Stereochemistry of the incorporation of valine methyl groups into methylene groups in cephalosporin C

Chi-Pui PANG,* Robert L. WHITE,* Edward P. ABRAHAM,* David H. G. CROUT,† Max LUTSTORF,† Phillip J. MORGAN† and Andrew E. DEROME‡

*Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K.,

†Department of Chemistry, University of Exeter, Stocker Road, Exeter EX4 4QD, U.K., and ‡The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.

(Received 27 March 1984/Accepted 27 April 1984)

'Chiral methyl valines', i.e. samples of valine labelled stereospecifically in the methyl groups with ^2H and ^3H , were incorporated into cephalosporin C by a suspension of washed cells of *Cephalosporium acremonium*. Analysis by ^3H n.m.r. of the cephalosporin C produced showed that the conversion of the 3-*pro-S*-methyl group of valine into the acetoxymethyl side-chain was a highly stereospecific process. By contrast, conversion of the 3-*pro-R*-methyl group into the endocyclic methylene group of the dihydrothiazine ring was shown to proceed by a non-stereospecific process.

The biosynthesis of cephalosporin C (5) from L- α -aminoadipic acid, L-cysteine and L-valine and acetate in *Cephalosporium acremonium* has been shown to occur via the tripeptide (L- α -amino- δ -adipyl)-L-cysteinyl-D-valine (2), which is cyclized to isopenicillin N (3, R = L- α -amino- δ -adipyl) (Fawcett *et al.*, 1976; O'Sullivan *et al.*, 1979; Konomi *et al.*, 1979; Baldwin *et al.*, 1980a). Isopenicillin N is epimerized to penicillin N (3, R = D- α -amino- δ -adipyl) (Jayatilake *et al.*, 1981). The latter undergoes ring expansion to yield

deacetoxycephalosporin C (4) (Baldwin *et al.*, 1980b), which is the precursor of deacetylcephalosporin C and of cephalosporin C itself.

Experiments with (3*S*)- and (3*R*)-[4- ^{13}C]valine (1) have shown that closure of the thiazolidine ring in the biosynthesis of penicillin is stereospecific and occurs with overall retention of configuration (Kluender *et al.*, 1973; Neuss *et al.*, 1973; Aberhart & Lin, 1974). They have also shown that the ring expansion of the dihydrothiazine ring of cephalosporin C is stereospecific, the 3-*pro-S*-methyl

group of valine (* in 1) becoming the exocyclic methylene group at C-3' and the 3-*pro-R*-methyl group (▲ in 1) becoming the endocyclic methylene group. Studies with methyl-deuterated valines have demonstrated that all six valine ^2H atoms are retained during the biosynthesis of penicillin and have indicated that two ^2H atoms are retained in the endocyclic methylene group and two in the exocyclic methylene group of cephalosporin C during formation of the latter from penicillin N (Kluender *et al.*, 1974; Aberhart *et al.*, 1974; Baldwin *et al.*, 1981).

Crout *et al.* (1981, 1983) described the synthesis of 'chiral methyl valines' that are labelled stereospecifically with ^3H and ^2H in both diastereotopic methyl groups and in which the configurations of the methyl groups are linked to the configuration of the prochiral isopropyl methine centre. They suggested that the 'chiral methyl valines' could be used in biosynthetic experiments. The biosynthesis of cephalosporin C offered an opportunity to use such valines to throw light on the stereochemistry of the incorporation of valine methyl groups into methylene groups, since the endocyclic (C-2) and exocyclic (C-3') methylene groups of cephalosporin C are derived specifically from the 3-*pro-R*- and 3-*pro-S*-methyl groups of valine respectively and since it was expected that the distribution of ^3H between the diastereotopic positions at C-2 and C-3' in cephalosporin C (5) could be determined by ^3H n.m.r. The use of the 'chiral methyl valines' would thus permit the direct and simultaneous observation of the stereochemical events occurring during the conversion of the methyl groups of valine into the corresponding methylene groups in cephalosporin C.

This paper describes the results obtained from four experiments with two pairs of 'chiral methyl valines' having 3-*pro-S*- and 3-*pro-R*-methyl groups in one case with *R* and *S* configurations respectively (7), and in the other with *S* and *R* configurations respectively (9). A brief account of preliminary experiments with such valines has been published (Abraham *et al.*, 1983).

Experimental

Materials

Cephalosporin C (potassium salt) was provided by Glaxo Group Research, Greenford, Middx., U.K., and was recrystallized from aqueous acetone. Amberlite XE-58 was from Rohm and Haas Corp., Philadelphia, PA, U.S.A. Dowex 50W-X8 (H^+ form; 200–400 mesh; standard grade) was from BDH Chemicals, Poole, Dorset, U.K. Liquid Scintillator Unisolve I was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Other chemicals from BDH Chemicals were of AnalaR

grade. Samples of 'chiral methyl valine' (each about 5 mg) were prepared by the method of Crout *et al.* (1983). The samples used in four experiments were: (a) one containing equimolar amounts of (2*S*,3*S*,4*R*)- and (2*S*,3*R*,4*S*)-[4- ^2H , ^3H]valine (108 mCi) [all isopropyl groups labelled as in (7)] (Expt. A₁); (b) one containing equimolar amounts of (2*S*,3*S*,4*R*)-, (2*R*,3*S*,4*R*)-, (2*S*,3*R*,4*S*)- and (2*R*,3*R*,4*S*)-[4- ^2H , ^3H]valine (2.2 Ci) [all isopropyl groups labelled as in (7)] (Expt. A₂); (c) two containing equimolar amounts of (2*S*,3*S*,4*S*)-, (2*R*,3*S*,4*S*)-, (2*S*,3*R*,4*R*)- and (2*R*,3*R*,4*R*)-[4- ^2H , ^3H]valine, with radioactivities of 1.45 Ci (Expt. B₁) and 2.55 Ci (Expt. B₂) respectively [all isopropyl groups labelled as in (9)].

