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CEPHALOSPORIN BIOSYNTHESIS: A BRANCHED PATHWAY SENSITIVE TO AN ISOTOPE EFFECT

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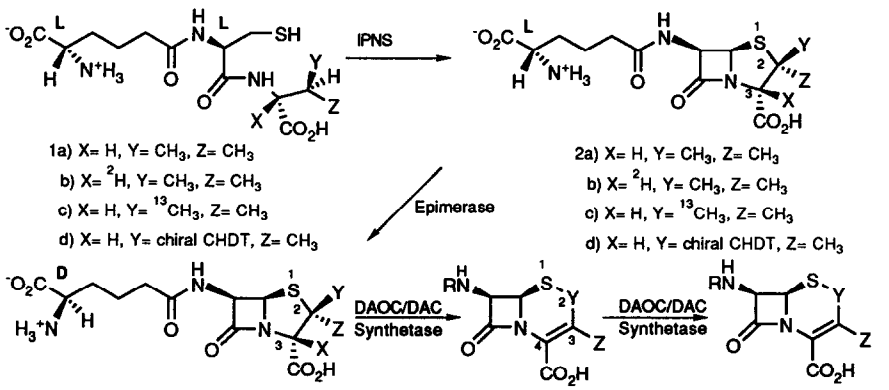
Abstract: Incubation of penicillin N (**3a**) with partially purified deacetoxy/deacetylcephalosporin C synthase (DAOC/DAC synthase) from *Cephalosporium acremonium* CO 728 gave in addition to the expected products, deacetoxycephalosporin C and deacetylcephalosporin C, a third β-lactam metabolite as a 3β-hydroxy-3α-methylcepham (**9a**). Production of the 3β-hydroxycepham was promoted from [3-^{2}H]penicillin N (**3b**) which was rationalised by the operation of a kinetic isotope effect on a branched pathway in the enzymic process. The oxygen of the 3β-hydroxy group was shown to be derived in part from molecular oxygen. In addition, the 2β-methyl group of penicillin N was shown to be incorporated into C2 of the 3β-hydroxy-3α-methylcepham, a result in stereochemical accord with the equivalent transformation of the 2β-methyl group of penicillin N into C2 of deacetoxycephalosporin C[1]. A mechanistic interpretation, consistent with these observations, is offered.

Penicillin N (**3a**), which is derived from δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (L,L,D-ACV, **1a**) *via* isopenicillin N (**2a**) (Scheme 1), was first rigorously proven to be the immediate precursor of the cephalosporins in 1980[2] and since then, mechanistic investigations concerning the ring expansion process have been in progress. *In vivo* labelling experiments utilising either stereospecifically labelled (2RS, 3R)-[4-^{13}C]valine[3] or (2S, 3S)-[4-^{13}C]valine[4] have been used to investigate the stereochemistry of incorporation of the diastereotopic methyl groups during the thiazolidine ring closure of isopenicillin N and subsequent ring expansion of penicillin N (3) to the dihydrothiazine system of the cephalosporins.



1a) X= H, Y= CH_3, Z= CH_3
b) X= ^{2}H, Y= CH_3, Z= CH_3
c) X= H, Y= $^{13}CH_3$, Z= CH_3
d) X= H, Y= chiral CHDT, Z= CH_3

2a) X= H, Y= CH_3, Z= CH_3
b) X= ^{2}H, Y= CH_3, Z= CH_3
c) X= H, Y= $^{13}CH_3$, Z= CH_3
d) X= H, Y= chiral CHDT, Z= CH_3

3a) X= H, Y= CH_3, Z= CH_3
b) X= ^{2}H, Y= CH_3, Z= CH_3
c) X= H, Y= $^{13}CH_3$, Z= CH_3
d) X= H, Y= chiral CHDT, Z= CH_3
e) X= ^{2}H, Y= CH_3 , Z= $^{13}CH_3$

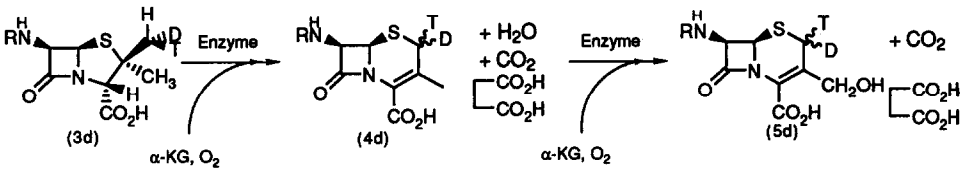
4a) Y= CH_2, Z= CH_3
b) Y= CH_2, Z= CH_3
c) Y= $^{13}CH_2$, Z= CH_3
d) Y= scrambled CDT, Z= CH_3
e) Y= CH_2, Z= $^{13}CH_3$

R= δ-D-α-Aminoadipoyl

5a) Y= CH_2 , Z= CH_2OH
b) Y= CH_2 , Z= CH_2OH
c) Y= $^{13}CH_2$, Z= CH_2OH
d) Y= scrambled CDT, Z= CH_2OH
e) Y= CH_2, Z= $^{13}CH_2OH$
f) Y= CH_2, Z= CH_2OAc
g) Y= $^{13}CH_2$, Z= CH_2OAc
h) Y= scrambled CDT, Z= CH_2OAc

Scheme 1

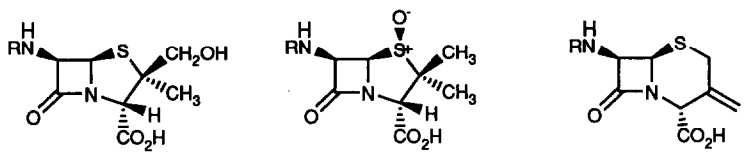
The results of these experiments indicated qualitatively that it is the β-methyl group of penicillin N (**3**) which undergoes incorporation into the cephem C2 position, (i.e **3c** to **4c**, Scheme 1). In *C. acremonium* the conversion of penicillin N (**3a**) to deacetoxycephalosporin C (DAOC, **4a**) and the subsequent hydroxylation of **4a** to deacetylcephalosporin C (DAC, **5a**) have been shown to be effected by a single bifunctional protein, deacetoxy/deacetylcephalopsorin C synthase (DAOC/DAC synthase)[5,6,7]. Further experiments using chiral methyl valines have indicated that the ring expansion process occurs with the complete loss of chirality of the β-methyl groups[8,9] and that both catalytic steps require dioxygen and α-ketoglutarate as co-substrates and ascorbate for optimal activity (Scheme 2)[7].



R = δ-D-α-aminodipoyl
α-KG = α-ketoglutarate

Scheme 2

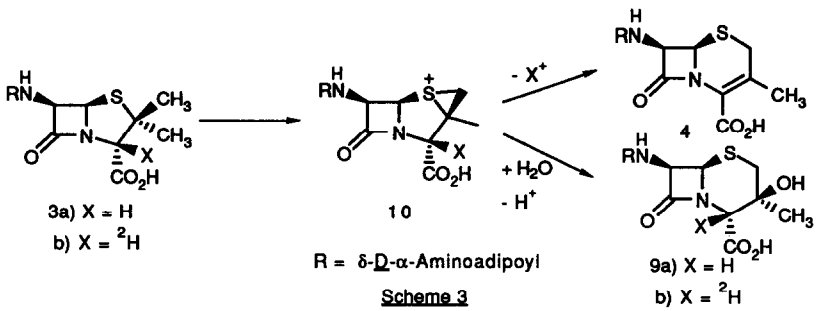
We have examined the 3β-methylenehydroxypenam (**6**) and the β-sulphoxide (**7**) as potential intermediates in the ring expansion process, neither however were converted to cephem products by DAOC/DAC synthase.[6] Somewhat surprisingly the 3-exomethylene cepham (**8**) was shown to give DAC (**5a**) without the apparent intermediacy of DAOC (**4a**) or any other β-lactam containing species[10].



(6) (7) (8)

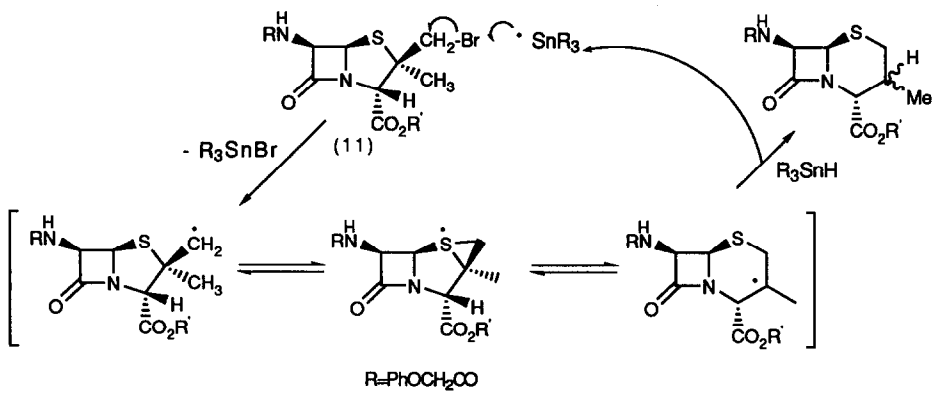
R = δ-D-α-Aminoadipoyl

The isolation of 7β-[(5R)-5-amino-5-carboxypentanoyl]-3β-hydroxy-3α-methyl-4α-carboxylic acid (**9a**) from a filtered broth of *C. acremonium* led to mechanistic speculation[11] regarding the involvement of an episulphonium ion intermediate (**10**) in the ring expansion process. Thus it was proposed that direct collapse of **10** *via* proton loss could give **4**, and that interception of **10** by water would give **9a** (Scheme 3).



