Cephalosporin C Biosynthesis; Stereochemistry of the Incorporation of D,L,D-α-Aminodipoyl-cysteinyl-(3*S*)-[2-^{2}H,4-^{13}C]valine into β-Lactam Compounds

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Incubation of D,L,D-α-aminoadipoyl-cysteinyl-(3*S*)-[2-^{2}H,4-^{13}C]valine (**8b**) with partially purified isopenicillin N synthetase from *Cephalosporium acremonium* CO 728 gave stereospecifically labelled (2*R*,3*S*)-[2′-^{13}C, 3-^{2}H]penicillin N (**9**): further incubation of the product with partially purified deacetoxycephalosporin C/deacetylcephalosporin C synthetase from *C. acremonium* CO 728 gave ring expanded products in which the ^{13}C-label was incorporated exclusively into the C3′-exocyclic positions.

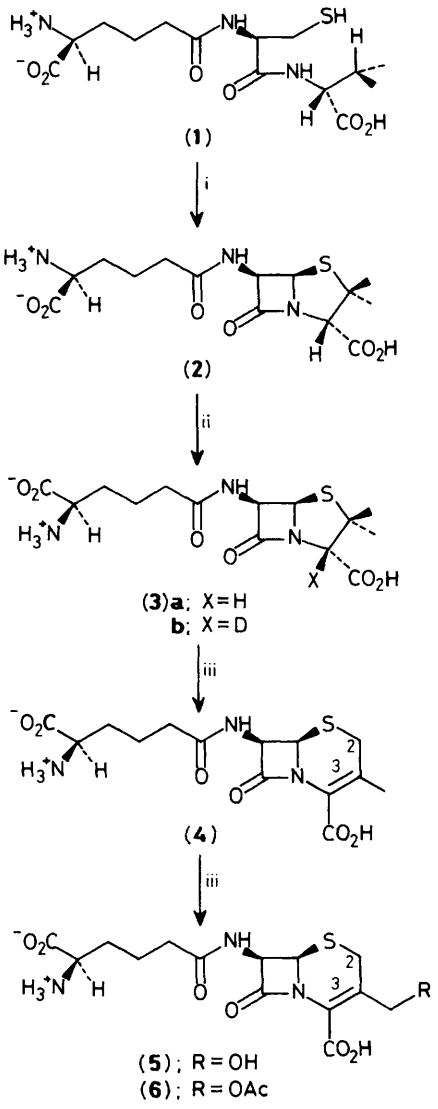
Within the β-lactam biosynthetic pathway, isopenicillin N (**2**) is formed from the L-amino acids L-α-aminoadipic acid, L-cysteine, and L-valine *via* the so-called Arnstein tripeptide L,L,D-α-aminoadipoyl-cysteinyl-valine [L,L,D-ACV] (**1**) (Scheme 1). Inversion of configuration of the L-α-aminoadipoyl side chain of isopenicillin N (**2**) then gives penicillin N (**3a**) which, in *Cephalosporium* and *Streptomyces Sp.*, undergoes ring expansion to deacetoxycephalosporin C [DAOC] (**4**) and subsequent hydroxylation to deacetylcephalosporin C [DAC] (**5**), the immediate biosynthetic precursor to cephalosporin C (**6**).[1]

The ring expansion process, (**3**) to (**4**), involves the abstraction of two hydrogen atoms and is catalysed by a bifunctional enzyme deacetoxycephalosporin C/deacetylcephalosporin C synthetase (DAOC/DAC synthetase).[2,3] Recent results have indicated that the first hydrogen abstraction occurs at the β-methyl group[4] giving rise to an intermediate possessing radical character.[5] Deuteriation at C-3 of penicillin N (**3b**) promotes bifurcation of the natural pathway to give enhanced levels of a 3β-hydroxycepham shunt metabolite (**7b**).[6]

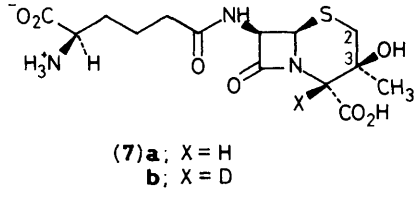
The fate of the valinyl isopropyl group during cephalosporin C biosynthesis has been the subject of several intact cell experiments.[7,8] These investigations utilised either stereospecifically labelled (2*RS*, 3*R*)-[4-^{13}C]valine[7] or (2*S*, 3*S*)-[4-^{13}C]-valine[8] and concentrated on the stereochemistry of incorporation of the diastereotopic methyl groups of valine during the thiazolidine ring closure of isopenicillin N (**2**) and subsequent ring expansion of penicillin N (**3**) to the dihydrothiazine system of the cephalosporins. 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 (**3a**) became the exocyclic C-3′ acetoxymethyl and endocyclic C-2 methylene of cephalosporin C (**6**) respectively. Recently Baldwin[9] has demonstrated that the direct conversion of ACV (**1**) into isopenicillin N (**2**) by the enzyme isopenicillin N synthetase (IPNS) proceeds with complete retention of the valinyl C-3 stereochemistry.

