SUBSTRATE SPECIFICITY OF CLONED DEACETOXYCEPHALOSPORIN C/ DEACETYLCEPHALOSPORIN C SYNTHETASE

JACK E. BALDWIN, ROBERT M. ADLINGTON, NICHOLAS P. CROUCH, JANICE B. COATES, JOHN W. KEEPING, CHRISTOPHER J. SCHOFIELD, WENDY A. SHUTTLEWORTH and JOHN D. SUTHERLAND

The Dyson Perrins Laboratory, South Parks Road, Oxford, OX1 3QY., UK

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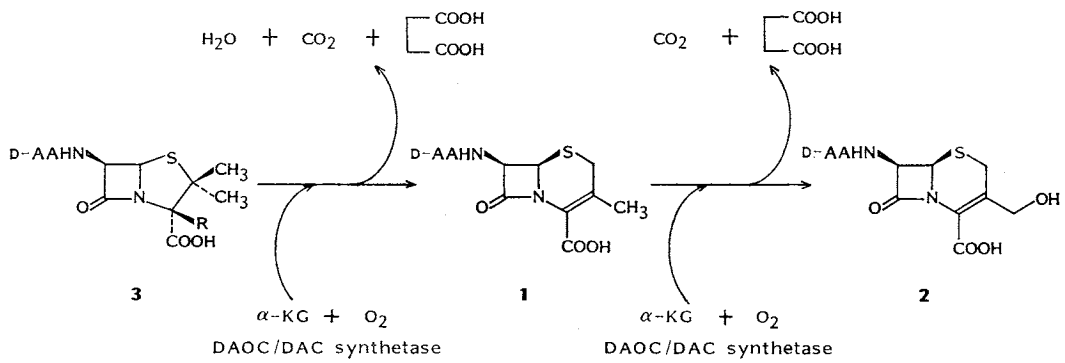
Recently the enzyme responsible for the biosyntheses of deacetoxycephalosporin C (DAOC, **1**) and deacetylcephalosporin C (DAC, **2**) in *Cephalosporium acremonium* has been purified to homogeneity (Scheme 1).[1,2] SAMSON *et al.*[3] provided proof of the bifunctionality of the enzyme (DAOC/DAC synthetase) by its subsequent cloning and expression in *Escherichia coli*; the recombinant enzyme was shown to convert penicillin N (**3**) to DAC (**2**) by HPLC analysis. We now report a comparative study of the substrate specificity of partially purified recombinant enzyme[3] with that of the wild type fungal enzyme.[1,2] In addition we have examined the Δ-2 cepham (**4**) as a novel substrate for the recombinant enzyme.

Initially we incubated penicillin N (**3**, R=H) with the partially purified recombinant enzyme† and obtained a mixture of DAOC (**1**), DAC (**2**) (and as a minor product the hydroxy cepham (**5**, R=H)) as assayed by ^{1}H NMR (500 MHz); in an independent experiment DAOC (**1**) was converted to DAC (**2**).

We have shown that incubation of [3-^{2}H]-penicillin N (**3**, R=D) with DAOC/DAC synthetase from *C. acremonium* gave, in addition to DAOC (**1**) and DAC (**2**), the 3β-hydroxy cepham (**5**) in a ratio **1**+**2**:**5** (R=D) of 65:35.[4] This material arises as a result of a deuterium isotope effect on a branched pathway in the ring expansion step; incubation of fully protiated penicillin N (**3**, R=H) gave a ratio of **1**+**2**:**5** (R=H) of 80:1. Hence, incubation of **3** (R=D) with DAOC/DAC synthetase should provide a subtle kinetic comparison with the fungal enzyme. Treatment of **3** (R=D) with recombinant enzyme and the appropriate cofactors gave a ratio of **1**+**2**:**5** (R=D) of 65:35, indicating essentially identical kinetic behaviour.

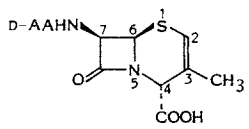
Secondly we incubated exomethylene cephalosporin C (**6**), which we have previously shown to be an unnatural substrate for the fungal enzyme,[5] with the recombinant enzyme. Analogous to the result obtained with the fungal enzyme, **6** was converted directly to DAC (**2**) without the detected intermediacy of DAOC (**1**). This observation has been confirmed by other

Scheme 1.



D-AA: δ-(D-α-Aminoadipoyl)
α-KG: α-Ketoglutarate

† Frozen competent cells of *E. coli* strain NM554[6] were transformed[7] with plasmid pIT511, which contained the DAOC/DAC synthetase gene and a gene which conferred tetracycline resistance. Purification of the enzyme was achieved essentially according to the protocol of J. E. DOTZLAFF and W. K. YEH (personal communication).



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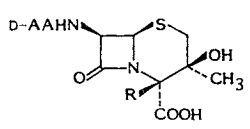

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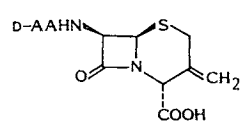

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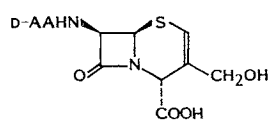

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studies (J. E. DOTZLAFF and W. K. YEH; personal communication).

Finally we examined the Δ-2 cephem (**4**) as a novel substrate for the recombinant DAOC/DAC synthetase. Thus incubation of **4**† gave the hydroxylated Δ-2 cephem (**7**) as the single observable (by 500 MHz ^{1}H NMR) product. Purification, by reverse phase HPLC (0.55% CH_3CN in H_2O, ODS column) gave **7**: Retention time 5.5 minutes; ^{1}H NMR (500 MHz, D_2O, TSP referenced) δ 1.63~1.98 (4H, m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca.* t, J=7 Hz, CH_2CH_2CO), 3.73~3.76 (1H, m, $CHCH_2CH_2$), 4.15, 4.24 (2H, ABq, J=13 Hz, CH_2OH), 4.85 (1H, s, CHCOO), 5.30, 5.40 (2×d, J=4 Hz, 6-H, 7-H), 6.34 (1H, s, CH=C); fast atom bombardment MS m/z 374 (MH^+). The biosynthetic **7** was identical (by ^{1}H NMR (500 MHz)) with an authentic synthetic sample.† It is of interest to note that no isomerisation of the cephem double bond to give either DAC (**2**) (*cf.* the conversion of exomethylene (**6**) to DAC (**2**)) or DAOC (**1**) was observed, in the biosynthetic transformation of **4** to **7**.

These studies clearly demonstrate that recombinant DAOC/DAC synthetase processes substrates in an identical manner to the fungal enzyme. The availability of large quantities of the cloned enzyme should permit the production of novel β-lactam metabolites, in addition to allowing more detailed analysis of the mechanism of this interesting and medicinally significant enzyme.

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† **4** and **7** were synthesised from DAOC (**1**) and cephalosporin C in 3 and 4 steps respectively. Full details will be reported elsewhere.

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