Purification and initial characterization of an enzyme with deacetoxycephalosporin C synthetase and hydroxylase activities

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Deacetoxycephalosporin C synthetase (expandase) from *Cephalosporium acremonium* (*Acremonium chrysogenum*) was purified to near homogeneity as judged by SDS/polyacrylamide-gel electrophoresis. The enzyme (M_r about 40000) exhibited a pH optimum around 7.5. It required 2-oxoglutarate (K_m 0.04 mM), Fe^{2+} and O_2 as cofactors, and ascorbate and dithiothreitol were necessary for maximum activity. It was stable for over 4 weeks at $-70^\circ C$ in the presence of 1 mM-dithiothreitol. Activity was inhibited by the thiol-quenching reagent *N*-ethylmaleimide, the metal-ion-chelating reagent bathophenanthroline, and NH_4HCO_3 . The highly purified enzyme also showed deacetoxycephalosporin C hydroxylase (deacetylcephalosporin C synthetase) activity, indicating that both expandase and hydroxylase activities are properties of a single protein. These activities could not be separated by ion-exchange, dye-ligand, gel-filtration or hydrophobic chromatography. A β -sulphoxide and a 3β -methylene hydroxy analogue of penicillin N were synthesized to test as potential intermediates in the ring-expansion reaction. Neither compound was a substrate for the enzyme. A synthetic analogue in which the 3β -methyl group and the 2-hydrogen atom of penicillin N were replaced by a cyclopropane ring was not a substrate but was a reversible inhibitor of the enzyme.

INTRODUCTION

Although the reactions catalysing the biosynthesis of penicillins and cephalosporins have been demonstrated in cell-free systems, it is only very recently that we have begun to understand the mechanisms of some of these reactions. The enzyme catalysing the cyclization of the tripeptide *L*- α -aminoadipoyl-*L*-cysteinyl-*D*-valine to form isopenicillin N (isopenicillin N synthetase) has been purified to homogeneity from *Cephalosporium acremonium* (*Acremonium chrysogenum*) (Pang *et al.*, 1984; Hollander *et al.*, 1984; Baldwin *et al.*, 1985) and this enzyme cloned into *Escherichia coli* (Samson *et al.*, 1985).

In *Cephalosporium* and *Streptomyces* the *L*- α -aminoadipoyl side chain of isopenicillin N is epimerized to the *D* form, yielding penicillin N (1) (Jayatilake *et al.*, 1981). This is further metabolized to cephalosporin C (for reviews see Abraham, 1985; Jensen, 1986). The five-membered thiazolidine ring of penicillin N (1) undergoes an oxidative expansion ('expandase reaction') to a six-membered cephem [deacetoxycephalosporin C (DAOC) (2)], which in turn is hydroxylated to form deacetylcephalosporin C (DAC) (3) (Scheme 1). In the prokaryote *Streptomyces clavuligerus* the two activities

were separated and partially purified by DEAE-Trisacryl chromatography (Jensen *et al.*, 1985). In the eukaryote *Cephalosporium acremonium* the activities were not separated chromatographically on the same resin (Scheidegger *et al.*, 1984), and it was suggested that they were associated with a single enzyme in this organism. In the present paper we describe methods for the assay and purification of an enzyme with both expandase and hydroxylase activities and some of the properties of the purified product.

By analogy with the chemical conversion of penicillins into cephalosporins (Morin *et al.*, 1963), the β -sulphoxide (4) has been proposed as a potential intermediate in the biosynthetic transformation of penicillin N (1) into DAOC (2) (Baldwin *et al.*, 1978; Queener & Neuss, 1982). Alternatively, the β -methylene hydroxy penam (5) has also been proposed as a feasible intermediate for the bioconversion (Abraham, 1977; Baldwin *et al.*, 1978), a transformation for which there is also a synthetic precedent (Stork & Cheung, 1965). The postulated intermediates are in stereochemical accord with the observation that the 3β -methyl group of penicillin N (1) is incorporated into the dihydrothiazine moiety of DAOC (2) (Kluender *et al.*, 1973; Neuss *et al.*, 1973;

Scheme 1.

Abbreviations used: DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C; DTT, dithiothreitol; f.p.l.c., fast protein liquid chromatography.

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J. E. Baldwin, R. M. Adlington, C. J. Schofield, N. P. Crouch & N. J. Turner, unpublished work). We therefore undertook the syntheses of compounds **4** and **5** in an attempt to evaluate these hypotheses.

MATERIALS AND METHODS

Assay of expandase and hydroxylase activities

(a) **Bioassay of expandase activity.** Reaction mixtures contained DTT (1 mM), ascorbic acid (1 mM), 2-oxoglutarate (0.8 mM), FeSO_4 (0.04 mM), $(\text{NH}_4)_2\text{SO}_4$ (100 mM) and penicillin N (0.14 mM) in 50 mM-Tris/HCl buffer, pH 7.4, in a final volume of 250 μl . Mixtures were incubated at 27 °C in an orbital incubator, shaking at 250 rev./min. Samples were taken at 20, 40 and 60 min, and the reaction was stopped by the addition of an equal volume of methanol. After centrifugation, 100 μl samples of supernatants were loaded into wells in bioassay plates (Smith *et al.*, 1967) containing supersensitive *E. coli* ESS 21/30 and β -lactamase I (from *Bacillus cereus*) (10000 units/ml), to destroy unconverted penicillin N (1). Plates were incubated overnight at 37 °C. The diameters of the inhibition zones were measured, and amounts of product estimated by reference to standards of cephalosporin C. One unit of enzyme is that amount catalysing the production of 1 μmol of product/min under the conditions used.

DAOC (2) and DAC (3) produced identical zone sizes in the bioassay plates. Thus, although there was some conversion of DAOC into DAC in enzyme incubations [judged by the h.p.l.c. assay (c)], the sum total of product (DAC+DAOC) reflected DAOC production in the expandase reaction.

(b) **T.l.c./bioassay of hydroxylase activity.** Reaction mixtures were as described above for (a), except that DAOC (2) (0.28 mM) was used as substrate and the final volume was 20 μl . Reaction mixtures were incubated at 27 °C for 60 min, when methanol (20 μl) was added to stop the reaction, and the samples were centrifuged to remove protein. Samples of supernatants (4 μl) were loaded on to reverse-phase t.l.c. plates (Opti-Up C_{18} ; Fluka) and developed with 25 mM- NH_4HCO_3 for approx. 30 min to separate DAOC (2) and DAC (3). Plates were dried and blotted on to bioassay plates containing *E. coli* ESS 21/30 for 30 min at room temperature. After incubation of bioassay plates at 37 °C overnight, the position and size of inhibition zones relative to DAOC (2) (R_F approx. 0.45) and DAC (3) (R_F approx. 0.65) standards were recorded. This method could also be used to monitor expandase activity, by replacing DAOC (2) with penicillin N (1) as the substrate in the enzyme incubation, and using bioassay plates containing β -lactamase I (from *B. cereus*) as described in (a).

