Bioorganic & Medicinal Chemistry Letters, Vol.1, No.8, pp. 421-424, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

THE ROLE OF SULPHUR IN CEPHALOSPORIN BIOSYNTHESIS

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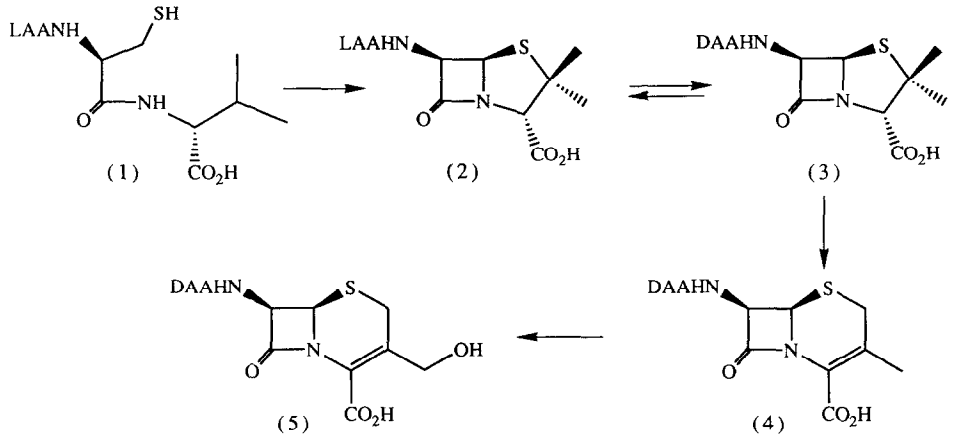
(*Received* 26 *July* 1991)

Abstract: *The role of sulphur in cephalosporin biosynthesis was probed by evaluation of a carbocyclic analogue of deacetoxycephalosporin C as a substrate for DAOC/DAC synthase.*

Key steps in the biosynthesis of the cephalosporin antibiotics are the desaturative cyclisation of the Arnstein tripeptide, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV, 1) to isopenicillin N (2) and the subsequent conversions of penicillin N (3) to deacetoxycephalosporin C (DAOC, 4) and deacetylcephalosporin C (DAC, 5) (Scheme 1)[1]. Each of the three oxidative steps are catalysed by ferrous dependent non-haem oxygenases. In the pathway in *C. acremonium*, both the ring expansion and hydroxylation steps are carried out by a single bifunctional enzyme (DAOC/DAC synthase)[2], whereas in *Streptomyces spp.* the ring expansion and hydroxylation steps are largely catalysed by separate enzymes (DAOC synthase and DAC synthase)[3].



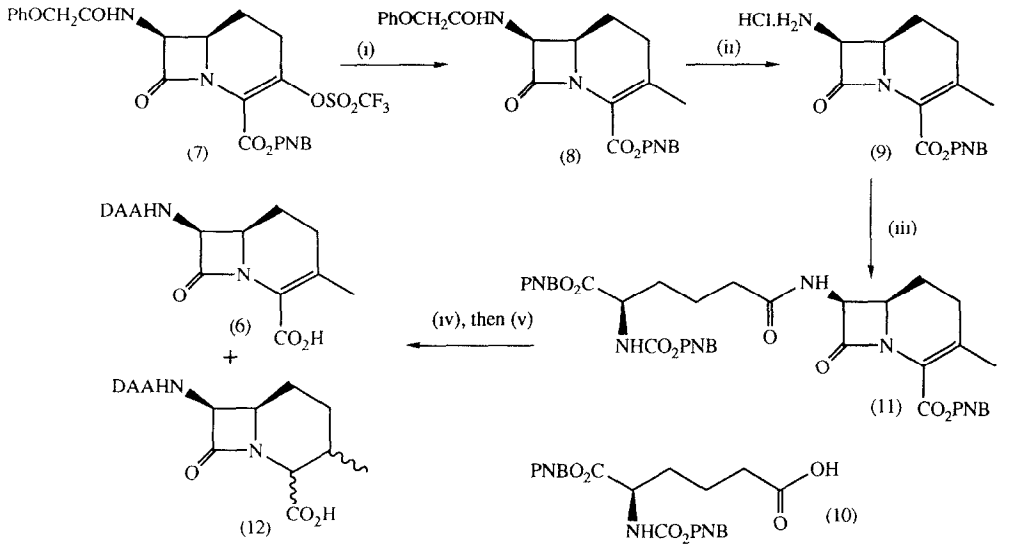
Scheme 1, LAAHN = L-δ-(α-aminoadipoyl), DAAHN = D-δ-(α-aminoadipoyl)