Approx. 60–85% of all molecules also contained a ^3H atom at C-3 and correspondingly 40–15% were labelled with ^3H at C-3 but not in the methyl groups. A ^3H -n.m.r. spectrum typical of the labelled valines produced has been published (Crout *et al.*, 1983). There is an obligatory loss of the label at C-3 during conversion into penicillin N.

The validity of the results obtained in the biosynthetic experiments depended on the stereochemical integrity of the 'chiral methyl valine'. This in turn depended on the stereospecificity of labelling of the deuterated isotopomers (6) and (8) (Scheme 1). Stereospecificity of the tritiation

Reagents; i, $^3\text{H}_2$, $[(\text{C}_6\text{H}_5)_3\text{P}]_3\text{RhCl}$; ii, AcOH , Ac_2O ; iii, H^+ , H_2O

* Mixture of 3*S*,4*R* and 3*R*,4*S* isomers.

† Mixture of 3*S*,4*S* and 3*R*,4*R* isomers.

Scheme 1

catalysed by Wilkinson's catalyst in the penultimate step of the synthesis was assumed. The partial 400 MHz spectra of the deuterated isodehydrovaline isotopomers are shown in Fig. 1. The spectra in Figs. 1(b) and 1(c) are of the precursors of the 'chiral methyl valines' used in Expts. A₂ and B₂ respectively (cf. Scheme 1). Signals attributable to the vinyl protons near δ 5 p.p.m. in the deuterated compounds are shifted upfield slightly relative to the signals in the undeuterated material. The spectrum of Fig. 1(b) reveals the presence of 12% undeuterated material and of approx. 5% of the *Z*-isomer. The spectrum of Fig. 1(c) reveals the presence of 12% unlabelled material. No signals attributable to the *E*-isomer are visible. The observed signal intensities in the spectra of biosynthesized cephalosporin C (Table 2) were corrected to take account of the presence of tritiated valines derived from these minor components.

General methods

Penicillin N and cephalosporin C were assayed by the hole-plate method (Smith *et al.*, 1967) with cephalosporin C (potassium salt) (10 units/mg) as the standard. The test organisms used were *Alcaligenes faecalis* A.T.C.C. 8750 (on plates containing β -lactamase I) for cephalosporin C activity and *Salmonella typhi* (strain 'Mrs S') for the combined activities of cephalosporin C and penicillin N. Cephalosporin C isolated after incorporation of tritium from 'chiral methyl valine' was also assayed by measurement of its absorbance at 260 nm (Abraham & Newton, 1961).

The radioactivity of compounds labelled with ³H was determined with a Packard Tri-Carb model 3375 liquid-scintillation spectrometer. Samples were dispersed in Unisolve I. ³H-labelled hexadecane (Amersham International) was used as the standard. Counting of the radioactivities of labelled compounds after paper electrophoresis was carried out as described by Smith *et al.* (1967).

³H-n.m.r. spectra were recorded with a Bruker WH-300 spectrometer at 320 MHz. ³H-n.m.r. spectra were 'ghost'-referenced from the p.m.r. spectrum of the sample. Chemical shifts are reported relative to tetramethylsilane.

Preparation of mycelium

Cephalosporium acremonium C91 was grown in a chemically defined medium in 500 ml baffled shake flasks at 27°C as described by Smith *et al.* (1967), except that the flasks were shaken at 160 rev./min. After 72 h the mycelium was harvested by filtration, washed with water and dried between filter paper. The damp-dry mycelium was divided into 8 g batches, which were stored at -20°C and used within 15 h. The culture filtrate

was assayed to check that penicillin N and cephalosporin C production had been normal.

Addition of 'chiral methyl valine' to resuspensions of mycelium

Each batch of mycelium was resuspended in 72 ml water in each of nine 500 ml baffled shake flasks. The resuspensions were incubated (27°C) at 200 rev./min on an orbital shaker enclosed in a thermostatically controlled box through which a steady stream of air was drawn by a water pump to convey any volatile ³H into a drain. A sample of 'chiral methyl valine' was diluted to 16 ml with water, and 0.25 ml of the resulting solution was added hourly to each of eight flasks during 8 h. The production of cephalosporin C and penicillin N was monitored by bioassays of samples removed at hourly intervals from the ninth flask, to which no valine had been added. After 10 h, the mycelium of the first eight flasks was filtered and washed with water. The filtrate and wash were combined, and the solution (about 700 ml) was concentrated on a rotary evaporator *in vacuo* to about 200 ml and then freeze-dried.

