OXIDATION OF DEACETYLCEPHALOSPORIN C BY DEACETOXYCEPHALOSPORIN C/DEACETYLCEPHALOSPORIN C SYNTHASE

JACK E. BALDWIN, KEE-CHUAN GOH and CHRISTOPHER J. SCHOFIELD

The Dyson Perrins Laboratory and the Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, United Kingdom

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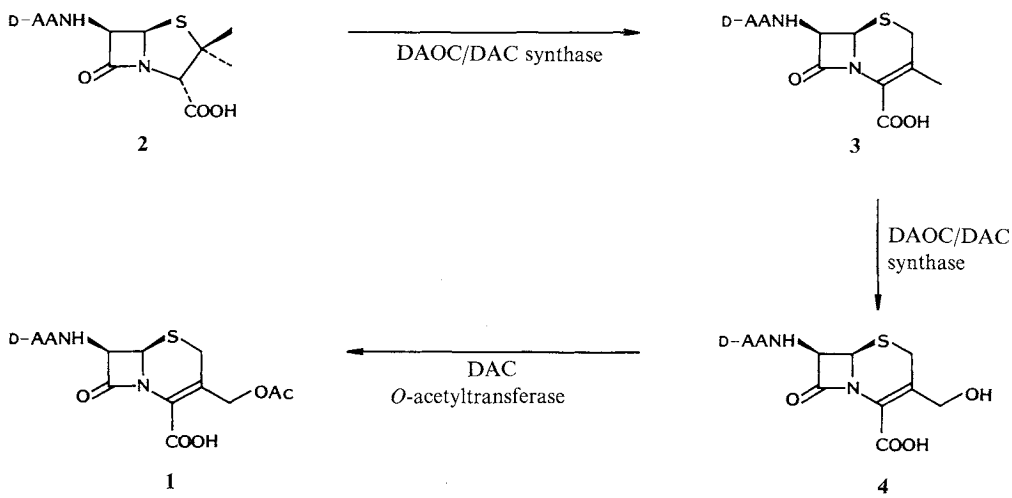
The final three steps in the biosynthesis of cephalosporin C (**1**) in *Cephalosporium acremonium* consist sequentially of the oxidative ring expansion of penicillin N (**2**) to deacetoxycephalosporin C (DAOC: **3**), the hydroxylation of DAOC to deacetylcephalosporin C (DAC: **4**), followed by the acetylation of DAC (**4**) to cephalosporin C (**1**) (Fig. 1).[1,2] In this fungus, the first two of these steps are apparently catalysed by a single bifunctional enzyme, DAOC/DAC synthase.

FUJISAWA and co-workers have reported the generation of mutants, derived from *C. acremonium* ATCC 14553, in which the terminal acetyl transfer step was blocked. Some of these mutants were shown to result in the accumulation of DAC (**4**) in the culture broth.[3] Subsequently,[4] a new metabolite, the cephalosporoate aldehyde (**5**), was isolated from one of the mutants defective in the acetyl transfer step. It was proposed that the new metabolite arised from intracellular oxidation of DAC (**4**) to the bicyclic aldehyde (**6**) (Fig. 2), which underwent facile β-lactam ring cleavage to give the cephalosporoate (**5**) as the final excreted product.[4~6] In this paper, we demonstrate that, *in vitro*, DAOC/DAC synthase is capable of catalysing the oxidation of DAC (**4**) to (**6**), consistent with its operation as a trifunctional enzyme in the mutants lacking the acetyltransferase activity.

Initially, we investigated the efficiency of DAC (**4**) as a substrate for both native[7] and recombinant[8] DAOC/DAC synthase, using standard incubation conditions.[7] Thus, a typical reaction mixture (5 ml) contained the following components, in 50 mM Tris-HCl buffer (pH 7.5): 1 mg/ml enzyme, 1 mM DAC, 1 mM ascorbate, 2 mM dithiothreitol, 2 mM α-ketoglutarate, 50 μM ferrous sulfate and 50 mM ammonium sulfate. Reaction mixtures were incubated at 28°C for 2 hours in an orbital shaker operating at 200 rpm, and were terminated by quenching with 2 volumes of acetone. Precipitated protein was removed by centrifugation at 48,000 × g for 10 minutes. Acetone was removed from the supernatant by rotary evaporation, and the remaining aqueous portion filtered through a 0.22 μm filter. Examination of the ^{1}H NMR (500 MHz) of the crude incubation mixture indicated the appearance of a new resonance at *ca.* 9.05 ppm, pointing to the possible production of an aldehyde. Initial purification by HPLC (Hypersil ODS

Fig. 1. Last three steps in the biosynthesis of cephalosporin C in *Cephalosporium acremonium*.