The availability of partially purified IPNS and DAOC/DAC synthetase, both from *Cephalosporium acremonium* CO 728, and our observation of a bifurcated pathway during the ring expansion of penicillin N (**3a**) to deacetoxycephalosporin C (**4**)[6] has prompted us to re-examine† the stereospecificity of

† Previous intact cell experiments suffered from a lack of sensitivity owing to low levels of labelled valine incorporation, low recovery of product from the fermentation broth, and because ^{13}C n.m.r. spectroscopic analysis was limited to low field spectrometers.

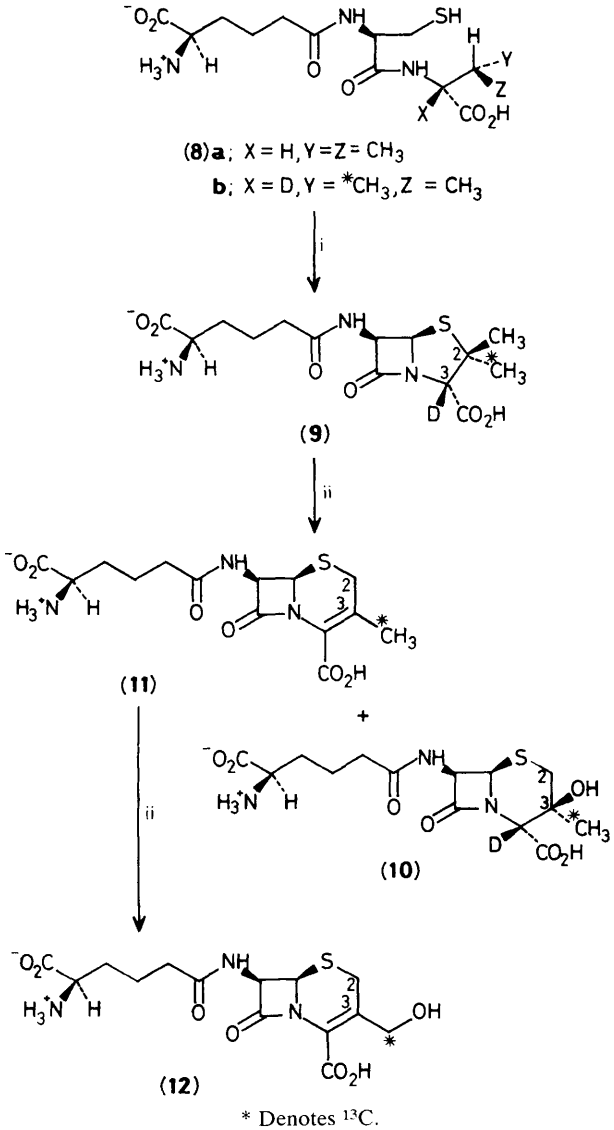


Scheme 1. *Reagents*: i, IPNS, O_2, Fe^{2+}, L-ascorbate, dithiothreitol; ii, epimerase; iii, DAOC/DAC synthetase, α-ketoglutarate, O_2, Fe^{2+}, L-ascorbate, D, L-dithiothreitol.



valinyl methyl group incorporation into the products penam (**3**), cephems (**4,5**), and 3β-hydroxycepham (**7**).

Studies on the substrate specificity of IPNS have shown that this enzyme is capable of converting the unnatural substrate D,L,D-ACV (**8a**) directly into penicillin N (**3a**), thereby providing easy access to an otherwise synthetically difficult target.[10] We now report that the thiazolidine ring closure in the conversion of the unnatural substrate, D,L,D-ACV (**8b**) to penicillin N (**9**) and the subsequent ring expansion by DAOC/DAC synthetase to the shunt metabolite 3β-hydroxycepham (**10**) and the usual products, deacetoxycephalosporin C (**11**) and deacetylcephalosporin C (**12**), are highly stereospecific processes.



* Denotes ^{13}C.

Scheme 2. Reagents: i, IPNS, O_2, Fe^{2+}, L-ascorbate, dithiothreitol; ii, DAOC/DAC synthetase, α-ketoglutarate, O_2, Fe^{2+}, L-ascorbate, D, L-dithiothreitol.

Thus reaction of D-α-aminoadipoyl-L-cysteinyl-(2*R*,3*S*)-[2-^{2}H,4-^{13}C]valine (**8b**)‡ with partially purified IPNS from *C. acremonium* CO 728[1] gave stereospecifically labelled (2*R*,3*S*)-[2′-^{13}C, 3-^{2}H]penicillin N (**9**)§ as shown by ^{13}C n.m.r. spectroscopy of the crude incubation mixture. Subsequent treatment with DAOC/DAC synthetase under the usual incubation conditions then gave, in addition to the expected cephems (**11**) and (**12**), the shunt product 3β-hydroxycepham (**10**) (Scheme 2). After isolation and purification (h.p.l.c., reverse phase octadecylsilane, 25 mM NH_4HCO_3 buffer) each of the products and unconverted penicillin N (**9**) were examined by ^{13}C n.m.r. spectroscopy (broad band decoupled) with accumulation of approximately 40 000 transients.