Isolation of cephalosporin C

Step 1. The product from the freeze-dried culture filtrate was extracted with 70% (v/v) ethanol (5 × 20 ml) at 0°C. Ethanol was removed *in vacuo* from the combined extracts, and the remaining aqueous solution was diluted to 100 ml with water and then freeze-dried. The subsequent procedure was based on that used by Trown *et al.* (1962).

Step 2. The product obtained after extraction with 70% (v/v) ethanol was dissolved in 8 ml of 0.2 M-ammonium acetate solution, pH 5.0, and the pH of the solution was lowered to 3.3 by the step-wise addition of Dowex 50W-X8 resin (H⁺ form). After removal of the resin by filtration, the filtrate was kept at 37°C for 3.5 h to convert penicillin N into its penillic acid (Newton & Abraham, 1956).

Step 3. The pH was then raised to 5.0 with aq. 4 M-NH₃ and the solution was applied to a column of Amberlite XE 58 resin (46 cm × 1.5 cm diam.) equilibrated with 0.2 M-ammonium acetate buffer, pH 5.0, at 4°C. Elution was carried out with the same buffer at a flow rate of 0.5 ml/min, and the absorbance of the eluate was monitored at 280 nm. Fractions (4 ml) were assayed for cephalosporin C and for radioactivity, and those containing cephalosporin C (usually 110–150) were combined and freeze-dried.

Step 4. The product from the Amberlite column was dissolved in water (2 ml) and applied to a column (2 cm × 1 cm diam.) of Dowex 50W-X8 resin (H⁺ form). After the column had been quickly washed with water (25 ml) under air pressure, the resin was suspended in water (5 ml),

Fig. 1. Partial ^1H -n.m.r. spectra (400 MHz) of (a) 2-acetamido-3-methylbut-3-enoic acid (*N*-acetylisodehydrovaline), (b) (*E*)-[4- ^2H]-2-acetamido-3-methylbut-3-enoic acid and (c) (*Z*)-[4- ^2H]-2-acetamido-3-methylbut-3-enoic acid. The signals due to the vinyl protons appear near $\delta = 4.95$ p.p.m. The upfield (○) and downfield (+) signals are attributable to the *E*- and *Z*-vinyl protons respectively. (N.B. The upfield signals are reproduced to a different scale from the signals due to the vinyl protons.)

and the pH was raised to 5.0 by addition of 1 M-NaOH and then to 6.5 with 0.1 M-NaOH with vigorous stirring. The resin was removed by filtration, and the filtrate was freeze-dried to yield

cephalosporin C (sodium salt). For n.m.r. determinations, this product was dissolved in water (0.1 ml) in a capillary tube, which was then sealed and placed in an n.m.r. tube containing 98% $^2\text{H}_2\text{O}$.

Results and discussion

In all four experiments with 'chiral methyl valines', two with a mixture of 3*S*,4*R*- and 3*R*,4*S*-valine (7) and two with a mixture of 3*R*,4*R*- and 3*S*,4*S*-valine (9), the purification of [^3H]cephalosporin C formed in suspensions of *C. acremonium* followed a course similar to that shown in Table 1. Two culture filtrates from Expts. A₂ and B₂ contained about twice as much cephalosporin C (53 and 44 μmol) as penicillin N (24 and 22 μmol). The u.v.-absorption spectra of the preparations of isolated cephalosporin C were virtually identical with that of authentic cephalosporin C (λ_{max} , 260nm), and estimates of the cephalosporin C content of these products by bioassay and by measurement of absorbance at 260nm agreed within 10%. After electrophoresis on paper at pH 4.5, one major spot with high radioactivity was located with ninhydrin in a position corresponding to cephalosporin C, 13cm towards the anode. A minor ninhydrin-positive spot, which showed less than 10% of the radioactivity of that due to cephalosporin C, was in a position corresponding to that of deacetylcephalosporin C lactone, 1.5cm towards the cathode (Loder *et al.*, 1961).

The efficiency of incorporation into penicillin N and cephalosporin C of ^3H from [$4\text{-}^2\text{H}, ^3\text{H}$]valines in these biosynthetic experiments was low, being 0.2–0.3% on the assumption that both the added L-valine and the L-valine formed in the mycelium from the added D-valine were precursors of the antibiotic (Warren *et al.*, 1967). Since the molar amount of [^3H]valine added to each mycelial suspension (about 43 μmol) was less than the combined amounts of penicillin N and cephalosporin C produced (66–77 μmol), a large dilution with endogenous valine occurred before biosynthesis of the tripeptide.