(c) **H.p.l.c. assays for expandase and hydroxylase activities.** Incubations, with either penicillin N (1) or DAOC (2) as substrate, were as described above. Samples were taken at zero time and after 60 min. After removal of protein by filtration in the Centrifree system (Amicon Corp., Stonehouse, Glos., U.K.) (Petchey & Crabbe, 1984), samples (10–30 μl) were loaded on to a $\mu\text{Bondapak C}_{18}$ h.p.l.c. column (Waters Associates, Milford, MA, U.S.A.) and eluted isocratically with 25 mM- NH_4HCO_3 at 1 ml/min, with monitoring at 220 nm. In other

experiments protein was removed by precipitation with an equal volume of methanol, samples were loaded on to a $\mu\text{Bondapak C}_{18}$ h.p.l.c. column, the products were eluted isocratically with 50 mM-sodium phosphate buffer (pH 3.8)/methanol/water (12:1:7, by vol.), and the eluate was monitored at 254 nm.

In all incubations product formation was linear for up to 1 h, and initial velocity was proportional to enzyme concentration under the conditions used in these studies.

Growth of organism

C. acremonium C.O. 728 was grown in a chemically defined medium as previously described (Pang *et al.*, 1984). Growth was monitored at 550 nm in a Pye-Unicam SP6-550 spectrophotometer. Mycelia were harvested and stored as described previously (Pang *et al.*, 1984). For the determination of the time course of enzyme production, enzyme activity was assessed after precipitation of expandase and hydroxylase from crude extracts with $(\text{NH}_4)_2\text{SO}_4$ (Table 1). Antibiotic production was determined by bioassays with *E. coli* ESS and *Staphylococcus aureus* N.C.T.C. 6571 as test organisms.

Preparation of cell-free extracts

These were prepared in a Dyno-Mill grinder (Glen Creston, Stanmore, Middx., U.K.) essentially as described previously (Pang *et al.*, 1984), by using the continuous-flow method and 50 mM-Tris/HCl buffer, pH 7.4, containing DTT (2 mM) and NaN_3 (0.015%).

Amino acid analysis

Thrice-distilled HCl (5.7 M) was used for hydrolysis, samples were treated by phenyl isothiocyanate labelling and analysed on a Waters Pico-Tag amino acid analysis h.p.l.c. (reverse-phase) system and 840 data-control station with Expert software (revision 4).

SDS/polyacrylamide-gel electrophoresis

This was performed by the method of Laemmli (1970), in a slab-gel apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with 10% (w/v) polyacrylamide gels. Ovotransferrin (hen's-egg; 76–78 kDa), albumin (bovine serum; 66.2 kDa), ovalbumin (hen's-egg; 45 kDa), carbonic anhydrase (bovine erythrocyte; 30 kDa), myoglobin (horse; 17.2 kDa) and cytochrome *c* (horse; 12.3 kDa) were used as molecular-mass markers (BDH Chemicals, Poole, Dorset, U.K.).

Chromatography

Matrex Gel Red A (Procion Red HE3B-agarose) was obtained from Amicon Corp. Buffers for f.p.l.c. (Pharmacia, Milton Keynes, Bucks., U.K.) were made up in Milli-Q highly purified water prepared from previously distilled water. Other buffers were prepared in deionized distilled water.

Synthetic methods

Standard chemical procedures as previously described were used (Baldwin *et al.*, 1986).

Melting points were recorded on a Büchi 510 apparatus and are uncorrected.

I.r. spectra were recorded on a Perkin-Elmer model 681 spectrophotometer (s = strong, m = medium, w = weak, b = broad).

^1H -n.m.r. spectra were either recorded at 300 MHz on a Bruker WH 300 NMR spectrometer or at 500 MHz on a Bruker AM 500 spectrometer.

Mass spectra in the electron-impact mode or chemical-ionization mode were recorded on a VG Micromass 16F spectrometer. Samples requiring desorption chemical ionization or fast atom bombardment were run on VG Micromass 30F or ZAB 1F spectrometers.

(2*S*,5*R*,6*R*)-1-Aza-3,3'-dimethyl-6-[(5*R*)-5-*p*-nitrobenzyloxycarbonyl-5-*p*-nitrobenzyloxycarbonylamino-pentanamido]-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylic acid *p*-nitrobenzyl ester (7)

To a solution of 6-aminopenicillanic acid *p*-nitrobenzyl ester (6.60 g, 18.8 mmol) in dichloromethane (100 ml) was added compound 6 (Baldwin *et al.*, 1980) (8.93 g, 18.8 mmol) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (4.64 g, 18.8 mmol). The solution was stirred at 20 °C for 24 h and then evaporated to dryness. The residue was dissolved in ethyl acetate (200 ml), washed with 1 M-HCl (100 ml), 0.6 M-NaHCO₃ (100 ml) and satd. NaCl (100 ml), dried (over Na₂SO₄), filtered and evaporated to dryness. Chromatography [flash silica, with dichloromethane/ethyl acetate (1:0 to 1:1, v/v)] gave compound 7 (9.60 g, 63% yield). T.l.c. (ethyl acetate/hexane (1:1, v/v)) *R_F* 0.4. M.s. (electron impact) 808 (*M*⁺). Analysis: Found: C, 53.33; H, 4.69; N, 9.99; S, 3.89; calc. for C₃₆H₃₆N₆O₁₄S: C, 53.46; H, 4.45; N, 10.34; S, 3.90%. ¹H n.m.r. δ (p.p.m.) (300 MHz, [²H]chloroform) 1.43 (3H, s, CH₃), 1.62 (3H, s, CH₃), 1.69–1.95 (4H, m, CH₂CH₂CH₂CO), 4.38–4.46 (1H, m, NCHCH₂), 4.47 (1H, s, 2-*H*), 5.20 (2H, approx. s, CH₂Ar), 5.23–5.36 (4H, m, 2 × CH₂Ar), 5.52 (1H, d, *J* = 4.0 Hz, 5-*H*), 5.69 (1H, dd, *J* = 6.0 and 4.0 Hz, 6-*H*), 6.27 (1H, d, *J* = 9.0 Hz, NH), 7.47–7.58 (7H, m, 6 × Ar-*H* + NH) and 8.18–8.29 (6H, m, 6 × Ar-*H*). [α]_D²⁰ + 86.6° (*c* = 1 in chloroform). I.r. (chloroform) 3440 (m), 1790 (s) (C=O, β-lactam), 1730 (s) (C=O, ester), 1690 (s) (C=O, amide), 1610 (s), 1525 (m), 1510 (m) and 1360 (s) (C–NO₂) cm⁻¹.