Scheme 3

We have demonstrated in a chemical model with the β-bromomethyl penicillin V (**11**), that generation of a β-methylene radical provides a route for a ring expansion process[12] (Scheme 4). Such a radical type intermediate is attractive in respect of the chirality loss observed during the *in vivo* conversion of labelled penicillin N (**3d**) to cephalosporin C (**5h**) (Scheme 1).



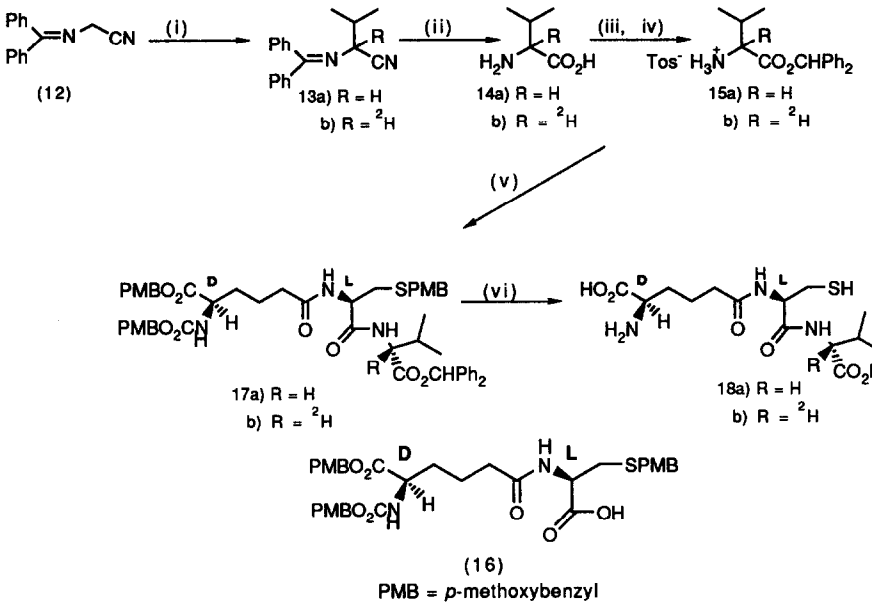
Scheme 4

However, trapping of such an expanded radical by water in the enzymatic reaction to give the 3β-hydroxy cephem (**9a**) is improbable. As the expansion of penicillin N (**3**) to deacetoxycephalosporin C (**4**) requires the formal loss of two hydrogen atoms, we investigated the effect of deuteration at these positions. We now report the results of the incubation of [3-^{2}H]penicillin N (**3b**), and related studies, on the course of the enzymic reaction.

Synthesis of [3-^{2}H]Penicillin N (3b)

As the total synthesis of [3-^{2}H]penicillin N (**3b**) would be a major undertaking, the biosynthesis of **3b** using isopenicillin N synthase (IPNS) *in vitro*, from an appropriately deuterated tripeptide, represented an attractive alternative. However, the side-chain of the natural substrate for IPNS, δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (L,L,D-ACV, **1a**) is of opposite configuration to that required for ring expansion by DAOC/DAC synthase.[6b] Additionally the epimerase enzyme responsible for the *in vivo*

inversion of the side-chain configuration [i.e isopenicillin N (**2**) to penicillin N (**3**)] (Scheme 1) was not available to us in sufficient quantities to prepare the desired milligram amounts of penicillin N (**3b**) required for *in vitro* studies with DAOC/DAC synthase. Tripeptides with D-configured side-chains are, however, substrates for IPNS[13,14] thus we proposed to incubate D,L,D-AC-[3-^{2}H]-valine (**18b**) with IPNS in order to enzymically synthesise (**3b**). Thus deuterated valine benzhydryl ester (**15b**) was prepared from the benzophenone imine (**12**)[15], coupled with the protected dipeptide (**16**) and deprotected *via* standard methodology[16] to give (**18b**) (Scheme 5).



Reagents: (i) 50% NaOH or NaOD, benzyltriethylammonium chloride, toluene, $(CH_3)_2CHBr$, 0°C, 24 hours; (ii) 1N HCl 12 hours, then 6N HCl, 12 hours, reflux; (iii) 1 eq. *p*- toluenesulphonic acid, THF/H_2O, then lyophilisation; (iv) Ph_2CN_2, MeCN, (v) EEDQ, DCM, (16); (vi) TFA, anisole, reflux 30 minutes.

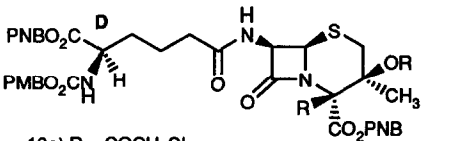
Scheme 5

Initially, the feasibility of preparing and purifying biosynthetic labelled penicillin N on a sufficient scale was shown by incubating D,L,D-ACV (**18a**) (4mg) with IPNS (*ca* 11 international units) and the appropriate cofactors. After purification by reverse phase h.p.l.c. (octadecylsilane column, 0.75% MeCN in 25mM NH_4HCO_3) *ca* 1mg of penicillin N (**3a**), pure by ^{1}H-n.m.r. (500 MHz) was obtained. Similarly we incubated (**18b**) (10mg) with IPNS (*ca* 25 I.U.) and obtained [3-^{2}H]penicillin N (**3b**) (2.1 mg, 27%).

Incubations with DAOC/DAC Synthase

Firstly, the product composition of an incubation of penicillin N (**3a**) (*ca* 1 mg) with partially purified DAOC/DAC synthase (ca 0.1 I.U.) from *C. acremonium* CO 728 and standard cofactors was carefully examined by 500 MHz ^{1}H-n.m.r. and was shown to contain three products [**4a** : **5a** : **9a**, 40 : 20 : 1].

Repeating the incubation with [3-^{2}H]penicillin N (**3b**) (*ca* 1 mg, with *ca* 0.2 I.U.) gave the same three products *but in substantially different* ratio, [**4a** : **5a** : **9b**, 40 : 25 : 35]. Compound (**9b**) was purified (HPLC, Water's system with an octadecylsilane column eluting with 25mM NH_4HCO_3) and it's structure determined from spectral data [Data for **9b**; δ_H (500MHz, D_2O, HOD suppressed) 1.36 (3H, s, CH_3), 1.63-1.94 (4H, m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca* t, *J* 7Hz, CH_2CO), 2.66, 3.56 (2H, ABq, *J* 14Hz, SCH_2), 3.75 (1H, m, $CHCH_2CH_2$), 5.29, 5.46 (2H, ABq, *J* 4Hz, CHCHS); *m/z* (F.A.B.) 377 (MH^+); consistent with literature values for **9a**[11] except for the presence of the C(3)-deuterium atom] and by chemical synthesis of **9a** from it's protected form, **19a/b**[17]. The hydroxycephams **9a** and **9b** were shown not to be substrates for cephem formation by DAOC/DAC synthase and they did not display antibacterial activity against *Staphylococcus aureus* N.C.T.C. 6571 or *Escherichia coli* ESS at a concentration of 100μg ml^{-1}.



19a) R = $COCH_2Cl$
b) R = H

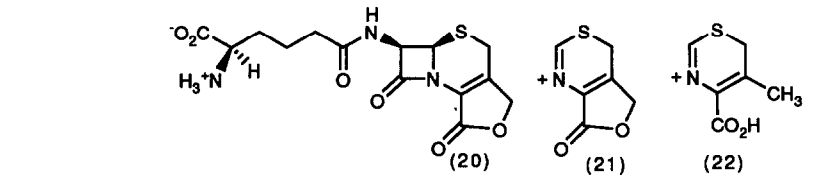
PNB = *p*-nitrobenzyl

Secondly we examined the origin of the oxygen of the 3β-hydroxy function of the cepham (**9b**). Thus incubation of [3-^{2}H]penicillin N (**3b**) with DAOC/DAC synthase and standard cofactors under an atmosphere of $^{18}O_2$ gas gave DAOC (**4a**), DAC (**5b**)[10,18] and hydroxycepham (**9b**) which were purified by h.p.l.c. Lactonisation of DAC (**5b**) to **20** (formic acid) gave a sample suitable for mass spectral analysis[10]. The samples of deacetoxycephalosporin C lactone (**20**) were then run under positive ion thermaspray reverse phase h.p.l.c. mass spectrometry conditions and those of **9b** using positive argon fast atom bombardment. These techniques revealed ^{18}O incorporation into both **5b** and **9b** (Table 1).

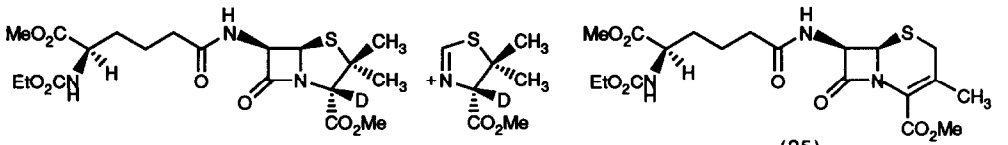
Table 1

Expt.	Conditions		Products (*m/z*; Relative intensity) 3β-Hydroxycepham (**9b**)						**20**(MH+)					
1	$H_2{}^{16}O$; $^{18}O_2$	*m/z*	376	377	378	379	380	381	355	356	357	358	359	360
		Found (%)	29	80	53	100	38	8	4	100	25	72	27	22
2	$H_2{}^{16}O$; $^{18}O_2$	*m/z*	376	377	378	379	380	381						
		Found (%)	-	69	19	100	23	15						
Synthetic **9a**		*m/z*	375	376	377	378	379	380						
		Found (%)	7	100	22	10	1	-						
		Calc. (%)	-	100	18	7	1	-						

DAOC (**4b**) and derivatised recovered [3-^{2}H]penicillin N (**3b**) [as (**23b**)][19, 20] from the incubation under $^{18}O_2$ were shown by mass spectrometry to contain no labelled oxygens. Analysis of mass spectral fragment ions (**21**), (**22**), (**24**) and derivative (**25**) confirmed these incorporations (Tables 3-6, experimental section).