A consequence of the poor incorporation was that the 43 μmol samples of 'chiral methyl valine' available for these experiments were required to have high specific radioactivity for the isolation of cephalosporin C containing sufficient ^3H to give

^3H -n.m.r. spectra with a desirably high signal-to-noise ratio. In the first experiment (A₁) the radioactivity of the [$4\text{-}^2\text{H}, ^3\text{H}$]valine added to the mycelial suspension (108mCi) was substantially less than had been expected, and this was subsequently found to be due to the rapid self-radiolysis of the sample while stored in the dry state between synthesis and use. In the subsequent experiments (B₁, A₂ and B₂) samples of [^3H]valine (1.43, 2.2 and 2.55Ci respectively) were kept in solution before use and remained virtually unchanged for several days. Lower-than-expected radiochemical yields of [^3H]valine in several experiments were also found to be due to the loss of $^3\text{H}_2$ gas during storage by self-radiolysis and adsorption of radiolysis products on the walls of the glass vials in which the $^3\text{H}_2$ was delivered. Once these problems had been identified, subsequent syntheses were carried out immediately on delivery of the $^3\text{H}_2$ gas and the biosynthetic experiments were carried out within 2–5 days of the preparation. The radioactivities of the cephalosporin C isolated from Expts. A₁, B₁, A₂ and B₂ were 0.25, 1.2, 3.04 and 3.15mCi respectively.

The principle underlying the n.m.r. analysis is illustrated in Fig. 2. This depicts the conversion of a chiral methyl group into a methylene group bearing diastereotopic hydrogen atoms, by insertion of a substituent X with retention of configuration. The assignments of the n.m.r. signals from diastereotopic protons in a normal methylene group are applicable also to the corresponding ^3H signals (Elvidge *et al.*, 1978). Let us assume that the ^3H -n.m.r. spectra corresponding to the products obtained by insertion of X into C– ^2H and C–H bonds are as shown in Figs. 2(a) and 2(b) respectively. [In principle spin–spin coupling to ^2H ($I=1$) should split the ^3H signal due to C $^2\text{H}^3\text{H}$ species into three lines of equal intensity. In practice, the coupling may be too small to be resolved, or may be absent because of rapid quadrupolar relaxation of the ^2H .] These would combine to give the resultant spectrum shown in Fig. 2(c). In general, the substitution process would

Table 1. Isolation of [^3H]cephalosporin C from suspension of *C. acremonium*

The values for cephalosporin C were obtained by bioassay and refer to about 500ml of culture filtrates. For details of the valine samples A₂ (2.2Ci) and B₂ (2.55Ci) used in Expts. A₂ and B₂ respectively, see the text.

Stage	Material in	Expt. A ₂		Expt. B ₂	
		Cephalosporin C (mg)	Radioactivity (mCi)	Cephalosporin C (mg)	Radioactivity (mCi)
	Culture filtrate	25.5	1027	21	1293
1	70%-ethanol extract	19.8	180	20.3	122
2	Solution incubated at pH 3.3	17.6	134	14.3	93
3	Amberlite XE-58 eluate	12.3	5.1	11.4	6.5
4	Dowex 50 eluate	7.1	3.04	3.7	3.15

Fig. 2. Analysis of the stereochemistry of substitution into a chiral methyl group by ^3H n.m.r.

be expected to exhibit a deuterium kinetic isotope effect, so that the $\text{C}^2\text{H}^3\text{H}$ product species would be more abundant than the $\text{C}^1\text{H}^3\text{H}$ species. The signals in the resultant spectrum would then have relative intensities in the sense indicated in Fig. 2(c). Substitution with inversion of configuration would give the complementary pattern (downfield doublet, upfield triplet). A complementary set of signals would be obtained starting from a chiral methyl group with the opposite configuration.

The application of such an analysis to the present problem required that both the endocyclic (C-2) and exocyclic (C-3') methylene groups of cephalosporin C (5) should appear as adequately resolved AB quartets. The ^1H -n.m.r. spectrum of cephalosporin C (Fig. 3) shows that this condition is fulfilled.

Assignment of the signals due to the endocyclic methylene group of cephalosporin C (5) were made by a nuclear-Overhauser-effect-difference experiment in which H-6 in cephalosporin C was irradiated, with observation of the AB quartet due to the C-2 protons. The difference spectrum (Fig. 4) clearly showed a nuclear Overhauser effect between H-6 and the C-2 proton giving rise to the downfield component of the AB quartet. Although accurate values are not available for cephalosporin C itself, accurate interproton distances for three cephem derivatives are available (Sweet & Dahl, 1970; Declercq *et al.*, 1977; Domiano *et al.*, 1978). In each case the H-6-H-2 α distance is shorter than the H-6-H-2 β distance; this average difference is 0.092 nm (0.92 Å). Accordingly, the downfield component of the AB quartet can be assigned to H-2 α and the upfield component to H-2 β . The signals due to the exocyclic methylene group have not

been assigned and are referred to in the following discussion as H-3'u (upfield) and H-3'd (downfield) respectively. Preliminary Expts. A₁ and B₁ (Abraham *et al.*, 1983) gave results indicating high stereospecificity for the side-chain hydroxylation. The operation of a significant ^2H -isotope effect was indicated by the absence of signals exhibiting ^1H - ^3H coupling in the ^3H -n.m.r. spectrum of the cephalosporin C produced, showing that the major tritiated species were formed with loss of ^1H rather than ^2H . An estimate of the stereospecificity of the hydroxylation was made by assuming that minor signals of an intensity 20% or more of that of the major signal would be significant. The limiting cases considered were that a non-stereospecific element in the reaction might or might not be associated with a significant ^2H -isotope effect. The absence of a visible singlet corresponding to H-3'u for the former case, or of a visible doublet corresponding to H-3'd (with corresponding singlet-doublet combination due to H-3'u) for the latter case, indicated a lower limit for the stereospecificity of the hydroxylation of 70%.