(2*S*, 5*R*, 6*R*)-1-Aza-3,3'-dimethyl-6-[(5*R*)-5-*p*-nitrobenzyloxycarbonyl-5-*p*-nitrobenzyloxycarbonylamino-pentanamido]-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylic acid *p*-nitrobenzyl ester β-sulphoxide (8)

To a solution of compound 7 (9.21 g, 11.4 mmol) in dichloromethane (50 ml) at 0 °C was added dropwise a solution of *m*-chloroperbenzoic acid (2.04 g, 14.8 mmol) in dichloromethane (50 ml). The reaction mixture was stirred at 0 °C for 45 min and at 20 °C for 1 h. The organic solution was then washed with 2.0 M-NaHSO₃ (2 × 50 ml) and satd. NaCl (50 ml), dried (over Na₂SO₄) and evaporated to dryness. Chromatography [flash silica, with ethyl acetate/light petroleum (b.p. 40–60 °C) (0:1 to 3:1, v/v)] gave compound 8 (8.80 g, 94% yield) as a foam. T.l.c. [ethyl acetate/dichloromethane (3:2, v/v)] *R_F* 0.2. Analysis: Found C, 50.30; H, 4.35; N, 9.43; calc. for C₃₆H₃₆N₆O₁₅S₂H₂O: C, 50.23; H, 4.18; N, 9.76%. ¹H n.m.r. δ (p.p.m.) (300 MHz, [²H]chloroform) 1.16 (3H, s, α-CH₃), 1.69 (3H, s, β-CH₃), 1.74–1.93 (4H, m, CH₂CH₂CH₂CO), 2.23–2.29 (2H, m, CH₂CO), 4.38–4.44 (1H, m, NCHCH₂), 4.70 (1H, s, 2-*H*), 5.04 (1H, d, *J* = 4.5 Hz, 5-*H*), 5.20 (2H, approx. s, CH₂Ar), 5.27 (2H, approx. s, CH₂Ar), 5.34 (2H, approx. s, CH₂Ar), 5.67 (1H, d, *J* = 8 Hz, NH), 6.05 (1H, dd, *J* = 10 and 4.5 Hz, 6-*H*), 7.01 (1H, d, *J* = 10 Hz, NH), 7.49–7.59 (6H, m, 6 × Ar-*H*) and 8.19–8.29 (6H, m, 6 × Ar-*H*). [α]_D²⁰ + 85.8° (*c* = 1 in chloroform). I.r.

(chloroform) 1800 (s) (C=O, β-lactam), 1750 (s) (C=O, ester), 1730 (s) and 1680 (s) cm⁻¹.

Penicillin N β-sulphoxide (4)

To a solution of compound 8 (71 mg, 0.091 mmol) in tetrahydrofuran (10 ml) were added water (10 ml) and NaHCO₃ (7.8 mg, 1 equivalent). The resultant solution was hydrogenated at 0.1 MPa (1 atm gauge) over 10% Pd on charcoal (100 mg) for 3 h. The solution was then filtered (Celite), washing with water (10 ml), and the filtrate was washed with ethyl acetate (2 × 10 ml). The aqueous layer was then freeze-dried to give crude compound 4. Chromatography (reverse-phase h.p.l.c., packing material Zorbax ODS C₁₈, eluent 25 mM-NH₄HCO₃, retention time 20 min) gave pure (by ¹H n.m.r.) compound 4 as the ammonium salt (25 mg, 73% yield). ¹H n.m.r. δ (p.p.m.) (300 MHz, ²H₂O) 1.11 (3H, s, α-CH₃), 1.50 (3H, s, β-CH₃), 1.45–1.59 and 1.63–1.77 (4H, 2 × m, CH₂CH₂CH₂CO), 2.19–2.23 (2H, m, CH₂CH₂CO), 3.60–3.33 (1H, m, NCHCH₂), 4.31 (1H, s, 2-*H*) and 5.18 and 5.67 (2 × 1H, 2 × d, *J* = 4.5 Hz, 5-*H* + 6-*H*). The stereochemistry of the sulphoxide was established by nuclear Overhauser spectroscopy in an analogous manner to that used for isopenicillin N β-sulphoxide (Bahadur *et al.*, 1981).

(2*R*, 3*R*)-2-(Benzothiazole-2-dithio)-1-[(1*R*)-1-isoprop-1-enylacetic acid *p*-nitrobenzyl ester]-3-[(5*R*)-5-*p*-nitrobenzyloxycarbonyl-5-*p*-nitrobenzyloxycarbonylamino-pentanamido]azetidin-4-one (9)

A solution of compound 8 (8.80 g, 8.92 mmol) in toluene (50 ml) was treated with 2-mercaptobenzothiazole (1.50 g, 8.9 mmol) and refluxed for 4 h. The solution was evaporated to dryness, after which chromatography [flash silica, with ethyl acetate/light petroleum (b.p. 40–60 °C) (0:1 to 3:1, v/v)] gave compound 9 as a foam (6.37 g, 62% yield). T.l.c. [ethyl acetate/light petroleum (b.p. 40–60 °C) (3:2, v/v)] *R_F* 0.3. ¹H n.m.r. δ (p.p.m.) (300 MHz, [²H]chloroform) 1.72–1.85 (4H, m, CH₂CH₂CH₂CO), 1.93 (3H, s, CH₃), 2.29–2.41 (2H, m, CH₂CH₂CO), 4.38–4.45 (1H, m, NCHCH₂), 5.01 and 5.07 (2H, 2 × s, C=CH₂), 5.17 (2H, approx. s, CH₂Ar), 5.19 (2H, approx. s, CH₂Ar), 5.21 (2H, approx. s, CH₂Ar), 5.22 (1H, s, NCHCC=CH₂), 5.37 (1H, dd, *J* = 8.0 and 5.0 Hz, 3-*H*), 5.51 (1H, d, *J* = 5.0 Hz, 2-*H*), 5.82 (1H, d, *J* = 8.0 Hz, NH), 7.08 (1H, d, *J* = 8.0 Hz, NH), 7.29–7.50 (8H, m, 8 × Ar-*H*), 7.73–7.85 (2H, m, 2 × Ar-*H*) and 8.10–8.22 (6H, m, 6 × Ar-*H*).

