Bioorganic & Medicinal Chemistry Letters, Vol.2, No.7, pp. 669-672, 1992
Printed in Great Britain

0960-894X/92 $5.00 + .00

SUBSTRATE SPECIFICITY OF SOLUBLE RECOMBINANT DEACETOXYCEPHALOSPORIN C/ DEACETYLCEPHALOSPORIN C SYNTHASE

Jack E. Baldwin, Robert M. Adlington, Nicholas P. Crouch, Richard J. Heath
Inês A.C. Pereira and John D. Sutherland.

The Dyson Perrins Laboratory and The Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, U.K..

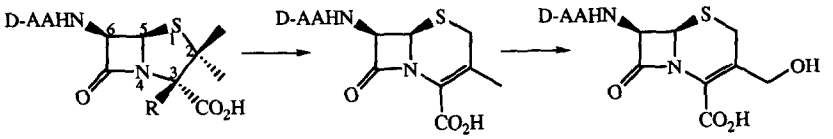
(*Received* 11 *March* 1992)

ABSTRACT.

The substrate specificity of a new soluble recombinant deacetoxycephalosporin C/ deacetylcephalosporin C synthase has been investigated and was found to be identical to that of the wild type fungal enzyme. Using the soluble enzyme, a new metabolite has been detected on incubation with [4-^{2}H]-exomethylene cephalosporin C.

INTRODUCTION.

The enzyme deacetoxycephalosporin C/deacetylcephalosporin C synthase (DAOC/DACS) is a bifunctional enzyme which catalyzes the ring expansion of penicillin N (1a) to deacetoxycephalosporin C (2) and the hydroxylation of this to deacetylcephalosporin C (3) (Scheme 1).



(1a) R=H (2) (3)
(1b) R=D

D-AA = δ-(D-α-Aminoadipoyl)

Scheme 1

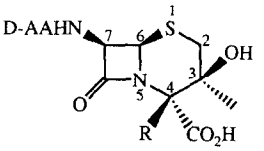
This enzyme has been purified to homogeneity from *Cephalosporium acremonium* [1,2], and a partial amino-acid sequence obtained which permitted the cloning and over-expression of the corresponding gene in *E. coli* [3]. Both ring expansion and hydroxylation activities were found in extracts of the *E. coli* cells, providing proof of the bifunctional nature

of DAOC/DACS in *C. acremonium*. Comparative studies on the activity and substrate specificity of the partially purified recombinant enzyme showed identical behaviour to the wild type enzyme[4]. Expression of this protein in *E. coli* under the control of the λP_L promoter resulted in the formation of inclusion bodies which required urea treatment for solubilization and partial recovery of activity. Unfortunately, a large part of the enzyme activity was lost during this process[5].

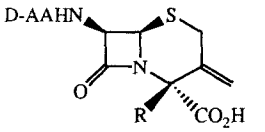
Recently, work carried out in this laboratory has resulted in the high level expression of *soluble, highly active* DAOC/DACS in *E. coli* under the control of a different promoter [6] (see preceding paper). We now wish to report results of the substrate specificity studies performed with this new recombinant enzyme.

RESULTS.

Incubations were carried out as previously described[7] with the purified soluble DAOC/DACS (*ca.* 0.1 I.U.) and *ca.* 1 mg of the substrate to be tested. Product composition was determined by ^{1}H-NMR (500 MHz). Thus, incubation of penicillin N (1a) resulted in the formation of DAOC (2), DAC (3) and the 3β-hydroxy cepham (4a) as a minor product [ratio (2):(3):(4a)=7:30:1]. Incubation of DAOC (2) gave DAC (3). Previously it was shown that incubation of [3-^{2}H]-penicillin N (1b) with partially purified DAOC/DACS from *C. acremonium* gave, in addition to DAOC (2) and DAC (3), the 3β-hydroxy cepham (4b)[7]. This result was rationalised by the operation of a kinetic isotope effect on a branched pathway in the ring expansion step. We incubated [3-^{2}H]-penicillin N (1b) and again observed the formation of the 3β-hydroxy cepham (4b) in addition to DAC (3)[8]. We also incubated exomethylene cephalosporin C (5a) which was previously shown to be an unnatural substrate for the wild type enzyme[9]. As before we observed direct conversion of (5a) to DAC (3).