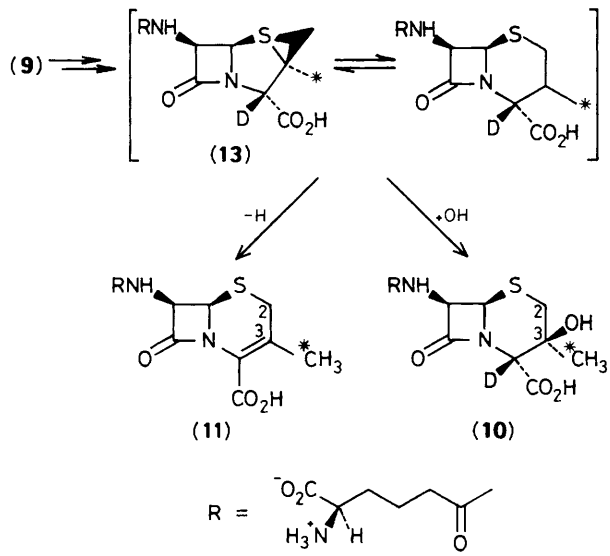
‡ Synthesised from (2*S*,3*S*)-[4-^{13}C]valine (*via* azalactone formation in deuteriated solvent) by methods analogous to those described previously, see ref. 9.

§ This result revealed that the cyclisation of D,L,D-ACV (**8b**) to penicillin N (**9**) by IPNS occurred with the same stereospecificity as the natural substrate.[9]

Table 1.

	^{13}C N.m.r. chemical shift[a]		Signal/noise intensity of enhanced signal
	Low field methyl	High field methyl	
D,L,D-ACV (**8a**)	19.22(q)	17.97(q)	
D,L,D-ACV (**8b**)	19.17[b]	Absent	>50 : 1
	2α-Me	2β-Me	
Penicillin N (**3a**)	27.22(q)[c]	31.11(q)	
2α-$^{13}CH_3$-Penicillin N (**9**)	27.22[b]	Absent	>40 : 1
	C-3-Me	C-2	
DAOC (**4**)	19.22(q)	29.17(t)[c]	
C-3-$^{13}CH_3$-DAOC (**11**)	19.12[b]	Absent	>9 : 1[e]
	C-3-CH_2OH	C-2	
DAC (**5**)	61.71(t)	26.15(t)[c]	
C-3-$^{13}CH_2OH$-DAC (**12**)	61.74[b]	Absent	>30 : 1
	3α-Me	C-2	
3β-OH Cepham (**7a**)[d]	25.84(q)	34.89(t)[c]	
3α-$^{13}CH_3$-3β-OH-Cepham (**10**)	25.79[b]	Absent	>30 : 1
	2α-Me	2β-Me	
Recovered penicillin N (**9**)	27.25[b]	Absent	≥8 : 1

[a] ^{13}C N.m.r. spectra were recorded at 125.77 MHz in D_2O and referenced to internal dioxan = 67.30 p.p.m. [b] Enhanced signal. [c] Assigned by selective ^{13}C-{^{1}H} irradiation experiments and ^{1}H nuclear Overhauser enhancement (n.O.e.) experiments performed upon authentic standards. [d] Prepared by analogous methods to those employed of Spry *et al.*[11] [e] A higher stereospecificity for (**11**) is implied from the ^{13}C n.m.r. spectrum of (**12**).



Scheme 3. * Denotes ^{13}C.

For each product a resonance was observed due to incorporation of the label exclusively into the C-3′-exocyclic position of the cephems (**11**) and (**12**) and the cepham (**10**). There was no signal corresponding to the C-2-methylene carbons (Table 1). The high degree of sensitivity obtained (*i.e.* high signal to noise ratio) for the ^{13}C n.m.r. spectra and prior determination of the chemical shifts of the C-2-methylene and C-3′-methyl and methylene resonances for (**10**), (**11**), and (**12**) (Table 1) enabled us to quantify the stereospecificity of the ring expansion process to greater than 95% stereospecific for each product (**10**—**12**). In addition unconverted penicillin N (**9**) was similarly analysed and, as anticipated, no evidence was obtained to suggest that scrambling of the labelled methyl group had occurred.

The stereochemical evidence presented herein is in accord with the suggestion[6] that the hydroxylated cepham (**10**) is a shunt metabolite arising from a common intermediate (**13**), either the cation or the radical, which in the majority of enzymatic cycles leads to the production of deacetoxycephalosporin C (**11**) (Scheme 3).

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