(2*S*, 3*R*, 5*R*, 6*R*)-1-Aza-3-chloroacetoxymethyl-3-methyl-6-[(5*R*)-5-*p*-nitrobenzyloxycarbonyl-5-*p*-nitrobenzyloxycarbonylamino-pentanamido]-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylic acid *p*-nitrobenzyl ester (10) and (2*R*,3*S*,6*R*,7*R*)-1-aza-3-chloroacetoxymethyl-3-methyl-7-[(5*R*)-5-*p*-nitrobenzyloxycarbonyl-5-*p*-nitrobenzyloxycarbonylamino-pentanamido]-8-oxo-5-thiabicyclo[4,2,0]octane-2-carboxylic acid *p*-nitrobenzyl ester (11)

A solution of compound 9 (4.00 g, 4.11 mmol) in dichloromethane (200 ml) was treated with silver chloroacetate (1.37 g, 8.22 mmol) and chloroacetic acid (17.47 g, 184 mmol) and stirred for 3 h at 20 °C. The reaction mixture was filtered (Celite), washing with dichloromethane (100 ml). The filtrate was washed with satd. NaHCO₃ (50 ml), water (50 ml) and satd. NaCl (50 ml), dried (over MgSO₄) and evaporated to

dryness. Chromatography [preparative t.l.c., with ethyl acetate/light petroleum (b.p. 40–60 °C) (3:2, v/v), three times] gave compounds **10** and **11**.

For compound **10** (654 mg, 18% yield): t.l.c. [ethyl acetate/light petroleum (b.p. 40–60 °C) (3:2, v/v)] R_F 0.30. Analysis: Found: C, 50.18; H, 4.47; N, 9.25; S, 3.01; calc. for $C_{38}H_{37}ClN_6O_{16}S$: C, 50.66; H, 4.11; N, 9.33; S, 3.55%. 1H n.m.r. δ (p.p.m.) (300 MHz, $[^2H]$ chloroform) 1.46 (3H, s, CH_3), 1.64–1.96 (4H, m, $CH_2CH_2CH_2CO$), 2.33–2.42 (2H, m, CH_2CH_2CO), 3.88, 4.58 (2H, ABq, $J = 11.5$ Hz, CH_2OCOCH_2Cl), 4.16 (2H, s, CH_2Cl), 4.46–4.49 (1H, m, $CHCH_2CH_2$), 4.68 (1H, s, 2- H), 5.21–5.39 (6H, m, $4 \times CH_2Ar$), 5.61 (1H, d, $J = 4$ Hz, 5- H), 5.63 (1H, obscured d, NH), 5.73 (1H, dd, $J = 9.5$ and 4 Hz, 6- H), 6.83 (1H, d, $J = 9.5$ Hz, NH), 7.49–7.59 (6H, m, $6 \times Ar-H$) and 8.63–8.73 (6H, m, $6 \times Ar-H$). $[\alpha]_D^{20} + 64.5^\circ$ ($c = 1$ in chloroform). I.r. (chloroform) 1790 (s) (C=O, β -lactam), 1750 (s) (C=O, ester), 1690 (s) (C=O, amide), 1610 (m) and 1360 (s) (C–NO₂) cm^{-1} .

For compound **11** (810 mg, 22% yield): t.l.c. [ethyl acetate/light petroleum (b.p. 40–60 °C) (3:2, v/v)] R_F 0.40. 1H n.m.r. δ (p.p.m.) (300 MHz, $[^2H]$ chloroform) 1.55 (3H, s, CH_3), 1.71–1.91 (4H, m, $CH_2CH_2CH_2CO$), 2.23–2.35 (2H, m, CH_2CH_2CO), 3.30, 4.04 (2H, ABq, $J = 15.5$ Hz, 4- H), 4.06, 4.16 (2H, ABq, $J = 11.5$ Hz, CH_2Cl), 4.87 (1H, s, 4- H), 4.89 (1H, d, $J = 4.5$ Hz, 6- H), 5.20 (2H, approx. s, CH_2Ar), 5.27 (2H, s, CH_2Ar), 5.30 (2H, s, CH_2Ar), 5.60 (1H, dd, $J = 9.5$ and 4.5 Hz, 7- H), 5.68 (1H, d, $J = 8$ Hz, NH), 6.50 (1H, d, $J = 9.5$ Hz, NH), 7.45–7.53 (6H, m, $6 \times Ar-H$) and 8.14–8.43 (6H, m, $6 \times Ar-H$).

(2S,3R,5R,6R)-1-Aza-3-hydroxymethyl-3-methyl-6-[(5R)-5-*p*-nitrobenzyloxycarbonyl-5-*p*-nitrobenzyloxycarbonylaminopentanamido]-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylic acid *p*-nitrobenzyl ester (12**)**

A solution of compound **10** (273 mg, 0.30 mmol) in acetone (10 ml) was treated with KI (151 mg, 0.91 mmol) and stirred for 2 h at 20 °C. *NN'*-Di-*n*-butylthiourea (284 mg, 1.52 mmol) was added and the reaction mixture was stirred for a further 20 h, after which it was evaporated to dryness (Kamiya *et al.*, 1973). Chromatography (Sephadex LH-20, with ethanol) gave compound **12**, as a white foam (72 mg, 29% yield). Analysis: Found: C, 50.19; H, 4.37; N, 9.58; calc. for $C_{36}H_{36}N_6O_{15}S_2H_2O$: C, 50.23; H, 4.18; N, 9.76%. M.s. (chemical ionization) 825 ($M^+ + H$). 1H n.m.r. δ (p.p.m.) (300 MHz, $[^2H]$ chloroform) 1.38 (3H, s, CH_3), 1.51–1.94 (4H, m, $CH_2CH_2CH_2CO$), 2.25–2.64 (2H, m, CH_2CH_2CO), 3.54, 3.74 (2H, ABq, $J = 11.5$ Hz, CH_2OH), 4.39–4.51 (1H, m, $CHCH_2CH_2$), 4.73 (1H, s, 2- H), 5.22–5.33 (6H, m, $3 \times CH_2Ar$), 5.60–5.74 (2H, m, 5- H + 6- H), 6.75 (1H, d, $J = 9$ Hz, NH), 7.50–7.63 (7H, m, $6 \times Ar-H$ + NH) and 8.17–8.33 (6H, m, $6 \times Ar-H$). $[\alpha]_D^{20} + 64.7^\circ$ ($c = 1$, in chloroform). I.r. (chloroform) 1790 (s) (C=O, β -lactam), 1745 (s) (C=O, ester), 1680 (s) (C=O, amide), 1615 (m) and 1365 (s) (C–NO₂).

(2S,3R,5R,6R)-1-Aza-6-[(5R)-5-carboxypentanamido-3-hydroxymethyl-3-methyl-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylate (5**)**

To a solution of compound **12** (9 mg, 0.01 mmol) in tetrahydrofuran (2 ml) was added a solution of

NaHCO₃ (1 mg, 0.01 mmol) in water (2 ml). The resultant solution was hydrogenated at 0.1 MPa (1 atm gauge) over 10% Pd on charcoal (25 mg) for 12 min. The solution was then filtered (Celite), washing with water (5 ml), and the filtrate was washed with ethyl acetate (2 $\times$ 10 ml). The aqueous layer was then evaporated to dryness to give crude compound **5**. Attempted purification of compound **5** (reverse-phase h.p.l.c., packing material μ Bondapak C₁₈, eluent 10 mM-NH₄HCO₃) resulted in decomposition of compound **5** and the isolation of compound **13**, which isomerized on standing in aqueous solution.