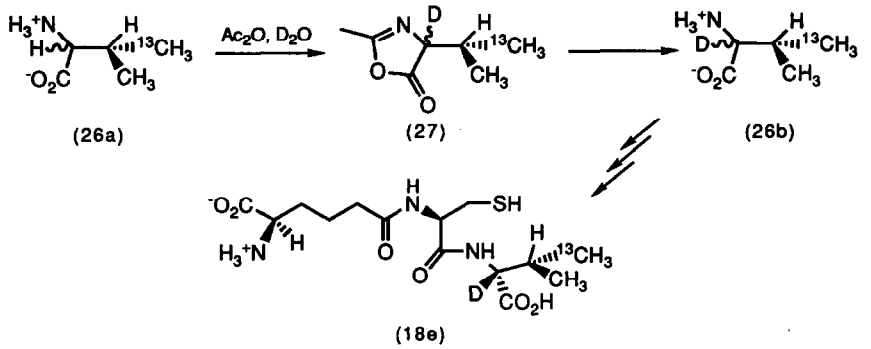
(20) (21) (22)



(23) (24) (25)

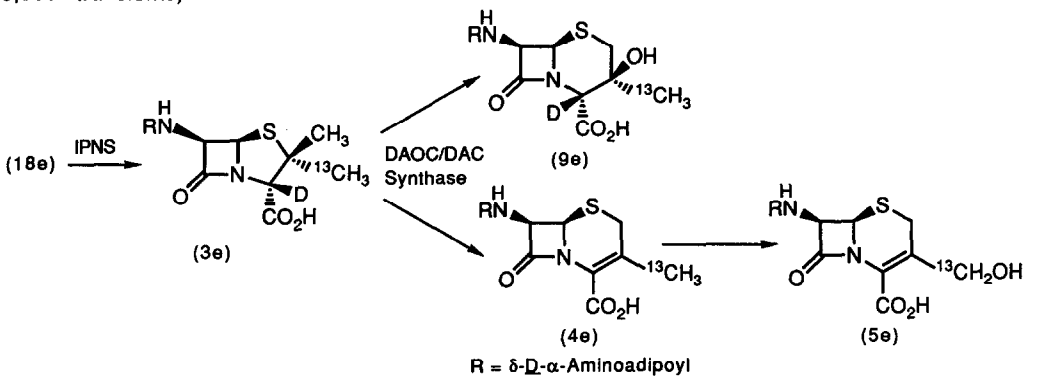
We next turned our attention to the stereochemistry of incorporation of the penicillin N (**3a**) methyl groups into DAOC (**4a**), DAC (**5a**) and the 3β-hydroxy cepham (**9a**). Previous *in vivo* studies with (2RS, 3R)-[4-^{13}C]valine[3] or (2S, 3S)-[4-^{13}C]valine[4] concentrated on the stereochemistry of incorporation of the diastereotopic methyl groups of valine during the thiazolidine ring closure to isopenicillin N (**2**) and subsequent ring expansion of penicillin N (**3**) to the dihydrothiazine system of the cephalosporins, the final analysis being carried out upon cephalosporin C. The results obtained indicated that the thiazolidine ring closure was largely stereospecific and that the pro-R and pro-S methyl groups of penicillin N (**3**) became the exocyclic C-3' acetoxymethyl and endocyclic C-2 methylene groups of cephalosporin C respectively. These experiments were limited with respect to their sensitivity due to the use of intact cells which resulted in low levels of label incorporation, combined with the availability of only comparatively low field NMR spectrometers. Recently, Baldwin has demonstrated that the direct conversion of ACV (**1**) to isopenicillin N (**2**) by IPNS *in vitro* proceeds with complete retention of the valinyl C-3 stereochemistry[21].

The availability of a sample of stereospecifically labelled 3S-^{13}C-valine and a convenient method for the enzymic synthesis of penicillin N possessing a potentially stereospecific ^{13}C-label in the α-methyl group prompted us to examine the stereospecificity of penicillin N methyl group incorporation into the 3β-hydroxy cepham (**9**) and to re-examine the stereospecificity of cephem formation. Thus (2RS, 3S)-[4-^{13}C]-valine (**26a**) was deuterated at the α-carbon *via* conversion to the azlactone **27** by treatment with acetic anhydride in D_2O using methods analogous to those previously reported (Scheme 6)[21]. A reasonable level of deuterium incorporation (*ca* 80-90%) was thus obtained and the resulting labelled valine then protected and coupled with appropriately protected δ-D-α-aminoadipoyl-L-cysteine (**16**) by standard methodology[16] to give a protected tripeptide. Deprotection by refluxing in trifluoroacetic acid then gave the required tripeptide **18e**.



Scheme 6

Treatment of δ-D-α-aminoadipoyl-L-cysteinyl-(2R,3S)-[2-^{2}H,4-^{13}C]-valine (**18e**) with partially purified isopenicillin N synthase from *Cephalosporium acremonium* CO 728[13] in the presence of the usual co-factors gave stereospecifically labelled (2R, 3S)-[2'-^{13}C, 3-^{2}H] penicillin N (**3e**) as shown by ^{13}C-nmr spectroscopy of the crude incubation mixture. The crude penicillin N (**3e**) was then incubated with DAOC/DAC synthase under the usual incubation conditions to give, in addition to the cephems **4e** and **5e**, the shunt product 3β-hydroxycepham (**9e**) (Scheme 7). These products were then isolated by chromatography (h.p.l.c. reverse phase octadecylsilane, 25mM NH_4HCO_3 buffer) and each of the products plus the unconverted penicillin N (**3e**) examined by ^{13}C-nmr spectroscopy (broad band decoupled, each 40,000 transients).



Scheme 7

For each of the isolated ring expanded products, a resonance due to incorporation of label exclusively into the C3'-exocyclic position of the cephems **4e** and **5e** and cepham **9e** and the complete absence of a signal at the position corresponding to the C2-methylene carbons was observed (Table 2). ^{13}C-nmr analysis of the stereospecificity of the ring expansion process was facilitated by preliminary assignment of the C2 and C3'-resonances of the ring expanded products by separate nmr experiments (Table 2), thus enabling non-stereospecific incorporation of label to be scrutinised. In addition unconverted penicillin N (**3e**) was similarly analysed and as anticipated no evidence was obtained to suggest that scrambling of the labelled methyl group had occurred. In these experiments the high signal to noise ratio obtained during accumulation of the ^{13}C-nmr spectra and prior determination of the chemical shifts of the C2-methylene

and C3-methyl and methylene resonances for **4**, **5** and **9**, enabled us to quantify the stereospecificity of the ring expansion process to be greater than 95% for the formation of each of the products.

Table 2

	^{13}C nmr Chemical Shift[a]		Signal/Noise Intensity of Enhanced Signal
	Low Field methyl	High Field methyl	
D,L,D- ACV **18a**	19.22 (q)	17.97 (q)	
D,L,D- ACV **18e**	19.17 (enhanced)	Absent	>50:1
	2α-methyl	2β-methyl	
Penicillin N **3a**	27.22 (q)[b]	31.11 (q)	
2α-$^{13}CH_3$-Pen N **3e**	27.22 (enhanced)	Absent	>40:1
	C3-Me	C2	
DAOC **4a**	19.22 (q)	29.17 (t)[b]	
C3-$^{13}CH_3$-DAOC **4e**	19.12 (enhanced)	Absent	>9:1[d]
	C3-CH_2OH	C2	
DAC **5a**	61.71 (t)	26.15 (t)[b]	
C3-$^{13}CH_2OH$-DAC **5e**	61.74 (enhanced)	Absent	>30:1
	3α-Me	C2	
3β-OH Cepham **9a**[c]	25.84 (q)	34.89 (t)[b]	
3α-$^{13}CH_3$-3β-OH Cepham **9e**	25.79 (enhanced)	Absent	>30:1
Recovered	2α-methyl	2β-methyl	
Penicillin N **3e**	27.25 (enhanced)	Absent	>8: 1

Footnotes.

(a) ^{13}C nmr spectra were recorded at 125.77MHz in D_2O and referenced to internal dioxan = 67.30 ppm.

(b) Assigned by selective ^{13}C-[^{1}H] irradiation experiments and ^{1}H-n.O.e experiments performed upon authentic standards.

