHz). A single β-lactam product was observed in the region δ 4.9-5.5. Purification by h.p.l.c. (25 mM ammonium bicarbonate) gave DAC (3), identical with an authentic sample, by ^1H n.m.r. (500 MHz, D_2O) 1.48-1.79(4H, m, $CH_2CH_2CH_2CO$), 2.22-2.28(2H, m, CH_2CO), 3.28, 3.58(2H, ABq, J 18Hz, 2 x 4-H), 4.07, 4.12(2H, ABq, J 15 Hz, CH_2OH), 4.96; 5.46(2H, 2 x d, J 4.5 Hz, 6-H, 7-H), H.p.l.c. [retention time ca 8 min.], electrophoresis (pH 3.5, 4 KV, 1hour, ca 21 cm from the origin) and bio-assay against E.coli ESS. In a boiled control experiment, the DAOC/DAC Synthetase was refluxed in TRIS-HCl for 10 minutes before incubation. After work-up as before, only starting material (10) was observed in the ^1H n.m.r. (500 MHz) of the crude incubation mixture.

Derivatisation of Biosynthetic 3 (Method a)

Biosynthetic 3 (ca 500 µg) was dissolved in formic acid (0.5 ml) and allowed to stand for 10 minutes, after which it was evaporated to dryness. The crude lactone (17) was dissolved in 1.5 ml (water), then diethyl pyrocarbonate (100 µl) was added to the solution. The reaction mixture was basified to pH 9-10 (aqueous sodium bicarbonate) and vigorously stirred for 50 minutes, when it was acidified to pH2. The aqueous solution was extracted with ethyl acetate (3 x 5 ml) and dried briefly (sodium sulphate). Excess diazomethane in ether was then added to the stirred solution. After stirring for 15 minutes, the solution was evaporated to dryness to give the crude derivative (18). Chromatography [preparative layer chromatography (ethyl acetate) Rf 0.35] gave 18, identical with an authentic sample. δ_H (500 MHz, $CDCl_3$) 1.24(3H, t, J 7 Hz, CH_2CH_3), 1.72-1.89(4H, m, $CH_2CH_2CH_2CO$), 2.29-2.40(2H, m, CH_2CH_2CO), 3.50, 3.76(2H, ABq, J 18 Hz, 4-H), 3.77(3H, s, CH_3) 4.08-4.12(2H, m, CH_2CH_3), 4.19, 4.96(2H, ABq, J 17 Hz, CH_2O), 5.05(1H, d, J 5 Hz, 6-H), 5.34(1H, d, J 7 Hz, NH), 5.94(1H, dd, J 8.5, 5 Hz, 7-H), 6.62(1H, J 8.5 Hz, NH); m/e (Desorption chemical Ionisation) 442 (MH^+); (electron impact, accurate mass measurement), found 441.1206, $C_{18}H_{23}N_3O_8S$ requires 441.1206.

Derivatisation of Biosynthetic 3 (Method b)

The crude lactone (17), obtained as in Method a, was purified by h.p.l.c. (ODS column, mobile phase = 5% acetonitrile/water) to give the purified lactone (17) (retention time = ca 14 minutes). δ_H (300 MHz, D_2O) 1.47-1.80(4H, m, $CH_2CH_2CH_2CO$), 2.22-2.29(2H, m, CH_2CH_2CO), 3.55, 3.72(2H, ABq, J 18 Hz, 4-H), 4.92, 4.95(2H, ABq, J 15 Hz, CH_2O), 5.06, 5.63(2H, 2 x d, J 4.5 Hz, 6-H, 7-H). m/e (positive argon fast atom bombardment) 356 (MH^+).

Incubation 2 or 10 with DAC/DAOC Synthetase under an atmosphere of $^{18}O_2$

A solution of 20 mmolar TRIS-HCl, pH 7.6 (15 ml), in a 2-necked 100 ml round bottomed flask was thoroughly degassed, after which the vessel was returned to atmospheric pressure with argon. This procedure was repeated three times. A sample of $^{18}O_2$ (99.8%, 40 ml) was then transferred to the incubation vessel by means of a gas tight syringe and introduced into the TRIS-HCl solution through a rubber spectrum. The solution was then stirred vigorously by means of an efficient magnetic stirrer for 3 hours. 5 ml of the oxygenated TRIS-HCl solution was then withdrawn from the vessel and was used to make up the cofactors solution, under an atmosphere of argon. The RE enzyme (ca 1 I.U.) in 4 ml of TRIS-HCl was then injected into the reaction flask. After stirring for 5 minutes, the cofactors (0.5 ml) were introduced and the vessel briefly degassed and returned to atmospheric pressure with argon. $^{18}O_2$ (40 ml) was then injected into the incubation solution, which was then stirred for 2 min., after which 10 or 2 (0.4 mg) in degassed TRIS HCl (0.25 ml) were added via a syringe. The incubation mixture was then shaken at 250 r.p.m. and 27°C for 2 hours, after which the protein was precipitated by injection of acetone and the incubation worked up as above to give 3, which was purified by h.p.l.c. as before and lactonised to give 17, which was analysed by h.p.l.c./mass spectrometry (see Tables 1 and 2).