For compound **5**: partial 1H n.m.r. δ (p.p.m.) (500 MHz, 2H_2O) 5.31 and 5.48 (2H, 2 $\times$ d, $J = 4.0$ Hz, 5- H + 6- H). Compound **5** showed antibacterial activity against *S. aureus* N.C.T.C. 6571.

For compound **13**: 1H n.m.r. δ (p.p.m.) (500 MHz, 2H_2O) 1.11 (3H, s, CH_3), 1.51–1.86 (4H, m, $CH_2CH_2CH_2CO$), 2.19–2.25 (2H, m, CH_2CH_2CO), 3.57, 3.72 (2H, ABq, $J = 11.0$ Hz, CH_2OH), 4.09 (1H, d, $J = 8.5$ Hz, CHS) and 4.80 (1H, d, $J = 8.5$ Hz, $NHCHS$). M.s. (fast atom bombardment) 394 ($M^+ + H$) and 416 ($M^+ + Na$).

(2S,4S,6R,7R)-1-Aza-4-methyl-7-[(5R)-5-*p*-nitrobenzyloxycarbonyl-5-*p*-nitrobenzyloxycarbonylaminopentanamido]-8-oxo-5-thiatricyclo-[4,2,0,0^{2,4}]octane-2-carboxylic acid *p*-nitrobenzyl ester (17**)**

To a solution of compound **15** (300 mg, 0.64 mmol) in dichloromethane (5 ml) and *NN*-dimethylaniline (0.30 ml) at 0 °C was added PCl₅ (180 mg, 0.87 mmol), and the mixture was allowed to warm to 20 °C over 1 h. The mixture was then cooled to 0 °C and treated with ethanol (2.0 ml), allowed to warm to 20 °C over 1 h, treated with water (40 ml) and stirred for 15 min. The solution was neutralized to pH 7 (1 M-NaOH) and extracted with ethyl acetate (3 $\times$ 100 ml). The organic extracts were combined, dried (over Na₂SO₄), filtered and evaporated to dryness. Chromatography [flash silica, with dichloromethane/ethyl acetate (1:0 to 0:1, v/v)] gave partially purified compound **16** (205 mg, 95% yield). This material was dissolved in diethyl ether (5 ml) and ethyl acetate (5 ml) and treated with anhydrous toluene-*p*-sulphonic acid (114 mg, 0.66 mmol) in diethyl ether/ethyl acetate (1:1, v/v) (10 ml). The resultant precipitate was filtered off, washed with diethyl ether (3 $\times$ 20 ml) and partitioned between ethyl acetate (150 ml) and 0.6 M-NaHCO₃ (50 ml). The organic layer was separated, re-extracted with satd. NaCl (2 $\times$ 50 ml), dried (over Na₂SO₄), filtered and evaporated to dryness, to yield compound **16** as an oil (60 mg, 26% yield), 1H n.m.r. δ (p.p.m.) (300 MHz, $[^2H]$ chloroform) 1.42 (3H, s, CH_3), 1.80–2.00 (2H, m, 3- H), 4.15 (1H, d, $J = 5.0$ Hz, 7- H), 5.05 (2H, approx. s, CH_2Ar), 5.95 (1H, d, $J = 5.0$ Hz, 6- H), 7.20–7.35 (2H, m, 2 $\times$ $Ar-H$) and 7.95–8.10 (2H, m, 2 $\times$ $Ar-H$). The oil was dissolved in dichloromethane (10 ml), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (51 mg, 0.21 mmol) and compound **6** (97 mg, 0.20 mmol) were added and the solution was stirred under argon for 36 h. The solution was evaporated to dryness, redissolved in ethyl acetate (100 ml), washed with 2 M-HCl (50 ml), 0.6 M-NaHCO₃ (50 ml) and satd. NaCl (50 ml), dried (over Na₂SO₄), filtered and evaporated to dryness. Chromatography [flash silica, with dichloromethane/ethyl acetate (1:0 to 1:4, v/v)] gave compound **17** as a foam (120 mg, 23% yield), m.p. 76–77 °C. T.l.c. [ethyl

Fig. 1. Growth profile of *C. acremonium* and time course for enzyme and antibiotic production

C. acremonium C.O. 728 was grown in standard medium as described in the text. $\square$, A_{550} ; $\triangle$, isopenicillin N synthetase specific activity; $\circ$, expandase specific activity; $\bullet$, cephalosporin C; $\blacktriangle$, penicillin N. For details see the text.

acetate/dichloromethane, 1:1, v/v) R_F 0.45. Analysis: Found: C, 53.38; H, 4.31; N, 10.28; S, 4.17; calc. for $C_{36}H_{34}N_6O_{14}S$: C, 53.60; H, 4.25; N, 10.42; S, 3.97%. M.s. (field desorption) 806 (M^+). 1H n.m.r. δ (p.p.m.) (300 MHz, $[^2H]$ chloroform) 1.64 (3H, s, CH_3), 1.65–2.00 (4H, m, $CH_2CH_2CH_2CO$), 2.10–2.24 (2H, ABq, $J = 12$ Hz, 3- H), 2.26 (2H, approx. t, $J = 7.0$ Hz, CH_2CONH), 4.40–4.42 (1H, m, $CHCH_2CH_2CH_2CO$), 5.21, 5.23, 5.31 (6H, 3 $\times$ approx. s, 3 $\times$ CH_2Ar), 5.49 (1H, dd, $J = 8.0$ and 4.0 Hz, 7-H), 6.19 (1H, d, $J = 8.0$ Hz, NH), 6.19 (1H, d, $J = 4.0$ Hz, 6- H), 6.44 (1H, d, $J = 8.0$ Hz, NH), 7.49–7.55 and 8.17–8.26 (12H, 2 $\times$ m, 12 $\times$ Ar- H). ^{13}C n.m.r. δ (p.p.m.) (62.5 MHz, 2H_2O) 20.0 (q, CH_3), 20.8 (t, $CH_2CH_2CH_2CO$), 31.5 (t, $CH_2CH_2CH_2CO$), 34.8 (t, CH_2CO), 41.5 (t, 3- C), 52.0, 55.3 (2 $\times$ s, 2- C +4- C), 53.7, 57.7 (2 $\times$ d, 7- C + $CHCH_2CH_2CH_2$), 65.5, 65.7, 66.2 (3 $\times$ t, CH_2Ar), 78.4 (d, 6- C), 123.8, 123.9, 124.0 (3 $\times$ d, m -Ar- C), 128.1, 128.5, 128.6 (3 $\times$ d, o -Ar- C), 142.2, 142.3, 143.6 (3 $\times$ s, $ipso$ -Ar- C), 147.7, 147.9, 147.9 (3 $\times$ s, p -Ar- C), 155.6, 167.3, 171.6, 171.7 and 173.5 (5 $\times$ s, CO). U.v. λ_{max} . (acetonitrile) 258 nm ($\epsilon = 34300$ M $^{-1}$ cm $^{-1}$). $[\alpha]_D^{25} +114^\circ$ ($c = 1$ in chloroform). I.r. (chloroform) 3690 (m), 1795 (m) (C=O, β -lactam), 1730 (s) (C=O, ester), 1685 (s) (C=O, amide), 1610 (m), 1420 (s), 1360 (s) (C-NO $_2$), 1060 (m), 1015 (m) and 930 (s) cm $^{-1}$.