D-AANH: δ-(D-α-aminoadipamido).

Fig. 2. Fate of DAC in cephalosporin C-negative mutants of *C. acremonium*.

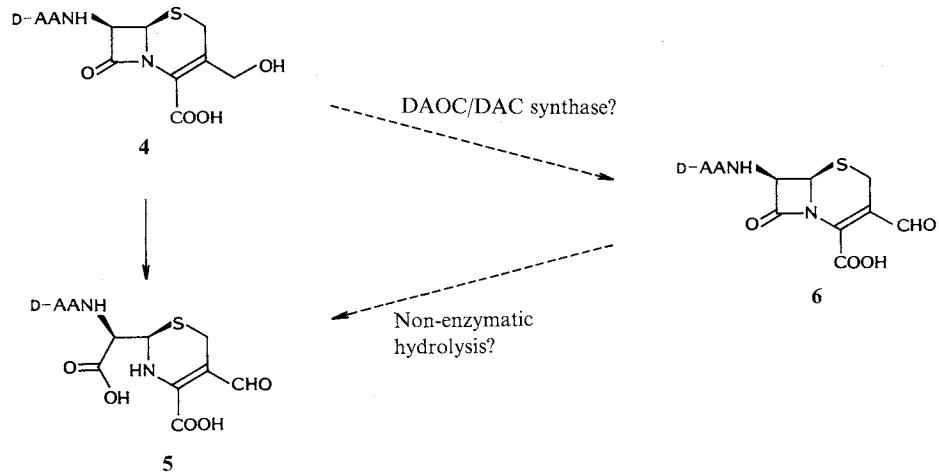
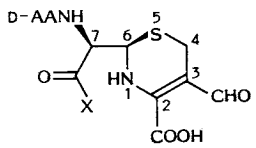


Fig. 3. Proposed structures of aldehyde products arising from action of DAOC/DAC synthase on DAC.



5 X=OH **8** X=NH–C(CH$_2$OH)$_3$

7 X=NH$_2$

column, 250×7 mm) was achieved using 0.1% formic acid as eluant. The eluate was monitored at 260 nm (for unreacted DAC (**4**), retention volume *ca.* 39 ml) and at 300 nm (for the aldehyde product (**7**), retention volume *ca.* 21 ml). Further HPLC purification on the same column, using 25 mM ammonium bicarbonate as eluant, gave an apparently pure (by ^{1}H NMR (500 MHz)) product (retention volume, *ca.* 8.1 ml). The product gave UV and NMR spectra similar to those reported for the 3-formyl cephalosporoate (**5**)[4]. However, electrospray mass spectrometry gave a value of *m/z* 389 for the protonated molecular ion $(M+H)^+$, as opposed to the anticipated value of 390 (for (**5**)), consistent with ring opening of the β-lactam by ammonia to give the amide (**7**) (Fig. 3); ^{1}H NMR (500 MHz, D_2O) δ 1.51~1.64 and 1.71~1.81 (4H, 2×m, C*H*$_2$C*H*$_2$CH$_2$CO), 2.28 (2H, t, *J*=7.0 Hz, C*H*$_2$CO), 3.33 and 3.50 (2H, ABq, *J*=15.5 Hz, 2×4-H), 3.62 (1H, t, *J*=6.0 Hz, HOOCC*H*(NH$_2$)CH$_2$), 4.67 (1H, d, *J*=7.5 Hz, 6-H), 4.80 (1H, d, *J*=7.5 Hz, 7-H), 9.05 (1H, s, CHO); UV absorption spectrum gave a single maximum at 300 nm.

Further incubations were then carried out in the absence of ammonium sulfate. The product, isolated as before, was found to have a larger retention volume (*ca.* 38 ml using 0.1% formic acid as eluant and *ca.* 17 ml using 25 mM ammonium bicarbonate as eluant). Electrospray mass spectrometric and NMR data, however, indicated that opening of the β-lactam ring by Tris ((HOCH$_2$)$_3$-CNH$_2$) had occurred, giving rise to (**8**) (Fig. 3); ^{1}H NMR (500 MHz, D_2O) δ 1.50~1.66 and 1.71~1.81 (4H, 2×m, C*H*$_2$C*H*$_2$CH$_2$CO), 2.28 (2H, t, *J*=7.0 Hz, C*H*$_2$CO), 3.36 and 3.50 (2H, ABq, *J*=15.0 Hz, 2×4-H), 3.62 (6H, s, NHC(C*H*$_2$OH)$_3$), 3.66 (1H, t, *J*=6.0 Hz, HOOCC*H*(NH$_2$)CH$_2$), 4.69 (1H, d, *J*=8.0 Hz, 6-H), 4.77 (1H, d, *J*=8.0 Hz, 7-H), 9.08 (1H, s, CHO); electrospray MS positive mode *m/z* 493 $(M+H)^+$.