The results with respect to the endocyclic methylene group were remarkably similar in both experiments and showed more nearly equal intensities for the signals attributable to ^3H -2 α and ^3H -2 β . However, because of the low signal-to-noise ratio in both spectra, the only conclusion that could be drawn was that, in both cases, the ratio of the signal due to ^3H -2 α to that due to ^3H -2 β appeared to lie between 1 and 2, and that, again, the absence of ^1H - ^3H coupling indicated the operation of a significant kinetic ^2H -isotope effect during sulphur migration.

Accordingly, further experiments were under-

Fig. 3. $^1\text{H-N.M.R.}$ spectrum of the sodium salt of cephalosporin C in $^2\text{H}_2\text{O}$ (400 MHz), showing the AB quartets due to the protons of the C-3' (+) and C-2 (o) methylene groups

Fig. 4. Nuclear-Overhauser-effect-difference spectrum of the signals due to the C-2 protons (+) of cephalosporin C (5) on irradiation of H-6

taken with 'chiral methyl valines' of higher ^3H content (Expts. A₂ and B₂). The ^3H -n.m.r. spectra of the samples of cephalosporin C obtained are shown in Figs. 5(a) and 5(b). The higher ^3H content of these samples of cephalosporin C permitted the acquisition of spectra of much improved signal-to-noise ratio without proton decoupling. The relative intensities of ^3H -3'd and ^3H -3'u are given in Table 2, and indicate a ratio of approx. 5:1 for the major and minor signals in each case. No indication can be seen in either case of a doublet flanking the minor signal, which could be attributed to the minor $\text{C}^1\text{H}^3\text{H}$ species corresponding to the $\text{C}^2\text{H}^3\text{H}$ species giving rise to the major signal. Taking into account the maximum intensity of the noise, it can be estimated that these signals would have been discernible if the intensities of the component doublets had exceeded 10% of that of the major signal in each case. It follows that

Fig. 5. Partial ^3H -n.m.r. spectra (320 MHz) of cephalosporin C (a) derived from a mixture of (2RS,3S,4R)- and (2RS,3R,4S)-[4- ^3H , ^2H]valine and (b) derived from a mixture of (2RS,3S,4S)- and (2RS,3R,4R)-[^3H , ^2H]valine

Table 2. ^3H -n.m.r. analysis of the distribution of ^3H between H-3'd and H-3'u and between H-2 α and H-2 β in cephalosporin C derived from 'chiral methyl valine'

Because relaxation times were expected to be closely similar for ^3H atoms in a given methylene group, intensities are compared rather than integrations. This makes it possible to estimate errors by comparing signal and noise intensities. In all cases, the relative signal strengths, based on intensity measurements, differed from those based on integrations by less than 2%. Relative intensities are corrected to take into account small contributions from minor isotopomers (see the Experimental section). The corrections to the intensity ratios due to ^3H in the endocyclic methylene group, C-2, were negligible. In Expt. A₂ the uncorrected ^3H -3' intensity ratio was 19:81 and in Expt. B₂ it was 83:17.

Expt.	Configuration of the precursor methyl group	Relative intensity of ^3H -n.m.r. signals	
		H-3'd	H-3'u
A ₂	R	10 $\pm$ 3	90 $\pm$ 3
B ₂	S	87 $\pm$ 3	13 $\pm$ 3
		H-2 α	H-2 β
A ₂	S	48 $\pm$ 3	52 $\pm$ 3
B ₂	R	57 $\pm$ 3	43 $\pm$ 3

associated with the C-3' hydroxylation there is a ^2H -isotope effect of at least 5.

Since the n.m.r. signals for the exocyclic methylene group have not been assigned, the foregoing results do not reveal whether the observed stereospecificity corresponds to predominant retention or inversion of configuration during hydroxylation. By analogy with similar hydroxylation reactions of known stereochemistry (see below), the former would appear to be the more likely. Further experiments will be required to clarify this point.

Table 2 indicates that with a precursor methyl group of the S-configuration (Expt. A₂) the ratio of intensities of the signals due to ^3H -2 α and ^3H -2 β is 48:52, and with a precursor methyl group of the R-configuration it is 57:43. From the estimated errors in these values it can be seen that the apparent complementarity of the signal intensities in the two experiments is not significant, and that the ratio of the intensities in each experiment is not essentially different from 50:50. As with the signals due to the exocyclic methylene group, and taking into account the signal-to-noise ratio, the absence of visible doublets attributable to ^1H - ^3H coupling indicates a ^2H -isotope effect of at least 3.

These results indicate that the sulphur migration step during ring expansion proceeds with a stereospecificity not significantly different from zero.

They also bear on possible mechanisms for this reaction. First, they do not favour a mechanism involving electrophilic substitution by electropositive sulphur into the C-2 β methyl group of penicillin N (3, R = D- α -amino- δ -adipyl) (Queener & Neuss, 1982). This would be expected to lead to retention of configuration, as in the analogous insertion of a carbocation into the C-6 methyl group during cycloartenol biosynthesis (Altman *et al.*, 1978). Secondly, they give no support to a mechanism in which hydroxylation of the methyl group is followed by activation (e.g. by phosphorylation), and substitution with sulphur migration followed by deprotonation (Queener & Neuss, 1982). By analogy with other hydroxylations (see below), the first step would be expected to proceed with retention of configuration. Formation of a free cation by departure of the leaving group would be extremely unlikely. It would be expected therefore that migration of the sulphur would be synchronized with departure of the leaving group and consequently proceed with inversion of configuration at the migration terminus (Lowry & Richardson, 1981). If this were so, the re-arrangement would occur with net inversion.