(2*S*,4*S*,6*R*,7*R*)-1-Aza-7-[(5*R*)-5-carboxypentanamido]-4-methyl-8-oxo-5-thiatricyclo-[4,2,0,0 2,4]octane-2-carboxylate (14)

To a solution of compound 17 (65 mg, 0.08 mmol) in tetrahydrofuran (10 ml) was added 8.20 ml of a solution of NaHCO $_3$ in water (8.3 mg in 10 ml). The resultant solution was hydrogenated at 0.1 MPa (1 atm gauge) over 10% Pd on charcoal (50 mg) for 1 h. The solution was then filtered (Celite), washing with water (10 ml), and the filtrate was washed with ethyl acetate (2 $\times$ 25 ml). The aqueous layer was then freeze-dried to give compound 14 (27 mg, 88% yield). 1H n.m.r. δ (p.p.m.) (300 MHz, 2H_2O) 1.42 (3H, s, CH_3), 1.42–1.71 (4H, m, $CH_2CH_2CH_2CO$), 1.71, 1.91 (2H, ABq, $J = 12$ Hz, 4- H), 2.19 (2H, approx. t, $J = 7.0$ Hz, CH_2CH_2CO), 3.50–3.54 (1H, m, $CHCH_2CH_2$), 5.10 (1H, d, $J = 4.0$ Hz, 7- H), 6.04 (1H, d, $J = 4.0$ Hz,

6- H). ^{13}C n.m.r. δ (p.p.m.) (62.5 MHz, 2H_2O) 20.3 (q, CH_3), 21.8 (t, $CH_2CH_2CH_2CO$), 30.7 (t, $CH_2CH_2CH_2CO$), 35.4 (t, CH_2CON), 40.4 (t, 3- C), 53.4, 55.1 (2 $\times$ s, 2- C +4- C), 55.4, 58.3 (2 $\times$ d, $CHCH_2CH_2$ +7- C), 77.9 (d, 6- C), 174.7, 175.4, 175.8 and 176.8 (4 $\times$ s, 4 $\times$ CO). I.r. (Nujol) 3650–2300 (b) (OH), 1770 (s) (C=O, β -lactam) and 1600 (b) cm $^{-1}$.

RESULTS

Growth profile and time course for enzyme and antibiotic production

Fig. 1 shows the growth profile of *C. acremonium* C.O. 728, together with the time course for production of expandase activity, isopenicillin N synthetase activity, penicillin N (1) and cephalosporin C, in standard medium [2.7% (w/v) glucose/3.6% (w/v) sucrose] with asparagine (0.75%) as the nitrogen source.

Raising the concentration of asparagine to 1.2% resulted in lower specific activity of the crude enzyme preparations.

Enzyme purification

Initial stages. All steps were carried out at 4 $^\circ$ C. Protamine sulphate was added to the cell-free extract to a final concentration of 0.5%. After centrifugation at 12000 g for 30 min, (NH $_4$) $_2$ SO $_4$ was added to the supernatant to a final concentration of 55% saturation. After equilibration and centrifugation (12000 g for 30 min), the supernatant was made up to 75% saturation with (NH $_4$) $_2$ SO $_4$, and the precipitate was collected by centrifugation at 12 000 g for 30 min. This material was redissolved in the extraction buffer (Pang *et al.*, 1984), and loaded on to a Sephadex G-75 gel-filtration column (130 cm $\times$ 5 cm) equilibrated with extraction buffer. After elution overnight at 4 $^\circ$ C, fractions were tested for expandase activity by bioassay. Active fractions were pooled and applied (flow rate 24 ml/h) to a column of DEAE-Sepharose (fast flow) (10 cm $\times$ 5 cm), pre-equilibrated with extraction buffer, at 4 $^\circ$ C. Expandase was eluted with a linear gradient of 0.05–0.3 M-NaCl in buffer, over 300 ml, at a flow rate of 24 ml/h. Active fractions, tested by bioassay, were pooled. The product (crude

Fig. 2. Purification of expandase from *C. acremonium*

(a) Dye-ligand chromatography on Matrex Gel Red A. $\circ$, A_{280} ; $\bullet$, expandase activity; $\blacktriangle$, conductivity. (b) Hydrophobic chromatography on phenyl-Sepharose (fast flow). $\circ$, A_{280} ; $\bullet$, expandase activity; $\blacktriangle$, conductivity. For details see the text.

enzyme) was used for study of some of the properties of the enzyme described below.

Dye-ligand chromatography, ion-exchange f.p.l.c., gel-filtration f.p.l.c. and hydrophobic chromatography. For purification to near homogeneity, as judged by SDS/polyacrylamide-gel electrophoresis, pooled active fractions after Sephadex G-75 chromatography were applied (flow rate 10 ml/h) to a column (30 cm $\times$ 2.5 cm) of Matrex Gel Red A (Procion Red HE3B-agarose). In preliminary experiments Procion

Red (0.1 mM) was found to inhibit reversibly expandase activity by virtually 100%. The column was washed with 350 ml of extraction buffer containing DTT (2 mM) at a flow rate of 24 ml/h, and enzyme was eluted with a linear gradient of 0–2.0 M-KCl in buffer over 500 ml, at the same flow rate, as shown in Fig. 2(a). Active fractions were free from isopenicillin N synthetase activity, since isopenicillin N synthetase did not bind to Matrex Gel Red A. Active fractions were pooled, exchanged into 20 mM-Tris/HCl buffer, pH 8.0, containing DTT (2 mM), and applied (flow rate

Table 1. Purification of deacetoxycephalosporin C synthetase (expandase) from *C. acremonium*

For details see the text. Values of percentage yield are calculated relative to step 1.