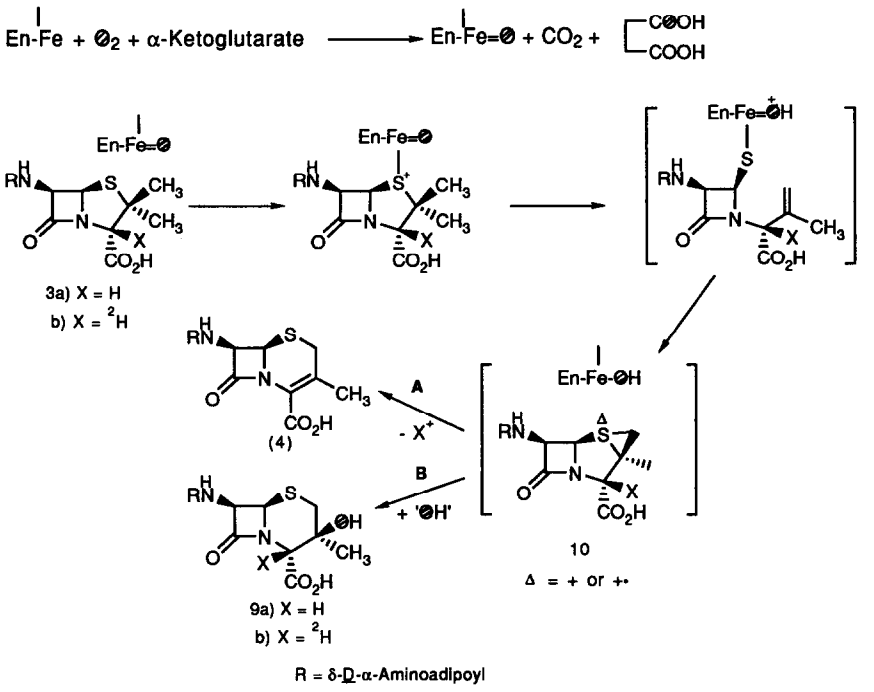
(c) Prepared by analogous methods to those employed of Spry et. al. [17]

(d) A higher stereospecificity for (**4e**) is implied from the spectrum of (**5e**).

When considered together the stereochemical evidence and the results obtained due to the operation of a kinetic isotope effect suggest that the 3β-hydroxy cepham (**9**) is a shunt product arising from a common enzyme bound intermediate which in the normal course of events during the ring expansion process gives rise to DAOC (**4**). We have also shown that a 1:1 mixture of penicillin N (**3a**) and [3-^{2}H]penicillin N (**3b**) is converted into DAOC (**4**), DAC (**5**), and hydroxycepham (**9**) with no isotopic enrichment in the pool of either penicillin N (**3a**) or (**3b**)[20]. This implies that the isotope effect responsible for changing the ratio of DAOC (**4**), DAC (**5**), and hydroxycepham (**9**) must occur subsequent to an irreversible step on DAOC/DAC synthase, i.e. the conversion of penicillin N (**3**) to DAOC (**4**) proceeds *via* a branched pathway, providing hydroxycepham (**9**) in addition to DAOC (**4**). Using fully protiated penicillin N (**3a**) the ratio of DAOC (**4**) and DAC (**5**), to hydroxycepham (**9**) is ca 60 : 1, thereby accounting for the low concentration of hydroxycepham (**9a**) observed in normal fermentations. The operation of a deuterium isotope effect on the cleavage of the C(3)-H bond substantially shifts the product ratio to *ca* 2 : 1. In addition the 3β-

hydroxycepham (**9**) is formed by specific incorporation of oxygen from dioxygen. These facts can be accommodated by a mechanism (Scheme 8) in which a bridged species **10**, either as the cation or the radical can decompose by loss of hydrogen at C3 to give the major product **4** (path A) or by the interception of the bridged species by a hydroxyl group (path B) derived from the α-ketoglutarate-penicillin coupled reduction of dioxygen (which produced **10** as part of the catalytic cycle). We are currently undertaking studies designed to differentiate between the heterocyclic and homolytic possibilities.

It is of interest to note that in Scheme 8 direct coordination between the ferryl [>Fe(IV)O] and the sulphur atom is postulated as a preliminary to the ring expansion event. Much work on the mechanism of the preceding enzyme of this pathway, IPNS, which makes isopenicillin N, suggests that the last event of this reaction is the dissociation of the sulphur from the iron species, releasing isopenicillin N. The considerable homology that exists between these enzymes is in accord with their close mechanistic relationship, i.e. that the ring expansion activity arose from isopenicillin N synthase.



Scheme 8

Acknowledgements: We thank the S.E.R.C. (for a studentship and research grant to N.P.C.), Eli Lilly & Co., Indianapolis, U.S.A. for financial support, Mr. G.C. Knight and Mr. J.W. Keeping for the preparation of enzyme extracts and Mr. R. Proctor for mass spectrometric analysis.

EXPERIMENTAL

General

Standard chemical procedures as previously reported[10] were used. Flash chromatography was performed with Merck Kieselgel 60, 230-400 mesh. Preparative plate chromatography was performed with silica gel (HF254) coated onto glass plates. Thin layer chromatography was performed with Merck

silica gel 60 F254 pre-coated onto aluminium plates. Infra red spectra were recorded on a Perkin Elmer 681 infra red spectrometer or Perkin Elmer 1750 fourier-transform spectrometer (absorbances recorded as: s strong, m medium, w weak and b broad).

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

^{1}H-n.m.r. spectra were either recorded at 300MHz on a Bruker WH 300 spectrometer or at 500MHz on a Bruker AM 500 spectrometer and are referenced internally to either TMS (samples in $CDCl_3$ $\delta_{ref.}$ = 0.0 ppm) or TSP (samples in D_2O, $\delta_{ref.}$ = 0.0 ppm) unless otherwise indicated. ^{13}C-n.m.r. spectra were either recorded at 62.38 MHz on a Bruker AM 250 spectrometer or at 125.77MHz on a Bruker AM 500MHz spectrometer and are referenced internally to either $CDCl_3$ ($\delta_{ref.}$ = 77.0ppm) or 1,4-dioxan (for samples in D_2O, $\delta_{ref.}$ = 67.3ppm).

Mass spectra in the electron-impact (E.I.) mode or chemical ionisation (C.I.) mode were recorded on a VG Micromass 30F spectrometer. Samples requiring field desorption chemical ionization (F.D.) were run on either a ZAB 1F spectrometer or VG 20-250 spectrometer. Fast atom bombardment (F.A.B.), thermaspray (T.S.P.) and h.p.l.c. mass spectra were run on a VG 20-250 spectrometer.

High performance liquid chromatography (h.p.l.c.) of crude incubation mixtures was performed with two Waters M-510 A pumps, a Rheodyne 7125 injector, a Waters 441 detector set at 218nm (unless otherwise stated) and columns packed with Hypersil 5 ODS (250 x 4.6mm internal diameter). Preparative scale h.p.l.c. was performed using two Gilson 303 pumps, a Rheodyne 7125 injector, a Gilson HM holochrome variable wavelength detector set at 220nm and columns packed with Zorbax ODS (250 x 9.4mm internal diameter).

2-[N-(Diphenylmethylene)amino]-3-methylbutyronitrile (13a)[15]

N-(Diphenylmethylene)aminoacetonitrile (**12**) (1.00 g, 4.55 mmol), benzyltriethylammonium chloride (100 mg, 0.44 mmol), NaOH (1.10 g as a 50% aqueous solution) and toluene (1ml) were stirred together at 0°C in a round bottom flask sealed with a rubber septum. 2-Bromopropane (540 mg, 4.39 mmol) in toluene (1ml) was added *via* a syringe over a period of 1-2 hours at 0°C. The solution was stirred at room temperature overnight and was then poured into a separating funnel containing dichloromethane (60ml) and water (80ml). The layers were separated and the aqueous layer extracted with dichloromethane (3 x 30ml). The organic layers were combined, washed with water (20ml), brine (20ml), dried (anhydrous Na_2SO_4), filtered and the solvent evaporated. Chromatography [flash silica, with petrol/diethyl ether (9:1, v/v)] gave **13a** (800 mg, 3.0 mmol, 69%) as an oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.25; δ_H (500MHz, $CDCl_3$) 1.00 and 1.12 (2 x 3H, 2 x d, *J* 7.5 Hz, 2 x CH_3), 2.11-2.16 (1H, m, C<u>H</u>Me$_2$), 3.98 (1H, d, *J* 6Hz, C<u>H</u>CHMe$_2$), 7.20-7.82 (10H, m, Ar<u>H</u>); δ_C(125.77MHz, $CDCl_3$); 18.22 and 18.65 (2 x q, 2 x CH_3), 33.26 (d, <u>C</u>HMe$_2$), 59.18 (d, <u>C</u>HCHMe$_2$), 118.51(s, CN), 127.16-138.37 (m, Ph), 172.57 (s, Ph$_2$<u>C</u>=N); υ_{max} ($CHCl_3$) 2948(s), 2177(w, CN), 1620(s), 1455(m), 1243(s); *m/z* (E.I.) 261 (5%), 262 (M^+, 8), 263 (MH^+, 12), 264 (3).

2-[N-(Diphenylmethylene)amino]-(2-^{2}H)-3-methylbutyronitrile (13b)[15]

The above procedure was repeated with N-(diphenylmethylene)aminoacetonitrile (**12**) (887 mg, 4.03 mmol), benzyltriethylammonium chloride (100 mg, 0.44 mmol), NaOD

(1.80 g as a 50% aqueous solution), toluene (1ml) and 2-bromopropane (500 mg, 4.03 mmol) in toluene (1ml). Chromatography [flash silica, with petrol/diethyl ether (9:1, v/v)] gave (**13b**) (674 mg, 2.55 mmol, 63%) as an oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.25; δ_H (500MHz, $CDCl_3$), 1.00 (3H, d, *J* 7Hz, $CHCH_3$), 1.12 (3H, d, *J* 7Hz, $CHCH_3$), 2.11-2.16 (1H, m, $CHMe_2$), 7.20-7.66 (10H, m, ArH); υ_{max} ($CHCl_3$) 2950 (s), 2890 (m), 2177 (w, CN), 1605 (s), 1455 (m), 1307 (m), and 1243 (s); *m/z* (E.I.) 263 (M^+, 10%), 264 (19), 265 (5), 266 (1).