Finally, incubations were carried out in 50 mM sodium phosphate buffer (pH 7.5), in the absence of ammonium sulfate, other conditions being identical to the previous incubations. Reverse phase HPLC (Hypersil ODS, 250×10 mm) gave a different aldehyde product (retention volume *ca.* 37 ml (0.1% formic acid) and *ca.* 12 ml (25 mM ammonium bicarbonate)). ^{1}H NMR and mass spectrometric data were consistent with the production of the previously[4] observed aldehyde (**5**) as the product; ^{1}H NMR (500 MHz, D_2O) δ 1.54~1.64 and 1.75~1.82 (4H, 2×m, C*H*$_2$C*H*$_2$CH$_2$CO), 2.28 (2H, t, *J*=7.0 Hz, C*H*$_2$CO), 3.39 and 3.46 (2H, ABq, *J*=16.0 Hz, 2×4-H), 3.66 (1H, t, *J*=6.0 Hz, HOOCC*H*(NH$_2$)CH$_2$), 4.59 (1H, d, *J*=6.0 Hz, 6-H), 4.84 (1H, d, *J*=6.0 Hz, 7-H), 9.04 (1H, s,

CHO); electrospray MS positive mode m/z 390 $(M+H)^+$.

Control experiments either using boiled enzyme or in the absence of α-ketoglutarate did not result in the turnover of DAC (**4**) to any aldehydic products. Incubations under an $^{18}O_2$ atmosphere were conducted in order to determine the possible level of dioxygen incorporation into the aldehydic product (**5**). The mass spectrometric data revealed little (<10%) or no incorporation of the dioxygen label, in contrast to similar experiments for the conversion of DAOC (**3**) to DAC (**4**),[9] when significant incorporation of labelled oxygen from dioxygen into the product was observed. This apparent difference may be due either to the aldehyde undergoing exchange with $H_2{}^{16}O$, such that all initial label, if present, is eventually washed out, or may result from the mechanistic constraints of the reaction. Preliminary kinetic analysis of DAC (**4**) as a substrate for the soluble recombinant enzyme[8] for the conversion of DAC (**4**) to (**5**) in phosphate buffer gave values of $K_m = 1.33$ mM and $V_{max} = 180$ nmol/minute/mg protein. Incubations carried out with the native fungal enzyme also demonstrated turnover, further extending our previous observation of a similar substrate specificity for native and recombinant proteins.[10]

DAOC/DAC synthase is thus capable of further oxidising DAC (**4**) to cephem-3-carbaldehyde (**6**), which is then hydrolysed to give 3-formyl cephalosporoate (**5**). This finding is interesting in several respects. Firstly, to our knowledge, it is the second member in the family of α-ketoglutarate and ferrous dependent oxygenases to exhibit an ability to process a substrate through three successive oxidative stages, the first example being thymine-7-hydroxylase.[11] In addition, it is of interest to compare the K_m value obtained for the oxidation of DAC (**4**) with that reported for the acetylation of DAC (**4**) to cephalosporin C (**1**).[12] For the acetylation of DAC (**4**) as catalysed by DAC *O*-acetyltransferase, the reported K_m value of 40 μM indicates that the acetyltransferase has a higher substrate affinity for DAC (**4**) than has DAOC/DAC synthase. Thus, it would seem reasonable to suppose that, *in vivo*, DAC (**4**) is largely processed through the acetylation pathway to cephalosporin C (**1**) before further oxidation can occur. Since the oxidation of DAC (**4**) to (**6**) results in rapid destruction of the β-lactam ring, it is possible that the acetylation process has evolved to maintain the integrity of the cephalosporin. Lastly, the destruction of the β-lactam ring bears important considerations for the industrial production of cephalosporins. It is evident that DAC (**4**) levels should not be allowed to accumulate during fermentations in order to maximise biosynthetic throughput.

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