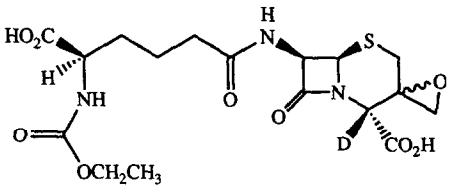
(4a) R=H
(4b) R=D



(5a) R=H
(5b) R=D

Finally we wish to report the observation of a new metabolite from incubation of [4-^{2}H]-exomethylene cephalosporin C (5b) with the soluble DAOC/DACS. Thus, analysis by ^{1}H-NMR of the crude product obtained on incubation of (5b) revealed, in addition to DAC (3) signals, two new β-lactam signals (δ 5.50 and 5.37 ppm). The ratio of DAC (3) to this second product varied between 4:1 and 8:1 depending on the level of conversion (higher conversions resulted in smaller amounts of the minor product). Attempts to isolate the new compound with several HPLC systems proved unsuccessful, due to co-elution with the main product DAC (3). N-Derivatization of a mixture of DAC (3) and the new metabolite with diethylpyrocarbonate at pH 8[10], followed by two HPLC purifications provided the N-

ethoxycarbonyl derivative of the unknown product in a sufficiently pure form for good ^{1}H-NMR and mass spectra to be obtained[11]. The spectroscopic data obtained thus far[12] are consistent with the spiro epoxide (6).



(6)

If correct, (6) represents the first example of epoxidase activity observed for DAOC/DACS. We believe the epoxide may be an intermediate in the conversion of (5) to (3), but it can only be observed if a deuterium isotope effect slows down its ring opening to give DAC (3). Intensive work is under way to isolate the underivatised epoxide structure in a pure state and to characterize it unequivocally.

In conclusion, we have demonstrated that the substrate specificity of the new soluble recombinant DAOC/DACS is identical to that of the wild type fungal enzyme and have used this new form of DAOC/DACS to detect a novel metabolite.

ACKNOWLEDGEMENTS.

We thank the SERC for financial support (to R.J.H. and I.A.C.P.) and also the Junta Nacional de Investigação Científica e Tecnológica/Programa Ciencia (Portugal) for a Scholarship (to I.A.C.P.). In addition we would like to thank Eli Lilly & Co., Indianapolis for financial and scientific support.

REFERENCES AND FOOTNOTES.

1. 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.
2. Dotzlaff, J.E. and Yeh, W.-K. *J. Bacteriol.*, **1987**, *169*, 1611.
3. Samson, S.M., Dotzlaf, J.E., Slisz, M.L., Becker, G.W., Van Frank, R., Veal, L.E., Yeh, W.-K., Miller, J.R., Queener, S.W. and Ingolia, T.D. *Biotechnology* **1987**, *5*, 1207.
4. 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. Antibiots.* **1988**, *41*, 1694.
5. Baldwin, J.E. and Sobey, W., unpublished results.
6. Baldwin, J.E., Blackburn, J.M., Heath, R.J. and Sutherland, J.D. preceding paper in this issue.
7. Baldwin, J.E., Adlington, R.M., Crouch, N.P., Schofield, C.J., Turner, N.J. and Aplin, R.T. *Tetrahedron*, **1991**, *47*, 9881.

8. The product ratio obtained on incubation of [3-^{2}H]-penicillin N (1b) with wild type enzyme or urea solubilised recombinant enzyme[4] was (2)+(3):(4b)=65:35. With the new soluble enzyme we observed formation of only DAC (3) and the 3β-hydroxy cepham (4b) in a ratio that varied between (3):(4b)=3:10 to (3):(4b)=1:2. We interpret this as a result of the high activity of the enzyme which converts all DAOC (2) to DAC (3) and is further processing this by oxidising the alcohol group in (3) to an aldehyde. DAOC/DACS has been shown to catalyze this reaction[13], and this was further supported by the observation of a signal at δ 9.18 ppm in the ^{1}H-NMR spectrum consistent with an aldehyde. A similar ratio of products could be observed with the urea solubilised recombinant enzyme if a large amount of enzyme was used.
9. Baldwin, J.E., Adlington, R.M., Crouch, N.P. and Schofield, C.J. *Tetrahedron*, **1988**, *44*, 643 and references therein.
10. Loder, P.B. and Abraham, E.P. *Biochem. J.*, **1971**, *123*, 471.
11. Isolation in this case was only successful when a large scale incubation of (5b) was attempted using the relatively larger quantities of enzyme from the soluble, higher activity DAOC/DAC synthase source.
12. ^{1}H-NMR (500 MHz, D_2O, HOD suppressed, TSP referenced) : 1.24 (3H, t, J 7 Hz, $CH_2C\underline{H}_3$), 1.65-1.86 (4H, m, $CHC\underline{H}_2C\underline{H}_2$), 2.34-2.45 (2H, m, $C\underline{H}_2CO$), 2.43 and 3.63 (2H, ABq, J 15 Hz, $C\underline{H}_2S$), 3.40 and 3.63 (2H, 2 x d, J 3.7 Hz, epoxide H's), 3.92-3.98 (1H, m, $HNC\underline{H}CO_2H$), 4.07-4.15 (2H, m, $C\underline{H}_2CH_3$), 5.36 and 5.49 (2H, 2 x d, J 4 Hz, β-lactam H's); m/z (Electrospray) : 447 ([MH^+], 20%), 469 ([MNa^+], 100%)
13 Baldwin, J.E.; Schofield, C.J.; Goh, K.-C.; in press 1992.