D/L-Valine (14a)[22]

To the imine **13a** (796 mg, 3.0 mmol) in diethyl ether (10 ml) was added 1N hydrochloric acid (25ml) and the mixture vigorously stirred for 12 hours. The organic and aqueous layers were separated and the aqueous phase washed with diethyl ether (3 x 10ml). Concentrated hydrochloric acid (25 ml) was added and the resulting solution refluxed for 12 hours. The solution was evaporated and the residue dissolved in a small quantity of water and purified by chromatography [ion-exchange, Dowex 1x8-400 acetate form (eluting with water)]. Column fractions were assayed with ninhydrin and the relevant fractions evaporated to dryness to give racemic (**14a**) (244 mg, 2.1 mmol, 67%) as white solid. δ_H (500MHz, D_2O, HOD suppressed) 0.99 (3H, d, *J* 8 Hz, $CHCH_3$), 1.04 (3H, d, *J* 8 Hz, $CHCH_3$), 2.25-2.31 (1H, m, $CHCHCMe_2$), 3.60 (1H, d, *J* 4Hz, $CHCHCMe_2$); *m/z* (C.I., NH_3) 118 (MH^+, 100%), 119 (6), 120 (1).

D/L-(2-^{2}H)-Valine (14b)

Repetition of the above procedure with 2-[N-(diphenylmethylene)amino]-(2-^{2}H),3-methylbutyronitrile (**13b**) (674 mg, 2.55 mmol) gave (2-^{2}H)-Valine (**14b**) (251 mg, 2.1 mmol, 80%). δ_H (500MHz, D_2O, HOD suppressed) 1.03 (3H, d, *J* 7Hz, $CHCH_3$), 1.05 (3H, d, *J* 7Hz, $CHCH_3$), 2.30-2.38 (1H, m, $CHMe_2$); *m/z* (C.I., NH_3) 118 (4%), 119 (MH^+, 100), 120 (6), 121(1).

D/L-Valine Benzhydryl Ester Ammonium Tosylate Salt (15a)

To the free amino-acid **14a** (100 mg, 0.85 mmol) in water (10 ml) was added a solution of *p*-toluenesulphonic acid (163 mg, 0.85 mmol) in THF (10 ml). The resulting solution was evaporated to dryness and the solid residue suspended in MeCN (20 ml). To the stirred suspension was added diphenyldiazomethane (165 mg, 1 mmol) in MeCN (5 ml), until a pale pink colouration persisted. After 30 minutes the white precipitate was filtered off and washed with petrol (10 ml) to give the product (**15a**) (360 mg, 0.80 mmol, 94%). δ_H (500MHz, $CDCl_3$) 0.80-0.84 (6H, m, $CH(CH_3)_2$), 2.18-2.28 (1H, m, $CHCHMe_2$ and 3H, s, $ArCH_3$) 3.99 (1H, *ca* t, *J* 4Hz, $CHCHMe_2$), 6.85 (1H, s, $CHPh_2$), 6.98-7.65 (14H, m, ArH).

D/L-(2-^{2}H)Valine Benzhydryl Ester Ammonium Tosylate Salt (15b)

Repetition of the above procedure with the amino-acid **14b** (100 mg, 0.65 mmol) in water (10 ml), *p*-toluenesulphonic acid (124 mg, 0.65 mmol) and diphenyldiazomethane (1.0 eq) gave the title compound (186 mg, 0.41 mmol, 63%). m.p.169-70 °C (from EtOH/Et_2O/petrol). Found: C 65.60; H 6.62; and N 2.95: $C_{25}H_{28}DNSO_5$ requires: C 65.77; H 6.40; and N 3.07%); δ_H (500MHz,$CDCl_3$)

0.82-0.85 (6H, m, $CH(C\underline{H}_3)_2$), 2.21-2.28 (1H, m, $CDC\underline{H}Me_2$ and 3H, s, $ArC\underline{H}_3$), 6.86 (1H, s, $C\underline{H}Ph_2$), 6.96-7.64 (14H, m, Ar$\underline{H}$); *m/z* (F.A.B.) 285 (MH^+-TsO^-, 9%), 286 (2), 287 (1).

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-valine Benzhydryl Ester (17a)[16]

The ammonium tosylate salt of the D-valine benzhydryl ester [23] (77 mg, 0.17 mmol) was suspended in saturated aqueous $NaHCO_3$ (10 ml) and the free amine extracted into EtOAc (3 x 20 ml). The combined organic layers were dried (anhydrous Na_2SO_4), filtered and evaporated to dryness to give the free amine which was used without further purification. To the amine (46 mg, 0.17 mmol) in dichloromethane (2 ml) was added (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (**16**) (116 mg, 0.17 mmol), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (47 mg, 0.19 mmol) and anhydrous Na_2SO_4 (*ca* 5 mg). The resulting mixture was stirred at room temperature under argon for 24 hours. The solution was then evaporated to dryness and the residue partitioned between EtOAc (50 ml) and water (50 ml) in a separating funnel. The layers were separated and the organic phase washed with 2N HCl (20 ml), saturated aqueous $NaHCO_3$ (20 ml) and brine (20 ml). The solution was dried (anhydrous Na_2SO_4), filtered and evaporated to dryness. Chromatography [flash silica, with dichloromethane/ ethyl acetate (gradient 4:1 to 1:3, v/v)] gave **17a** (125 mg, 0.14 mmol, 82%) as a white amorphous solid. T.l.c. [ethyl acetate/ petrol (1:1, v/v)] Rf 0.45; δ_H (500MHz, $CDCl_3$) 0.76 (3H, d, *J* 7Hz, $CHC\underline{H}_3$), 0.87 (3H, d, *J* 7Hz, $CHC\underline{H}_3$), 1.62-1.86 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.05-2.25 (3H, m, CH_2CO and $C\underline{H}(CH_3)_2$), 2.67, 2.82 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.70 (2H, s, $SC\underline{H}_2Ar$), 3.74 (3H, s, $SCH_2ArOC\underline{H}_3$), 3.78 (6H,s, 2 x $CH_2ArOC\underline{H}_3$), 4.28-4.35 (1H, m, $C\underline{H}CH_2CH_2$), 4.51 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, $C\underline{H}CH_2S$), 4.62-4.64 (1H, m, $C\underline{H}CHMe_2$), 5.00, 5.02 (2H, ABq, *J* 12Hz, $OC\underline{H}_2Ar$), 5.07 (2H, s, $OC\underline{H}_2Ar$), 5.45 and 6.29 (2H, 2 x d, *J* 8Hz, 2 x NH), 6.79-6.89 and 7.16-7.31 (23H, 2 x m, $C\underline{H}Ph_2$ and Ar$\underline{H}$); δ_C (125.77MHz, $CDCl_3$); 17.24 (q, $CH\underline{C}H_3$) 19.02 (q, $CH\underline{C}H_3$), 21.24 (t, $CH_2\underline{C}H_2CH_2$), 31.09 (d, $\underline{C}HMe_2$), 31.77 (t, $\underline{C}H_2CH_2CH_2$), 33.18, 35.27, 35.82 (3 x t, $S\underline{C}H_2Ar$, $\underline{C}H_2S$, and $\underline{C}H_2CO$), 52.14 and 53.59 (2 x d, $NH\underline{C}HCH_2$ and $NH\underline{C}HCHS$), 55.17 (q, $ArO\underline{C}H_3$), 57.31 (d, $\underline{C}HCHMe_2$), 66.74 and 66.92 (2 x t, $\underline{C}H_2Ar$), 77.93 (d, $\underline{C}HPh_2$), 113.86, 113.96 and 114.04 (3 x d, Ar), 126.89-139.57 (m, Ar), 156.09, 158.76, 159.52 and 159.73 (4 x s, $\underline{C}OMe$ of ArOMe), 170.24, 170.56, 172.06 and 172.48 (4 x $\underline{C}$=O); $\upsilon_{max}(CHCl_3)$ 3020, 1736, 1682 and 1515 (all s); *m/z* (F.D.) 933 (M^+).

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H)-valine Benzhydryl Ester (17b)[16]

Similarly, repetition with the ammonium tosylate salt of the D/L-(2-^{2}H)valine benzhydryl ester (**15b**) (342 mg, 0.75 mmol), **16** (500 mg, 0.75 mmol) and EEDQ (207 mg, 0.83 mmol) gave after separation of the diastereomers by chromatography [preparative plate, with ethyl acetate/hexane (1:1 v/v)], **17b** (248 mg, 0.27 mmol, 36%). T.l.c. [ethyl acetate/ petrol (1:1, v/v)] Rf 0.45; δ_H (500MHz, $CDCl_3$) 0.77 (3H, d, *J* 7Hz, $CHC\underline{H}_3$), 0.88 (3H, d, *J* 7Hz, $CHC\underline{H}_3$), 1.55-1.85 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.00-2.26 (3H, m, CH_2CO and $C\underline{H}(CH_3)_2$), 2.65, 2.84 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.72 (2H, s, $SC\underline{H}_2Ar$), 3.76 (3H, s, $SCH_2ArOC\underline{H}_3$), 3.79 (6H,s, 2 x

$CH_2ArOC\underline{H}_3$), 4.28-4.35 (1H, m, $C\underline{H}CH_2CH_2$), 4.47 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, $C\underline{H}CH_2S$), 5.00, 5.02 (2H, ABq, J 12Hz, $OC\underline{H}_2Ar$), 5.46 and 6.29 (2H, 2 x d, J 8Hz, 2 x NH), 6.78-6.90 and 7.23-7.32 (23H, 2 x m, $C\underline{H}Ph_2$ and $Ar\underline{H}$); δ_C (125.77MHz, $CDCl_3$) 17.25 (q, $CH\underline{C}H_3$) 18.75 (q, $CH\underline{C}H_3$), 21.20 (t, $CH_2\underline{C}H_2CH_2$), 30.81 (d, $\underline{C}HMe_2$), 31.51 (t, $\underline{C}H_2CH_2CH_2$), 33.27, 35.12, 35.68 (3 x t, $S\underline{C}H_2Ar$, $\underline{C}H_2S$, and $\underline{C}H_2CO$), 52.08 and 53.55 (2 x d, $NH\underline{C}HCH_2$ and $NH\underline{C}HCHS$), 54.97 (q, $ArO\underline{C}H_3$), 57.29 (br.m, $\underline{C}DCH$), 66.52 and 66.69 (2 x t, CH_2Ar), 77.73 (d, $\underline{C}HPh_2$), 113.70, 113.80, and 113.84 (3 x d, Ar), 126.72-139.45 (m, $\underline{Ar}$), 156.09, 158.57, 159.35 and 159.55 (4 x s, $\underline{C}OMe$ of ArOMe), 170.29, 170.46, 171.97, and 172.48 (4 x $\underline{C}$=O); $\upsilon_{max}(CHCl_3)$ 3020(s), 1725(s), 1670(m) and 1520(s); *m/z* (F.A.B.) 934 (7%), 935 (MH^+, 100), 936 (62), 937 (29), 938 (13).