Step	Volume (ml)	Concn. of protein (mg/ml)	$10^3 \times$ Expandase activity (units/ml)	Total expandase activity (units)	$10^3 \times$ Specific activity (units/mg)	Yield (%)	Purification (fold)
1. Sephadex G-75 chromatography	109	5.90	8.26	0.9	1.4	100	—
2. Dye-ligand chromatography on Matrex Gel Red A	39	0.90	4.32	0.17	4.8	19	3.4
3. Ion-exchange chromatography on Mono Q f.p.l.c.	1.15	0.14	2.59	0.03	18.5	3.3	13.2
3a. Hydrophobic chromatography on phenyl-Sepharose (fast flow) after Matrex Gel Red A chromatography	37.0	0.05	0.81	0.03	16.1	3.3	11.5
4. Gel filtration on Superose 12 f.p.l.c.	1.03	0.10	3.85	0.004	38.5	0.4	27.5

Fig. 3. Effect of pH on partially purified expandase

Buffers were as follows: ○, Tris/HCl; □, sodium phosphate; ▲, diethanolamine; ■, Bistris/HCl. For details see the text.

8 ml/min) to a Mono Q 16/10 strong anion-exchange column for f.p.l.c. Enzyme was eluted with a linear gradient of 80–180 mM-NaCl in 20 mM-Tris/HCl buffer, pH 8.0, containing DTT (2 mM), at the same flow rate. Active fractions were pooled, desalted, concentrated and applied (flow rate 0.25 ml/min) to a Superose 12 f.p.l.c. gel-filtration column. Enzyme was eluted with 0.1 M-Tris/HCl buffer, pH 8.0, containing DTT (2 mM). Active fractions were pooled and concentrated. In alternative experiments, active fractions after Procion Red chromatography were pooled, concentrated, made 1.2 M with respect to $(\text{NH}_4)_2\text{SO}_4$, in 50 mM-Tris/HCl buffer, pH 7.4, containing DTT (2 mM) and applied to a column (28 cm $\times$ 1.5 cm) of phenyl-Sepharose (fast flow). Enzyme was eluted (flow rate 24 ml/h) with a linear gradient of 1.2–0 M- $(\text{NH}_4)_2\text{SO}_4$ in pH 7.4 buffer (Fig. 2b). Active fractions were pooled and concentrated. Details of a typical enzyme purification are shown in Table 1. At each stage fractions with less than 30% of maximum activity were discarded. A similar final specific activity of 0.04 unit/mg was obtained by chromatography on DEAE-Sepharose followed by DEAE-TSK 545 h.p.l.c.

Enzymic properties

The effect of pH on partially purified expandase activity in 0.1 M buffers, judged by bioassay, is shown in Fig. 3. Sodium phosphate and Bistris buffers (0.1 M) inhibited activity. Similar results were obtained in 50 mM buffers. Ascorbate was essential for activity, and in the presence of ascorbate DTT caused a 30% increase in activity up to 1 mM-DDT. D-Isoascorbate had the same effect as its isomer. However, the effect of DTT was approx. 25% greater with 'reduced' enzyme, where enzyme had been exposed to DTT (1 mM) throughout the purification procedure, than with 'oxidized' enzyme, where 'reduced' enzyme had been exchanged into buffer without DTT and incubated at 27 °C for 90 min in an orbital shaker before bioassay.

Partially purified enzyme was stable for over 4 weeks at -70 °C provided that DTT (1 mM) was present. At 4 °C the half-life of expandase activity was found to be 48 h in the presence of DTT (1 mM). Addition of ascorbic acid (1 mM), FeSO_4 (0.04 mM), 2-oxoglutarate (0.8 mM), $(\text{NH}_4)_2\text{SO}_4$ (100 mM) or bovine liver catalase (40000 units/ml) to the stored enzyme in the absence of DTT had little effect on stability.

2-Oxoglutarate was essential for activity (K_m 0.04 mM by bioassay), and could not be replaced by 2-oxoadipate or pyruvate. The addition of ATP was found not to enhance activity, in agreement with Scheidegger *et al.* (1984).

Addition of NH_4HCO_3 (100–500 mM) to the bioassay mixture completely inhibited expandase activity, whereas NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$ caused 2.8-fold enhancement of activity at 500 mM. NaCl, Na_2SO_4 and KCl had little effect on activity.

Expandase activity was inhibited by the thiol-quenching reagent *N*-ethylmaleimide (0.1 mM). The metal-ion-chelating reagent bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) inhibited activity by 50% at 0.02 mM, whereas the non-chelating 4',7'-isomer had no effect.

Chemical syntheses

Oxidation of tri-protected penicillin N (7) (Baldwin *et al.*, 1980) (*m*-chloroperbenzoic acid, 0 °C, dichloromethane) gave exclusively the β -sulphoxide (8) (94%), which was deprotected (H_2 , Pd/charcoal) to give compound 4 (Scheme 2).

The synthesis of compound 5 was achieved by utilizing mild interconversions established in model

Scheme 2.

studies (Baldwin & Singh, 1979). Thus treatment of the disulphide (**9**), prepared by the procedure of Kamiya *et al.* (1973) from compound **7**, with silver chloroacetate gave a mixture of the penam (**10**) (18%) and the cepham (**11**) (22%) (Scheme 3). Reaction of compound **10** with KI followed by *NN'*-di-*n*-butylthiourea gave compound **12**. Deprotection (H_2 , Pd/charcoal) gave the desired 3 β -hydroxypenam (**5**) (positive bioassay against *S. aureus* N.C.T.C. 6571) (Scheme 4). However, compound **5** was found [by 1H n.m.r. (500 MHz)] to be unstable in aqueous solution and rapidly decomposed to give the penicilloate (**13**), which equilibrated (at C-2) upon standing in 2H_2O .

The tricyclic penam (**14**) was also synthesized for evaluation as a substrate for the expandase reaction. Thus removal of the phenylacetyl side chain of compound **15** (*NN'*-dimethylaniline/ PCl_5) gave the amine (**16**), which was coupled with diprotected D- α -aminoadipic acid (**6**) to give compound **17**. Deprotection (H_2 , Pd/charcoal) gave the desired tricyclic penam (**14**) (Scheme 5).

Substrate specificity of expandase

Incubation of compounds **4** and **5** (in the case of the latter, immediately after deprotection of compound **12**) with the crude enzyme (under conditions that

Fig. 4. Ion-exchange chromatography on DEAE-Sephadex (fast flow) of material after Sephadex G-75 gel filtration (see Table 1)

○, A_{280} ; ●, expandase activity, measured by bioassay; ▲, conductivity. For details see the text. X and Y are peaks analysed for expandase and hydroxylase activities by h.p.l.c. (see Fig. 5).

permitted conversion of compound **1** into compounds **2** and **3** at a 1 mg testing level) did not give rise to cephem products, judged by ^1H n.m.r. (500 MHz) or bioassay as described above.