Stereochemically, the present findings resemble those with certain coenzyme B-12-dependent reactions for which radical intermediates have been proposed (Retey & Robinson, 1982). Thus migration of the thiolester group in the re-arrangement of ethylmalonyl-CoA to methylsuccinoyl-CoA occurs with only partial (75%) retention of configuration at the migration terminus (Retey *et al.*, 1978), and the low stereospecificity has been attributed to the possibility of rotation in the postulated radical intermediate (10). This interpretation is consistent with the observation that the analogous hydrogen migration in the ethanolamine ammonia-lyase reaction leads to 'racemic' product (Retey *et al.*, 1974). The barrier to rotation of a torsion-symmetric intermediate [e.g. (11)] in this reaction would be expected to be even lower than in the ethylmalonyl-CoA re-arrangement. A similar situation obtains in the conversion of 1,2-ethanediol into acetaldehyde catalysed by diol dehydratase (Arigoni, 1979).

The high stereospecificity of the side-chain hydroxylation may be compared with the stereospecific, or partly stereospecific, retention of

configuration observed in the hydroxylation of steroids (Bergstrom *et al.*, 1958; Corey *et al.*, 1958; Hayano *et al.*, 1958, 1959; Karlson & Hoffmeister, 1963; Roberts *et al.*, 1969; Galbraith *et al.*, 1970; Tomita & Sakurai, 1974), camphor (Gunsalus *et al.*, 1974), proline (Fujita *et al.*, 1964; Salzman *et al.*, 1965), 1-methyl-2-phenylethylamine (Taylor, 1974), 2-(3,4-dihydroxyphenyl)ethylamine (Battersby *et al.*, 1974), ethylbenzene (McMahon *et al.*, 1969), norbornane (Groves *et al.*, 1978), fatty acids (Jones, 1968; Heinz *et al.*, 1969) and, most pertinently, in the ω -hydroxylation of lauric acid (Gautier, 1980).

The operation, in a single biosynthetic pathway, of two methyl group oxidations, the one stereospecific and the other non-stereospecific, is notable, particularly since the ring-expansion enzyme (Kupka *et al.*, 1983) and the C-3'-hydroxylating enzyme (Stevens *et al.*, 1975; Turner *et al.*, 1978) resemble each other in being 2-oxoglutarate-dependent dioxygenases with a requirement for iron(II) and a reducing agent. One possible inference is that the two processes differ fundamentally in mechanism and that the former is under stereo-electronic control, whereas the latter proceeds via a torsiosymmetric intermediate. Another possibility is that both reactions proceed through a torsiosymmetric intermediate but that the binding at the active site of the enzyme of the methyl group that becomes the exocyclic methylene group in compound (5) is such that free rotation of the intermediate cannot occur.

The ring-expansion enzyme is unusual in that, although it catalyses a two-electron oxidation of penicillin N, oxygen is not incorporated, as a result of its action, into the substrate (Stevens *et al.*, 1975). In this respect it is unique among the dioxygenases that have so far been described. Mechanistically, the sulphur migration strikingly resembles certain peroxidase-initiated re-arrangements of thioketals and sulphides (Scheme 2) (Terent'ev, 1965) (see also Beckwith & Ingold, 1980). These re-arrangements, with rare exceptions (Freidlina *et al.*, 1963), do not proceed in the absence of peroxide and are clearly free-radical reactions. They are accompanied by the formation of alkene and disulphide by-products and may proceed by an addition-elimination mechanism (Scheme 2,c) (Beckwith & Ingold, 1980).

These analogies suggest that possible mechanisms for the ring expansion would be those shown in Scheme 3. Formation of an initial primary free radical from the 2-*pro-S*-methyl group of penicillin N (which could undergo rotation) would be followed by re-arrangement to a tertiary radical, as in the analogous peroxide-initiated sulphide re-arrangements of Scheme 2. In one pathway to cephalosporin C direct electron transfer to the

Scheme 2

Scheme 3

oxidizing enzyme might take place, followed by, or concomitant with, proton loss (Scheme 3, pathway a). In an alternative pathway (Scheme 3, pathway b) there would be ligand transfer to the radical from an enzyme-bound iron-oxo species to give a hydroxylated intermediate, as is presumed to occur in normal dioxygenase reactions (Hamilton, 1974; Groves, 1980). Subsequent elimination of water would lead to the cephem structure. A 3 β -hydroxycepham (Miller *et al.*, 1981) was not converted into cephalosporin C by a cell-free extract of *C. acremonium* that transformed penicillin N into cephalosporin C (Queener *et al.*, 1980), but a 3 α -

hydroxycepham has not been eliminated as an intermediate. The 3 α -hydroxy compound would have the *trans* arrangement of 3-OH and 4-H appropriate for the elimination of water (Queener & Neuss, 1982).