[(5R)-5-Amino-5-carboxypentanoyl]-L-cysteinyl-D-valine (18a)

To the dry fully protected tripeptide (**17a**) (70 mg, 0.08 mmol) under argon was added anisole (0.4 ml) and freshly distilled trifluoroacetic acid (2.0 ml). Immediately upon addition of the acid, the stirred solution turned pink and the solution was heated under reflux for 30 minutes. Evaporation of this mixture gave a residue which was re-dissolved in dry toluene and once more evaporated to dryness. The solid material was partitioned between EtOAc (5 ml) and water (5 ml). After separation, the aqueous phase was washed with EtOAc (2 x 5 ml) and lyophilised to give the deprotected tripeptide (**18a**) as its ammonium trifluoroacetate salt (26 mg, 0.07 mmol, 90%). δ_H (500MHz, D_2O, $\underline{H}OD$ suppressed) 0.92-0.99 (6H, m, $CH(C\underline{H}_3)_2$), 1.65-2.02 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.16-2.24 (1H, m, $C\underline{H}(CH_3)_2$), 2.41 (2H, *ca* t, J 7Hz, CH_2CO), 2.88, 2.94 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.98 (1H, *ca* t, J 6Hz, $C\underline{H}CH_2S$), 4.28 (1H, d, J 6Hz, $C\underline{H}CHMe_2$), 4.57 (1H, *ca* t, J 7Hz, $CH_2C\underline{H}NH_3^+$); δ_C(125.77MHz, D_2O) 18.02 (q, $CH\underline{C}H_3$), 19.23 (q, $CH\underline{C}H_3$), 21.45 (t, $CH_2\underline{C}H_2CH_2CO$), 26.15 (t, $\underline{C}H_2CH_2CH_2CO$), 30.00 (t, $CH_2\underline{C}H_2CO$), 30.68 (d, $\underline{C}H(CH_3)_2$), 35.29 (t, CH_2SH), 53.74 (d, $\underline{C}HCH_2S$), 56.28 (d, $CHNH_3^+$), 59.18 (d, $\underline{C}HCO_2H$), 172.64, 172.95, 175.62 and 176.37 (4 x s, 4 x $\underline{C}$=O); *m/z* (FAB) 362 (6%), 363 (10), 364 (MH^+,100), 365 (23), 366 (10).

[(5R)-5-Amino-5-carboxypentanoyl]-L-cysteinyl-D-(2-^{2}H)valine (18b)

The above procedure was repeated with (**17b**) (96 mg, 0.10 mmol) to give (**18b**) as its ammonium trifluoroacetate salt (32 mg, 0.09 mmol, 90%). δ_H (500MHz, D_2O, $\underline{H}OD$ suppressed) 0.92-0.99 (6H, *ca* t, J 7Hz, $CH(C\underline{H}_3)_2$), 1.66-2.01 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.17-2.24 (1H, m, $C\underline{H}(CH_3)_2$), 2.42 (2H, *ca*. t, J 7Hz, CH_2CO), 2.98, 2.95 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.97 (1H, *ca*. t, J 6Hz, $C\underline{H}CH_2S$), 4.57 (1H, *ca*. t, J 7Hz, $CH_2C\underline{H}NH_3^+$); δ_C(125.77MHz, D_2O); 17.96 (q, $CH\underline{C}H_3$), 19.20 (q, $CH\underline{C}H_3$), 21.45 (t, $CH_2\underline{C}H_2CH_2CO$), 26.18 (t, $\underline{C}H_2CH_2CH_2CO$), 30.00 (t, $CH_2CH_2\underline{C}H_2CO$), 30.58 (d, $\underline{C}H(CH_3)_2$), 35.26 (t, CH_2SH), 53.78 (d, $\underline{C}HCH_2S$), 56.28 (d, $CHNH_3^+$), 58.91 (m, $\underline{C}DCO_2H$), 172.73, 173.06, 175.66 and 176.34 (4 x s, 4 x $\underline{C}$=O); *m/z* (F.A.B.) 363 (2%), 364 (5), 365 (MH^+,100), 366 (18), 367 (10), 368 (2).

Procedure for Isopenicillin N Synthase Incubations

Partially purified IPNS enzyme[24] (2-3 ml, *ca* 1.0 International Units) in TRIS-HCl buffer (50

mM ; pH 7.4) was exchanged into ammonium bicarbonate buffer (3.5 ml, 50 mM, pH 7.8) on a pre-equilibrated sephadex column (PD-10) in a cold-room at 4°C. To an aqueous solution of the tripeptide (720 ml, *ca* 10 mM) was added dithiothreitol (80 ml, 2 mM), L-ascorbate (80 ml, 1 mM, ferrous sulphate (80 ml, 0.1 mM) and catalase (40 ml). The pH was adjusted to 7.8 by the addition of 1N NaOH solution and the enzyme (3.5 ml) added giving a total volume of 4.5 ml. This solution was divided into two aliquots, each of *ca* 2.3 ml and incubated at 27°C and 250 rpm for 10 minutes after which time, more dithiothreitol (10 ml, 2 mM) was added to each aliquot. After a further 30 minutes, the incubation was quenched by the addition of acetone to 70% (v/v). The precipitated protein was spun down by centrifugation (15Krpm, 2 minutes, 0°C), the supernatant evaporated to dryness and the residue dissolved in D_2O (0.5 ml). After filtration (ACRO LC13 disposable filter assembly, Product No. 4450 Gelman Sciences), the incubation mixture was examined by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed).

N.M.R. Calibration of Penicillin N Samples

The sample to be calibrated was dissolved in D_2O (1.0 ml) containing 1,4-dioxan (0.10 $\mu l ml^{-1}$, 1.17 $\mu mol\ l^{-1}$). The ^{1}H-n.m.r. spectrum (500MHz, D_2O, HOD suppressed) was recorded over at least 40 transients and the resonances due to the dioxan, the CH_2CO of 5-amino-5-carboxypentanoic acid side chain and the β-lactam protons integrated. The concentration of the penicillin was then calculated from the equations :

Penicillin Concentration = { 1/2 x ∫CH_2CO / ∫Dioxan } x 8 x 1.17 $\mu mol\ l^{-1}$

= { 1/2 x ∫combined β-lactams / ∫ Dioxan } x 8 x 1.17 $\mu mol\ l^{-1}$

Procedure for DAOC/ DAC synthase Incubations.

Partially purified DAOC/ DAC synthase (2 ml, ca 0.1 International Units) in TRIS-HCl buffer (pH 7.4, 50 mM) was pre-incubated for 5 minutes at 27°C and 250 rpm with 200 µl of cofactor solution prepared from α-ketoglutarate (14.6 mg), L-ascorbate (17.6 mg), dithiothreitol (30.8 mg), iron (II) sulphate (1.4 mg) and ammonium sulphate (1.32 g) in distilled water (10 ml). The substrate (1 mg) in TRIS-HCl (1.8 ml, 50 mM, pH 7.4) was added and the pH adjusted to pH 7.4 by the addition of 1N NaOH solution. The resulting solution was incubated at 27°C and 250 rpm for 2 hours after which time the protein was precipitated by the addition of acetone to 70% (v/v). After centrifugation (15 Krpm, 2 minutes, 0°C) the supernatant was evaporated to dryness and the residue dissolved in D_2O (0.5 ml). After filtration (ACRO LC13 disposable filter assembly, Product No. 4450 Gelman Sciences) the crude incubation mixture was examined by ^{1}H-n.m.r. (500MHz, D_2O, HOD suppressed).

Penicillin N (3a)

D,L,D-ACV (**18a**) (4mg) was incubated with partially purified IPNS (*ca* 11 I.U.) according to the general procedure with the appropriate co-factors concentrations. The resulting mixture was purified by chromatography (h.p.l.c., Gilson system, solvent; 0.75% MeCN in 25mM aqueous NH_4HCO_3; flow rate 4.0 ml min^{-1}, retention time 8.0 min) to give pure penicillin N (**3a**) (*ca* 1mg); δ_H (500MHz, D_2O, HOD suppressed) 1.51 (3H, s, CH_3), 1.63 (3H, s, CH_3) 1.64-1.95 (4H, 2 br. m,

$CH_2CH_2CH_2CO$), 2.40 (2H, *ca* t, *J* 7Hz, CH_2CO), 3.77 (1H, ca t, *J* 6Hz, $CHCH_2CH_2$), 4.34 (1H, s, $CHCO_2H$), 5.47, 5.56 (2H, ABq, *J* 4.5Hz, CHCHS). Irradiation at δ_H 1.51 gave 6% n. O. e. to 5-H (δ_H 5.56) and 3.5% to 3-H (δ_H 4.34) whereas irradiation at δ_H 1.63 gave 13% n.O.e. to 3-H but no n.O.e. to 5-H. δ_C (125.77MHz, D_2O) 21.67 (t, $CH_2CH_2CH_2CO$), 27.22 (q, α-CH_3), 30.80 (t, $CHCH_2CH_2$), 31.11 (q, β-CH_3), 32.27 (t, CH_2CO), 55.28 , 57.43 (2 x d, CHCHS), 58.74 (d, $^+H_3NCHCO_2^-$), 73.91 (d, $CHCO_2H$), 161.05, 175.21, 175.58 and 176.63 (4 x s, C=O). Selective irradiation at ^1H-frequency of high field α-methyl group in the off resonance decoupled ^{13}C-n.m.r, with low decoupling power collapsed the 27.22 ppm quartet to a broad singlet.