References

1. This work has been reported in a preliminary form: R.M. Adlington, J.E. Baldwin, B. Chakravarti, M. Jung, S.E. Moroney, J.A. Murphy, P.D. Singh, J.J. Usher, and C. Vallejo, J.Chem.Soc., Chem.Commun., 1983, 153; J.E. Baldwin, R.M. Adlington, C.J. Schofield, N.P. Crouch, and Hong-Hoi Ting, J.Chem.Soc., Chem.Commun., 1987, 1556.
2. M. Kohsaka, and A.L. Demain, Biochem.Biophys.Res.Commun., 1976, 70, 465.
3. J.E. Baldwin, P.D. Singh, M. Yoshida, Y. Sawada, and A.L. Demain, Biochem.J., 1980, 186, 889.
4. M.K. Turner, J.E. Farthing, and S.L. Brewer, Biochem.J., 1978, 173, 839.
5. Y. Fujisawa, H. Shirafuji, M. Kida, K. Nara, M. Yoneda, and T. Kanzaki, Nature (London), New Biol., 1973, 246, 154.
6. N. Neuss, C.H. Nash, J.E. Baldwin, P.A. Lemke, and J.B. Grutzner, J.Am.Chem.Soc., 1973, 95, 3797.
7. H. Kluender, C.H. Bradley, C.J. Sih, P. Fawcett, and E.P. Abraham, J.Am.Chem.Soc., 1973, 95, 6149.
8. C.A. Townsend, A.B. Theis, A.Scott Neese, E.B. Barrabee, and D. Poland, J.Am.Chem.Soc., 1985, 107, 4760.
9. C.A. Townsend, and E.B. Barrabee, J.Chem.Soc., Chem.Commun., 1984, 1586.
10. C.-P. Pang, R.L. White, E.P. Abraham, D.H.G. Crout, M. Lutstorf, P.J. Morgan, and A.E. Derome, Biochem.J., 1984, 222, 777.
11. J.E. Baldwin, E.P. Abraham, R.M. Adlington, J.D. Coates, M.J. Crabbe, N.P. Crouch, J.W. Keeping, G.C. Knight, C.J. Schofield, M. Thornilley, H.-H. Ting, C.A. Vallejo, and T. Willis, Biochem.J., 1987, 245, 831.
12. A. Scheidegger, M.T. Küenzi, and J. Nüesch, J.Antibiot., 1984, 37, 522.
13. S.E. Jensen, D.W.S. Westlake, and S. Wolfe, J.Antibiot., 1985, 38, 263.
14. J.E. Baldwin, S.R. Herchen, and P.D. Singh, Biochem.J., 1980, 186, 881.
15. P.B. Loder, and E.P. Abraham, Biochem.J., 1971, 123, 471.
16. J.E. Baldwin, R.M. Adlington, A.E. Derome, H.-H. Ting, and N.J. Turner, J.Chem.Soc., Chem.Commun., 1984, 1211.
17. J.E. Baldwin, R.M. Adlington, A. Basak, S.L. Flitsch, A.K. Forrest, and H.-H. Ting, J.Chem.Soc., Chem.Commun., 1986, 273.

18. J.E. Baldwin, R.M. Adlington, S.L. Flitsch, H.-H. Ting, and N.J. Turner, J.Chem.Soc., Chem.Commun., 1986, 1305.

19. C.M. Stevens, E.P. Abraham, F.-C. Huang, and C.J. Sih, results reported at the Annual meeting of the Federation of American Society for experimental Biology and Medicine, (American Society for Biological Chemistry), April, 1975.

20. J.E. Baldwin in the Proceedings of the 15th International Symposium on the Chemistry of Natural Products, Kyoto, Japan, 1986.

21. See for example: B. Siegel, Bio-Org.Chem., 1979, 8, 219; H.M. Hanauske-Abel, and V. Gunzler, J.Theoret.Biol., 1982, 94, 421; J.S. Blanchard, and S. Englard, Biochem., 1983, 22, 5922.

22. J.T. Groves, and D.V. Subramanian, J.Am.Chem.Soc., 1984, 106, 2177.

23. J.T. Groves, and W.J. Kruper Jr., J.Am.Chem.Soc., 1979, 101, 7613.

24. J.T. Groves, R.C. Haushalter, M. Nakumara, T.E. Nomo, and B.J. Evans, J.Am.Chem.Soc., 1981, 103, 2884. See also; G.D. Nordblom, R.E. White, and M.J. Coon, Arch.Biochem.Biophys., 1976, 175, 524.

25. R.Kent Murmann, J.Am.Chem.Soc., 1974, 96, 7836.

26. D. Fujimoto, and N. Tamiya, Biochem.Biophys.Act, 1963, 69, 559.

27. D. Prockop, A. Kaplan, and S. Udenfriend, Arch.Biochem.Biophys., 1963, 101, 499.

28. R.R. Chauvette, and P.A. Pennington, J.Org.Chem., 1973, 38, 2994.

29. J. Heilmann, J. Barrollier, and E. Watzke, Hoppe-Seyler's Z.Physiol.Chem., 1957, 309, 219.