We thank Professor J. E. Baldwin for providing ^3H -n.m.r. facilities, Lady Richards for determining ^3H -n.m.r. spectra, Mr. T. J. Beesley for the preparation of mycelium Dr. J. D. Wallis for providing crystallographic data, Professor J. R. Knowles for helpful comments on the manuscript, and the Science and Engineering Research Council and the British Technology Group for financial support.

References

- Aberhart, D. J. & Lin, J. (1974) *J. Chem. Soc. Perkin Trans. 1* 2320–2326
- Aberhart, D. J., Chu, J. Y.-R., Neuss, N., Nash, C. H., Occolowitz, J., Huckstep, L. L. & Higuera, N. D. L. (1974) *J. Chem. Soc. Chem. Commun.* 564–565
- Abraham, E. P. & Newton, G. G. F. (1961) *Biochem. J.* **79**, 377–393
- Abraham, E. P., Pang, C.-P., White, R. L., Crout, D. H. G., Lutstorf, M., Morgan, P. J. & Derome, A. E. (1983) *J. Chem. Soc. Chem. Commun.* 723–724
- Altman, L. J., Choong Yol Han, Bertolino, A., Handy, G., Laungani, D., Muller, W., Schwartz, S., Shanker, D., de Wolf, W. & Yang, F. (1978) *J. Am. Chem. Soc.* **100**, 3235–3237
- Arigoni, D. (1979) in *Vitamin B₁₂ (Proc. Eur. Symp. Vitamin B₁₂ and Intrinsic Factor 3rd)* (Zagalak, B. & Friedrich, W., eds.), pp. 389–411, Walter de Gruyter, Berlin
- Baldwin, J. E., Johnson, B. L., Usher, J. J., Abraham, E. P., Huddleston, J. A. & White, R. L. (1980a) *J. Chem. Soc. Chem. Commun.* 1271–1272
- Baldwin, J. E., Singh, P. D., Yoshida, M., Sawada, Y. & Demain, A. L. (1980b) *Biochem. J.* **186**, 889–895
- Baldwin, J. E., Jung, M., Usher, J. J., Abraham, E. P., Huddleston, J. A. & White, R. L. (1981) *J. Chem. Soc. Chem. Commun.* 246–247
- Battersby, A. R., Sheldrake, P. W., Staunton, J. & Williams, D. C. (1974) *J. Chem. Soc. Chem. Commun.* 566–567
- Beckwith, A. L. & Ingold, K. U. (1980) in *Rearrangements in Ground and Excited States* (de Mayo, P., ed.), vol. 1, pp. 161–310, Academic Press, New York, London, Toronto, Sydney and San Francisco
- Bergstrom, S., Lindstedt, S., Samuelson, B., Corey, E. J. & Gregoriou, G. A. (1958) *J. Am. Chem. Soc.* **80**, 2337–2338
- Corey, E. J., Gregoriou, G. A. & Peterson, D. H. (1958) *J. Am. Chem. Soc.* **80**, 2338
- Crout, D. H. G., Lutstorf, M., Morgan, P. J., Adlington, R. M., Baldwin, J. E. & Crimmin, M. J. (1981) *J. Chem. Soc. Chem. Commun.* 1175–1176
- Crout, D. H. G., Lutstorf, M. & Morgan, P. J. (1983) *Tetrahedron* **39**, 3457–3469
- Declercq, J. P., Germain, G., Moreaux, C. & Van Meerssche, M. (1977) *Acta Crystallogr. Sect. B* **33**, 3868–3871
- Domiano, P., Nardelli, M., Balsamo, B., Macchia, F. & Meinardi, G. (1978) *J. Chem. Soc. Perkins Trans. 2* 1082–1087
- Elvidge, J. A., Jones, J. R., Chambers, V. M. A. & Evans, E. A. (1978) in *Isotopes in Organic Chemistry* (Buncel, E. & Lee, C. C., eds.), pp. 1–49, Elsevier Scientific Publishing Co., Amsterdam, Oxford and New York
- Fawcett, P. A., Usher, J. J., Huddleston, J. A., Bleaney, R. C., Nisbet, J. J. & Abraham, E. P. (1976) *Biochem. J.* **157**, 651–660
- Freidlina, R. Kh., Terent'ev, A. B. & Petrova, V. G. (1963) *Dokl. Akad. Nauk. SSSR* **149**, 866–868
- Fujita, Y., Gottlieb, A., Peterkofsky, B., Udenfriend, S. & Witkop, B. (1964) *J. Am. Chem. Soc.* **86**, 4709–4716
- Galbraith, M. N., Horn, D. H. S., Middleton, E. J. & Thomson, J. A. (1970) *J. Chem. Soc. Chem. Commun.* 179–180
- Gautier, A. E. (1980) Ph.D. Dissertation (no. ETH 6583), Eidgenossischen Technischen Hochschule, Zürich
- Groves, J. T. (1980) in *Metal Ion Activation of Dioxygen* (Spiro, T. G., ed.), pp. 125–162, John Wiley and Sons, New York, Chichester, Brisbane and Toronto
- Groves, J. T., McClusky, G. A., White, R. E. & Coon, M. E. (1978) *Biochem. Biophys. Res. Commun.* **81**, 154–160
- Gunsalus, I., Mecks, J., Lipscomb, L., Debrunner, P. & Munck, E. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayashi, O., ed.), pp. 559–613, Academic Press, New York and London
- Hamilton, G. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 405–451, Academic Press, New York and London
- Hayano, M., Gut, M., Dorfman, R. I., Sebek, O. K. & Peterson, D. H. (1958) *J. Am. Chem. Soc.* **80**, 2336–2337
- Hayano, M., Gut, M., Dorfman, R. I., Schubert, A. & Siebert, R. (1959) *Biophys. Acta* **32**, 269–270
- Heinz, E., Tulloch, A. P. & Spencer, J. F. T. (1969) *J. Biol. Chem.* **244**, 882–888
- Jayatilake, G. S., Huddleston, J. A. & Abraham, E. P. (1981) *Biochem. J.* **194**, 645–647
- Jones, D. F. (1968) *J. Chem. Soc.* 2827–2833
- Karlson, P. & Hoffmeister, H. (1963) *Hoppe-Seyler's Z. Physiol. Chem.* **331**, 298–300
- Kluender, H., Bradley, C. H., Sih, C. J., Fawcett, P. & Abraham, E. P. (1973) *J. Am. Chem. Soc.* **95**, 6149–6150
- Kluender, J., Huang, F. C., Fritzberg, A., Schnoes, H., Sih, C. J., Fawcett, P. A. & Abraham, E. P. (1974) *J. Am. Chem. Soc.* **96**, 4054–4055
- Konomi, T., Herchen, S., Baldwin, J. E., Yoshida, M., Hunt, N. & Demain, A. L. (1979) *Biochem. J.* **184**, 427–430
- Kupka, J., Shen, Y. Q., Wolfe, S. & Demain, A. L. (1983) *FEMS Microbiol. Lett.* **16**, 1–6
- Loder, B., Newton, G. G. F. & Abraham, E. P. (1961) *Biochem. J.* **79**, 408–416
- Lowry, T. H. & Richardson, K. S. (1981) in *Mechanism and Theory in Organic Chemistry*, 2nd edn., pp. 387–392, Harper and Row, New York