The tricyclic penam **14** was not found to be a substrate for expandase activity. However, in a competitive experiment with an equimolar amount of compound **1** (under standard incubation conditions), it was found to be a potent reversible inhibitor of the transformation of compound **1** into compound **2**, causing over 90% inhibition at a concentration of 40 μM .

Deacetoxycephalosporin C hydroxylase activity

Throughout the purification procedure fractions were analysed for both expandase and hydroxylase activities by using h.p.l.c. or t.l.c. methods. Figs. 4 and 5 show analysis of both activities after DEAE-Sephadex (fast flow) ion-exchange chromatography. Two peaks of expandase activity were separated on DEAE-Sephadex (Fig. 4). Both peaks contained hydroxylase activity, as judged by h.p.l.c. (Fig. 5) and t.l.c. assays. Fig. 6 shows a typical t.l.c. bioassay analysis of hydroxylase activity in fractions eluted from phenyl-Sephadex (fast flow) chromatography, where elution of hydroxylase activity corresponded exactly to elution of expandase activity, as judged by the standard bioassay.

In further experiments, enzyme after the Sephadex

Fig. 5. H.p.l.c. analysis of fractions from peaks X and Y after DEAE-Sephadex (fast flow) chromatography (see Fig. 4)

(i) Standards [A, deacetylcephalosporin C (DAC); B, penicilloate; C, deacetoxycephalosporin C (DAOC); D, penicillin N]; (ii) blanks without substrates at $t = 0$; (iii) penicillin N as substrate at $t = 0$; (iv) penicillin N as substrate at $t = 60$ min; (v) DAOC as substrate at $t = 0$; (vi) DAOC as substrate at $t = 60$ min. For details see the text.

Fig. 6. T.l.c. analysis of hydroxylase activity in fractions after phenyl-Sepharose (fast flow) chromatography

Fractions eluted from hydrophobic chromatography on phenyl-Sepharose (fast flow) (Fig. 2b) were analysed directly for hydroxylase activity by t.l.c. assay. L, Sample loaded onto column (after chromatography on Matrex Gel Red A); 36–46, fraction numbers (fraction volume 10 ml); S, DAC and DAOC standards. For details see the text.

Fig. 7. SDS/polyacrylamide-gel electrophoresis of expandase/hydroxylase after Superose 12 f.p.l.c.

M, Molecular-mass markers; pre Superose, pooled concentrated enzyme after Mono Q f.p.l.c.; 12–15, fraction numbers (fraction volume 1 ml) eluted from Superose 12 f.p.l.c. For details see the text.

G-75 step was chromatographed on a hydroxyapatite (100 mm × 8 mm) h.p.l.c. column (4.8 ml bed volume), and eluted with a gradient of 10–350 mM-sodium phosphate buffer, pH 6.9, at a flow rate of 0.5 ml/min (Waters gradient programme 3). A single major protein peak was obtained, corresponding to co-elution of expandase and hydroxylase activities, judged by h.p.l.c. and t.l.c. assays. This pattern of co-elution of activities judged by bioassay and t.l.c. assays was maintained throughout all chromatographic steps, up to that of stage 4 in Table 1 (Fig. 7). The amino acid composition of this enzyme is shown in Table 2. The molecular mass of the expandase/hydroxylase protein was estimated to be 40000 Da by SDS/

Table 2. Amino acid composition of expandase/hydroxylase

For details see the text.

Amino acid	Composition (residues/40000 Da)
Asx (Asp + Asn)	37
Thr	21
Ser	22
Glx (Glu + Gln)	37
Pro	20
Gly	31
Ala	31
Val	28
Met	7
Ile	12
Leu	30
Tyr	13
Phe	18
His	6
Lys	21
Arg	25
Total	359

polyacrylamide-gel electrophoresis. Gel filtration on Superose 12 f.p.l.c., by comparison with markers of known molecular mass, showed no evidence for enzyme polymerization in the native state.

DISCUSSION

The lack of utilization of compounds 4 and 5 as substrates for expandase activity is evidence against their formation as free intermediates in the reaction mechanism. This does not, however, rule out the possibility of the formation of one of them as an enzyme-bound intermediate. Although the tricyclic penam (14) was not a substrate for the expandase, it may bind to the substrate-binding site, causing the potent inhibition of activity that was observed.

Even allowing for discarding of activity before pooling fractions, the yield at each stage of enzyme purification was low. This may reflect instability of the protein in solution at 4 °C, although at –70 °C it was stable for over 4 weeks.

The protein appears to have at least one catalytically important thiol group. DTT and ascorbate may be required to ensure that the iron is in a reduced form and/or to maintain protein thiol groups in their reduced state. Expandase activity was maximal at pH 7.4–8.0 in Tris/HCl buffer. Phosphate buffer was inhibitory, and loss of activity in Bistris buffer may be due to a metal chelation effect of the buffer. Expandase activity required 2-oxoglutarate, Fe²⁺ ions and O₂ for activity, with stimulation by ascorbate and DTT. These are similar requirements to those exhibited by the oxygenases thymidine 2-hydroxylase (Bankel *et al.*, 1972) and collagen hydroxylases (Majamaa *et al.*, 1985), in which the 2-oxoglutarate is stoichiometrically decarboxylated to succinate. In the fungus *Neurospora crassa* thymine 7-hydroxylation is accomplished by a 2-oxoglutarate-requiring system that involves three discrete steps, all of which appear to be associated with a single enzyme (Liu *et al.*, 1973). By using a coupled

assay system with succinate thiokinase, pyruvate kinase and lactate dehydrogenase, we have shown that both expandase and hydroxylase activities produce succinate from 2-oxoglutarate stoichiometrically with the production of cephem (Baldwin & Crabbe, 1987). This accords with the findings that 2-oxo[1-¹⁴C]glutarate was oxidatively decarboxylated to radiolabelled CO₂ by a partially purified preparation of expandase (Kupka *et al.*, 1983).

The purification to near SDS/polyacrylamide-gel-electrophoretic homogeneity of a 40 000 Da protein that exhibits both deacetoxycephalosporin C synthetase ('expandase') and hydroxylase activities suggests that a single protein, deacetoxycephalosporin C synthetase/hydroxylase can catalyse both reactions. This would be in agreement with the proposal by Scheidegger *et al.* (1984), who failed to separate expandase and hydroxylase activities from *Cephalosporium acremonium* by chromatography on DEAE-Trisacryl M and by isoelectric focusing, but in contrast with the separation of the two activities in *Streptomyces clavuligerus* (Jensen *et al.*, 1985). However, we have not ruled out the possibility that two very similar proteins that co-purify under normal conditions may catalyse each reaction independently. This question may only be answered by cloning of the 40 000 Da protein into *E. coli* and determining whether both activities are expressed.

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