[3-^{2}H]Penicillin N (3b)

This procedure was repeated with (**18b**) (10 mg) using partially purified IPNS (*ca* 25 I.U.) to give after h.p.l.c. isolation (Gilson system, solvent; 0.75% MeCN in 25mMolar aqueous NH_4HCO_3, retention time 8 min), (**3b**) (2.1 mg, 27%). δ_H (500MHz, D_2O, HOD suppressed) 1.52 (3H, s, CH_3), 1.63-1.96 (3H, s, CH_3 and 4H, 2 x m, $CH_2CH_2CH_2CO$), 2.40 (2H, *ca* t, *J* 7Hz, CH_2CO), 3.77 (1H, *ca* t, *J* 6Hz, $CHCH_2CH_2$), 5.47, 5.56 (2H, ABq, *J* 4.5Hz, CHCHS). *m/z* (NH_3 C.I.) of N-ethoxycarbonyl, dimethyl ester derivative[19] (**23b**) 461 (100, MH^+)., 462 (25), 463 (10).

Incubation of Penicillin N (3a) with DAOC/DAC Synthase Enzyme

Penicillin N (**3a**) (*ca*.1mg) was incubated with partially purified DAOC/DAC synthase according to the general procedure. Examination of the crude incubation mixture by ^1H-n.m.r (500MHz, D_2O, HOD suppressed) in the region δ 4.9-5.5 ppm indicated that three β-lactam compounds had been formed in addition to unconverted penicillin N (**3a**). These ring expanded products were identified as DAOC (**4**), DAC (**5**) and the 3β-hydroxycepham (**9a**) by comparison to authentic samples. Integration of this region indicated that the ratio of these products **4** : **5** : **9a**, was *ca* 40 : 20 : 1.

Incubation of [3-^{2}H]Penicillin N (3b) with DAOC/DAC Synthase Enzyme

[3-^{2}H]Penicillin N (**3b**) (*ca* 1mg) was incubated with partially purified DAOC/DAC synthase according to the general procedure. Examination of the crude incubation mixture by ^1H-n.m.r (500MHz, D_2O, HOD suppressed) in the region δ 4.9-5.5 ppm indicated that the products were DAOC (**4**), DAC (**5**) and the [4-^{2}H]-3β-hydroxycepham (**9b**) as judged by comparison with authentic samples. Integration of this region indicated that the ratio of these products **4a** : **5a** : **9b** was *ca*. 40: 25: 35. Purification [h.p.l.c. Waters' system, 25 mMolar aqueous NH_4HCO_3] gave **4a**, **5a**, **9b** and unconverted **3b**. Data for **9b**; δ_H (500MHz, D_2O, HOD suppressed) 1.39 (3H, s, CH_3), 1.63-1.94 (4H, m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca* t, *J* 7Hz, CH_2CO), 2.66, 3.56 (2H, ABq, *J* 14Hz, SCH_2), 3.75 (1H, m, $CHCH_2CH_2$), 5.29, 5.46 (2H, ABq, *J* 4Hz, CHCHS); *m/z* (F.A.B.) 377 (MH^+); Identical to (**9a**) except for the presence of the deuterium atom at C3.

Incubation of [3-^{2}H]Penicillin N (3b) with DAOC/DAC Synthase Enzyme Under an Atmosphere of $^{18}O_2$ Gas

A solution of 50 mM TRIS-HCl, pH7.4 (12ml) in a 2-necked 100ml round bottom flask was thoroughly degassed by evacuation, the vessel returned to atmospheric pressure with argon, and the procedure repeated a further three times. A sample of $^{18}O_2$ gas (99.8 % atom ^{18}O, 40ml) was

transferred to the flask by means of a gas tight syringe and introduced into the TRIS-HCl solution through a rubber septum. The solution was vigorously stirred by means of an efficient magnetic stirrer for 3 hours. DAOC/DAC synthase enzyme (*ca.*1 I.U.) in TRIS-HCl (4ml) was injected into the flask and stirred for 5 minutes. Cofactors [160 μl containing α-ketoglutarate (14.6 mg), L-ascorbate (17.6 mg), $FeSO_4$ (1.4 mg) and dithiothreitol (30.8 mg) prepared in a sample of the oxygenated TRIS-HCl solution (2ml) previously removed from the flask with a syringe] were added and the flask briefly degassed and returned to atmospheric pressure with argon. $^{18}O_2$ gas (40ml) was injected into the enzyme solution which was then stirred for 2 minutes after which time [3-^{2}H]penicillin N (**3b**) (*ca* 3mg) in degassed TRIS-HCl (1ml) was added *via* a syringe. The resulting solution was then shaken at 250 rpm, 27°C for 2 hours, the protein was then precipitated by the injection of acetone to 70% (v/v) and the incubation worked up as in the general procedure. Purification as before gave the β-lactam compounds deacetoxycephalosporin C (**4**), deacetylcephalosporin C (**5**), [4-^{2}H]-3β-hydroxycepham (**9b**) and unconverted [3-^{2}H]penicillin N (**3b**). Mass spectral analysis upon derivatised deacetoxycephalosporin C (**4**) [as (**25**) prepared by treatment of an aqueous solution of (**4**) at pH 8 with excess diethyl pyrocarbonate to give a N-ethoxycarbonyl compound which was acidified and extracted into ethyl acetate and then methylated with diazomethane][19] and [3-^{2}H]penicillin N (**3b**) [as (**23b**) prepared in the same manner as (**25**)] indicated no incorporation of ^{18}O. Analysis of [4-^{2}H]-3β-hydroxycepham (**9b**) and of deacetylcephalosporin C (**5**) as its lactone **20** [prepared by stirring (**5**) in freshly distilled formic acid[10] followed by chromatographic purification [h.p.l.c Gilson system] indicated *ca.* 50% incorporation of label had occurred (Tables 3 and 4).

Table. 3; [4-^{2}H]-3β-hydroxycepham (**9b**)

Expt.	*m/z* (MH$^+$)	*376*	*377*	*378*	*379*	*380*	*381*
1	Found (%)	29	80	53	100	38	8
2	Found (%)	-	69	19	100	23	15
	Calculated (%)	-	100	18	7	1	-
Synthetic (**9a**)	*m/z* (MH$^+$)	*375*	*376*	*377*	*378*	*379*	*380*
	Found (%)	7	100	22	10	1	-
	Calculated (%)	-	100	18	7	1	-

Table. 4; Deacetylcephalosporin C lactone (**20**)

Expt.	*m/z* (MH$^+$)	*355*	*356*	*357*	*358*	*359*	*360*
1	Found (%)	4	100	25	72	27	22
	Calculated (%)	-	100	18	7	1	-
	m/z (Fragment **21**)	*155*	*156*	*157*	*158*	*159*	*160*
1	Found (%)	4	100	14	70	10	6
2	Found (%)	4	100	13	54	7	6
	Calculated (%)	-	100	8	5	-	-

Table.5; [3-^{2}H]Penicillin N derivative (**23b**)

Expt.	*m/z* (Fragment **24b**)	*174*	*175*	*176*	*177*	*178*	*179*
1	Found (%)	20	100	18	15	12	8
	Calculated (%)	-	100	9	5	-	-

Table.6; Deacetoxycephalosporin C (**4**)

Expt.	*m/z* (MH^+)	*357*	*358*	*359*	*360*	*361*	*362*
1	Found (%)	3	100	26	52	14	10
	Calculated (%)	-	100	18	7	1	-
2	*m/z* deriv. **25**(MH^+)	*457*	*458*	*459*	*460*	*461*	*462*
	Found (%)	6	100	34	9	7	5
	Calculated (%)	-	100	24	9	2	-
	m/z (Fragment **22**)	*171*	*172*	*173*	*174*	*175*	*176*
	Found (%)	1	100	10	10	16	3
	Calculated (%)	-	100	9	5	-	-

Footnote. Calculated values (%) based upon natural isotopic distributions.