Each of the synthases has been isolated from wildtype sources and has been cloned and expressed[4]. A significant degree of sequence homology was found between DAOC/DAC synthase, DAOC synthase and DAC synthase and also with the oxygenase which catalyses the conversion of (1) to (2)[3b,4][isopenicillin N synthase (IPNS)]. Mechanistic investigations have indicated that the chemistry of the DAOCS/DACS enzymes bears a strong similarity to that of IPNS, and the substrate analogue and kinetic studies have been rationalised in both

cases by invoking a ferryl-oxo intermediate[1]. A major difference is in the stoicheometry of dioxygen utilisation by the two enzymes. For IPNS, an overall four electron oxidation of ACV (1) is achieved, concomitant with conversion of one molecule of dioxygen into two molecules of water. In the case of DAOCS/DACS, for each consecutive step, only a two electron oxidation of the β-lactam substrate is achieved for each molecule of dioxygen consumed, since in each step one molecule of α-ketoglutarate is transformed into carbon dioxide and succinate[5]. Thus, DAOCS/DACS, like other α-ketoglutarate dependent oxygenases, utilises only the ferryl state in its attack on the substrate, having produced this by initial splitting of α-ketoglutarate and dioxygen.

Spectroscopic studies with IPNS have indicated that one equivalent of ferrous iron is bound per mol. of enzyme and that in the resting enzyme the metal is in the high spin ferrous state[6]. Further studies are consistent with the proposal[1] that binding of ACV (1) to IPNS results in the formation of a metal-sulphur bond[7]. Site-specific mutagenesis of the two cysteines of IPNS from *C. acremonium* to serine, both separately and together, produced proteins that were still active[8], indicating that the formation of the metal-sulphur bond probably results from interaction of the cysteinyl residue of ACV (1) directly with the metal. The formation of such a bond is an integral part of our proposed mechanism for IPNS[1].

Given the pivotal role assigned to the thiol of ACV (1) in the formation of isopenicillin N (2) we believed that it would be of interest to explore the role of sulphur in the ring expansion and hydroxylation steps. Herein, we report the synthesis and results of the incubation of a carbocyclic analogue (6) of DAOC (4) with DAOC/DAC synthase



Scheme 2, Reagents: (i) LiCl/DMF then $(MeCN)_2PdCl_2$ (83%); (ii) PCl_5/CH_2Cl_2, then ^tBuOH; (iii) EEDQ/Et_3N/CH_2Cl_2 [43% from (8)]; (iv) H_2/Pd/C/THF:H_2O, 1:1, 1eq x $NaHCO_3$[>95% to (6) and (12)]; (v) HPLC see text [50% from (11) to (6)] ; PNB = *para*-nitrobenzyl

Thus, the diprotected 3-triflate carbacephem (7) was prepared according to the method of Evans and Sjogren[9,10] and efficiently converted to the 3-methyl cephem (8) [LiCl/DMF , then $(MeCN)_2PdCl_2$ (83%)] using

a modified Stille coupling. Selective removal of the phenoxyacetyl amino protecting group gave the amine hydrochloride (9), which was coupled with diprotected α-(δ-aminoadipic acid) (10)[11] to give the desired compound in protected form (11). Deprotection by catalytic hydrogenation gave a mixture of carba-DAOC (6) and the carba-cephams (12) [(6) : (12) = 3 : 2], from which (6) was purified by HPLC (25mM aqueous NH_4HCO_3, 250 x 10 mm C_{18} column, retention time = 13 min at flow rate of 4 ml/min)[12].