- McMahon, R. E., Sullivan, H. R., Cymerman Craig, J. & Pereira, W. E. (1969) *Arch. Biochem. Biophys.* **132**, 575-577
- Miller, R. D., Huckstep, L. L., McDermott, J. P., Queener, S. W., Kukolja, S., Spry, D. O., Elzey, T. K., Lawrence, S. W. & Neuss, N. (1981) *J. Antibiot.* **34**, 984-993
- Neuss, N., Nash, C. H., Baldwin, J. E., Lemke, P. A. & Grutzner, J. B. (1973) *J. Am. Chem. Soc.* **95**, 3797-3798; *ibid.*, p. 6511 (correction)
- Newton, G. G. F. & Abraham, E. P. (1956) *Biochem. J.* **62**, 651-658
- O'Sullivan, J., Bleaney, R. C., Huddleston, J. A. & Abraham, E. P. (1979) *Biochem. J.* **184**, 421-426
- Queener, S. W. & Neuss, N. (1982) in *Chemistry and Biology of β -Lactam Antibiotics* (Morin, R. B. & Gorman, M., eds.), vol. 3, pp. 1-81, Academic Press, New York
- Queener, J., McDermott, J., Miller, R., Huckstep, L., Spry, D., Kukolja, S. & Neuss, N. (1980) *Am. Soc. Microbiol. Conf. Genet. Mol. Biol. Indust. Microorgn. 2nd, Bloomington*, Session 2, Abstr. 1
- Retey, J. & Robinson, J. (1982) *Stereospecificity in Organic Chemistry and Enzymology*, pp. 185-207, Verlag Chemie, Stuttgart
- Retey, J., Suckling, C. J., Arigoni, D. & Babior, B. M. (1974) *J. Biol. Chem.* **249**, 6359-6360
- Retey, J., Smith, E. H. & Zagalak, B. (1978) *Eur. J. Biochem.* **83**, 437-451
- Roberts, K. D., Bandy, L. & Lieberman, S. (1969) *Biochemistry* **8**, 1259-1270
- Salzman, L. A., Weissbach, H. & Katz, E. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 542-546
- Smith, B., Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967) *Biochem. J.* **103**, 877-890
- Stevens, C. M., Abraham, E. P., Huang, F. C. & Sih, C. J. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 625
- Sweet, R. M. & Dahl, L. F. (1970) *J. Am. Chem. Soc.* **92**, 5489-5507
- Taylor, K. B. (1974) *J. Biol. Chem.* **249**, 454-458
- Terent'ev, A. B. (1965) *Izv. Akad. Nauk SSSR Ser. Khim.* 1258-1260
- Tomita, Y. & Sakurai, E. (1974) *J. Chem. Soc. Chem. Commun.* 434-435
- Trown, P. W., Abraham, E. P., Newton, G. G. F., Hale, C. W. & Miller, G. A. (1962) *Biochem. J.* **84**, 157-166
- Turner, M. K., Farthing, J. E. & Brewer, S. J. (1978) *Biochem. J.* **173**, 839-850
- Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967) *Biochem. J.* **103**, 902-912