(2R,3S,6R,7R)-1-Aza-3-methyl-3-hydroxy-7-[(5R)-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylate (9a)

Thiourea (16 mg, 0.21 mmol) and **19a**[17] (100 mg, 0.12 mmol) were dissolved in freshly distilled ethanol (2 ml) and the resulting solution stirred at 60°C under argon for 1 hour. The solvent was evaporated and the residue re-suspended in EtOAc (50 ml). The resulting organic layer was washed with water (3 x 20 ml), brine (20 ml), dried (anhydrous Na_2SO_4), and filtered. The solvent was evaporated in *vacuo* to give the crude alcohol **19b**. Hydrogenation of the crude alcohol in THF/water [1:1, v/v (20 ml)] with 10% Pd/C (*ca* 100mg) under an atmosphere of H_2 for 5 hours, followed by filtration through celite, washing with EtOAc (3 x 5 ml) and freeze-drying gave the deprotected 3β-hydroxycepham (**9a**). Chromatography [h.p.l.c., Gilson system, flow rate 4 ml min^{-1}, with 0.75% MeCN in 5mM aqueous NH_4HCO_3] gave 3β-hydroxycepham (**9a**) (13mg). δ_H (500MHz, D_2O, HOD suppressed) 1.39 (3H, s, CH_3), 1.63-1.94 (4H, m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca* t, *J* 7Hz, CH_2CO), 2.66, 3.56 (2H, ABq, *J* 14Hz, SCH_2), 3.75 (1H, m, $CHCH_2CH_2$), 4.16 (1H, s, $CHCO_2H$), 5.29, 5.45 (2H, ABq, *J* 4Hz, CHCHS); δ_C (125.77MHz, D_2O); 21.67 (t, $CH_2CH_2CH_2CO$), 25.84 (q, CH_3), 30.57 (t, $CHCH_2CH_2$), 34.89 (t, SCH_2), 35.63 (t, CH_2CO), 55.08 , 55.27 (2 x d, CHCHS), 59.39 (d, $CHCH_2CH_2$), 64.42 (d, $CHCO_2H$), 65.57 (s, COH), 167.79, 174.97, 172.09 and 177.30 (C=O) Individual selective irradiations at the ^{1}H-frequency of the high-field and low-field doublets of SCH_2 ABq in the off resonance decoupled ^{13}C-n.m.r, with low decoupling power collapsed the 34.89 ppm triplet to a doublet.; *m/z* (FAB) 376 (MH^+, 100%). The sample had identical ^{1}H-n.m.r. and mass spectroscopy to previously reported data[11]. Pure 3β-hydroxycepham (**9a**) was assayed against *Escherichia coli*. ESS(+) and *Staphylococcus aureus* at a concentration of (100μg ml^{-1}) and no anti-bacterial activity was observed.

Incubation of 7β-[(5R)-5-Amino-5-carboxypentanamido]-3β-hydroxy-3α-methyl[4-^{2}H]cepham-4-carboxylic Acid (9b) with Partially Purified DAOC/DAC Synthase Enzyme

Compound **9b** (ca 60μg) was incubated with partially purified DAOC/DAC synthase enzyme using the general incubation procedure. The incubation mixture was bio-assayed against *E.coli*. ESS(+) and *S. aureus* at a concentration of 100μg ml^{-1}. No anti-bacterial activity was observed.

[(2R,3S)-(2'-^{13}C,3-^{2}H)]Penicillin N, (2S,3R,5R,6R)-1-Aza-(2-^{2}H)-3-methyl-3-(^{13}C-methyl)-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo [3.2.0]heptane-2-carboxylate (3e)

δ-D-α-aminoadipoyl-L-cysteinyl-D-(3S)-(2-^{2}H,4-^{13}C)valine (**18e**)[16,21] (*ca* 1.5mg) was incubated with partially purified IPNS enzyme according to the usual procedure. The incubation mixture was subjected to the usual work-up and the total crude sample examined by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) in the region δ5.0-5.5ppm (ABq, *J* 4.5Hz, β-lactam protons) . Partial δ_C (125.77MHz, D_2O); 27.22 ([2R] -^{13}CH$_3$, signal : noise ratio, *ca.* 43 : 1), no signal at 31.11 ([2S] -CH$_3$).

Incubation of [(2R,3S)-(2'-^{13}C,3-^{2}H)]Penicillin N (3e) with DAOC/DAC Synthase

Crude [(2R,3S)-(2'-^{13}C,3-^{2}H)]penicillin N (**3e**) (*ca* 1-1.5mg) was incubated with partially purified DAOC /DAC synthase in the presence of the usual cofactors. After work-up, examination by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed) in the region δ 4.9-5.5 ppm indicated good conversion to the products **4e**, **5e** and **9e**. The total crude incubation mixture was then examined by ^{13}C-n.m.r (125.77MHz, D_2O, *ca* 40-50 thousand transients) and the unconverted [(2R,3S)-(2'-^{13}C,3-^{2}H)]penicillin N (**3e**) and ring expanded products isolated by chromatography (reverse phase h.p.l.c., Waters system eluting with 25mM aqueous NH_4HCO_3). After examination by ^{1}H-n.m.r (500MHz, D_2O, HOD suppressed), the individual β-lactam compounds were all examined by ^{13}C-n.m.r (125.77MHz, D_2O, *ca* 40-50 thousand transients). No signals corresponding to incorporation of ^{13}C-label into the endocyclic C-2 position of the cephalosporin products were observed (see Table 2.).

3'-^{13}C-DAOC (**4e**): (from incubation of [(2R,3S)-(2'-^{13}C,3-^{2}H)]penicillin N (**3e**) with DAOC/DAC synthase) partial δ_C (125.77MHz, D_2O) 19.12 (^{13}CH$_3$, signal : noise ratio, *ca.* 9 : 1), no signal at 29.17 (SCH$_2$).

DAOC (**4a**): (from incubation of penicillin N (**3a**) with DAOC/DAC synthase) δ_H(500MHz, D_2O, HOD suppressed) 1.67-2.03 (4H, 2 x m, CH$_2$CH$_2$CH$_2$CO , and 3H, s, CH$_3$), 2.44 (2H, *ca*.t, CH$_2$CO), 3.38 and 3.61 (2 x 1H, 2 x d, *J* 18Hz, SCH$_2$), 4.02 (1H, *ca*.t, CHNH$^+$$_3$), 5.14 and 5.58 (2H, ABq, *J* 4Hz, CHCHS); δ_C(125.77MHz, D_2O) 19.22 (q, CH$_3$), 21.70 (t, CH$_2$CH$_2$CH$_2$CO), 29.17 (t, SCH$_2$), 30.61 (t, CHCH$_2$CH$_2$), 35.40 (t, CH$_2$CO), 55.23 , 57.53 (2 x d, CHCHS), 59.44 (d, $^+$H$_3$NCHCO$_2^-$), 122.90 and 127.39 (2 x s, C=CMe), 165.16, 170.66, 175.07 and 177.29 (4 x s, C=O). Selective irradiation at ^{1}H-frequency of the high-field doublet of SCH$_2$ methylene ABq in the off resonance decoupled ^{13}C-n.m.r, with low decoupling power collapsed the 29.17 ppm triplet to a doublet.

3'-^{13}C-DAC (**5e**): (from incubation of [(2R,3S)-(2'-^{13}C,3-^{2}H)]penicillin N (**3e**) with DAOC/DAC synthase) partial δ_C (125.77MHz, D_2O) 61.74 (^{13}CH$_2$OH, signal : noise ratio, *ca.* 30 : 1), no signal at 26.15 ppm (SCH$_2$).

DAC (**5a**): (from incubation of deacetoxycephalosporin C (**4a**) with DAOC/DAC synthase) δ_H (250MHz, D_2O, HOD suppressed) 1.64-1.94 (4H, 2 x m, CH$_2$CH$_2$CH$_2$CO), 2.44 (2H, *ca* t, *J* 7Hz, CH$_2$CO), 3.42 and 3.60 (2H, ABq, *J* 17Hz, SCH$_2$), 3.70 (1H, *ca* t, *J* 6Hz, CHNH$_3^+$), 4.79, (2H, s, CH$_2$OH), 5.07 and 5.58 (2H, ABq, *J* 4Hz, CHCHS); δ_C(125.77MHz, D_2O) 21.67 (t, CH$_2$CH$_2$CH$_2$CO), 26.15 (t, SCH$_2$), 30.60 (t, CHCH$_2$CH$_2$), 35.38 (t, CH$_2$CO), 55.21 , 57.97 (2 x d, CHCHS), 59.66 (d, $^+$H$_3$NCHCO$_2^-$), 61.71 (t, CH$_2$OH), 122.01 and 130.20 (2 x s, C=CMe), 165.66, 169.72, 175.11 and 172.27 (4 x s,

C=O). Selective irradiation at ^{1}H-frequency of the high-field doublet of SCH_2 ABq in the off resonance decoupled ^{13}C-n.m.r, with low decoupling power collapsed the 26.15 ppm triplet to a doublet.

(3'-^{13}C)-3β-Hydroxycepham (9e): (from incubation of [(2R,3S)-(2'-^{13}C,3-^{2}H)]penicillin N (3e) with DAOC/DAC synthase) δ_H (500MHz, D_2O, HOD suppressed, referenced to HOD δ4.64 ppm) 1.39 (3H, s plus d (90%), J ^{13}C-^{1}H 125Hz, $^{13}CH_3$), 1.63-1.94 (4H, m, $CH_2CH_2CH_2CO$), 2.42 (2H, *ca* t, J 7Hz, CH_2CO), 2.66, 3.56 (2H, ABq, J 14Hz, SCH_2), 3.75 (1H, m, $CHCH_2CH_2$), 5.29, 5.45 (2H, ABq, J 4Hz, CHCHS); partial δ_C(125.77MHz, D_2O) 25.79 ($^{13}CH_3$, signal : noise ratio, *ca* 30 : 1) no signal at 34.89 (SCH_2).

Recovered [(2R,3S)-(2'-^{13}C,3-^{2}H)]penicillin N (3e): (from incubation of [(2R,3S)-(2'-^{13}C,3-^{2}H)]penicillin N (3e) with DAOC/DAC synthase) Partial δ_C (125.77MHz, D_2O) 27.25 ([2R] -$^{13}CH_3$, signal : noise ratio, *ca* 8 : 1), no signal at 31.11 (β-CH_3).

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