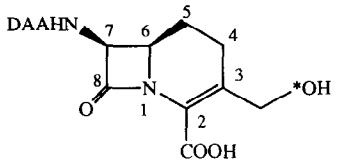
Incubation of (6) with recombinant DAOC/DAC synthase[13] resulted in the observation of a single β-lactam product in the crude incubation mixture by 500 MHz ^1H NMR spectroscopy. The product was isolated by HPLC (25 mM aqueous NH_4HCO_3, 250 x 4.6 mm C_{18} column, retention time = 5 min at flow rate of 1 ml/min) and identified as the carbocyclic analogue of DAC (13a): δ_H (D_2O, 500 MHz) ; 1.47-1.51 (1 H, m, 5-H), 1.57-1.62 (2 H, m, $C\underline{H}_2CH_2CO$), 1.72-1.87 (2 H, m, $C\underline{H}_2CH_2CH_2CO$), 1.88-1.96 (1 H, m, 5-H), 2.28-2.37 (4 H, $C\underline{H}_2CO$, 2 x 4-H), 3.62-3.67 (1 H, *m*, $NC\underline{H}CH_2CH_2$), 3.81-3.86 (1 H, m, 6-H), 4.13,4.18 (2 H, ABq, *J* = 13 Hz, $C\underline{H}_2OH$), 5,52 (1 H, d, *J* = 4.5 Hz, 7-H); a 2-D COSY experiment was consistent with the connectivity as indicated; m/z for MH^+(electrospray) - see Table 1.

		MH^+						
(13) $H_2{}^{16}O/{}^{16}O_2$	m/z	355	356	357	358	359	360	361
	%	0	100	19	6	2	2	3
(13) $H_2{}^{16}O/{}^{18}O_2$	m/z	355	356	357	358	359	360	361
	%	7	42	12	100	20	5	2

Table 1

Incubation of (6) under an atmosphere of $^{18}O_2$ gas produced the alcohol (13) with *ca.* 70% level incorporation of a single ^{18}O atom (Table 1). This less than stoicheometric incorporation has been previously remarked upon in the conversion of (4) to (5)[5] and in the case of other α-ketoglutarate dependent oxygenases and has been rationalised by invoking a water molecule bound to the iron atom of DAOC/DAC synthase. Kinetic analysis of the carbocyclic compound (6) as a substrate gave $K_m = 710 \pm 50$ μM and $V_{max} = 129 \pm 7$ mU/mg protein compared with values of $K_m = 37 \pm 3$ μM and $V_{max} = 400 \pm 20$ mU/mg protein for the conversion of natural substrate (4) to (5), obtained under analogous conditions[14].

(13a) *O = ^{16}O
(13b) *O = ^{18}O



The significant increase in K_m reflects a lower affinity of the enzyme for the unnatural substrate (6), than for the natural substrate (4), which can probably be attributed to conformational changes caused by substitution of the sulphur atom with a methylene group. The difference in V_{max} was, however, much less marked, and indicates that sulphur of DAOC (4) is not directly involved in the hydroxylation reaction. In future, it is hoped to probe the

role of the sulphur of the thiazolidine ring in the ring expansion step by the synthesis and incubation of the corresponding carbocyclic analogue of penicillin N.

Acknowledgement : We thank Robin Aplin for mass spectral analyses, Robert Field for discussions and the Singapore Economic Development Board for a scholarship to K. C. G.

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12. Compound (6) exhibited ~ 40% of the antibacterial activity of DAOC (4) against *Escherichia coli* X580; Compound (13) exhibited ~ 75% of the antibacterial activity of of DAC (5) against the same organism.
13. The enzyme used in this study was refolded recombinant material prepared according to a modified procedure of that previously reported; (a) S. M. Samson, J. E. Dotzlaf, M. L. Slisz, G. W. Becker, R. M. Van Frank, L. E. Veal, W.-K Yeh, J. R. Miller, S. W. Queener, and T. D. Ingolia, *Bio/Technology*, **1987**, 5, 1207;(b) J. E. Baldwin, R. M. Adlington, J. B. Coates, N. P. Crouch, J. W. Keeping, C. J. Schofield, W. A. Shuttleworth, and J. D. Sutherland, *J. Antibiot.*, **1988**, 41, 1694
14. One unit of enzyme activity refers to one micromole of product formed in one minute.[3b]