Stereochemical Fate of Chiral-Methyl Valine in the Ring Expansion of Penicillin N to Deacetoxycephalosporin C

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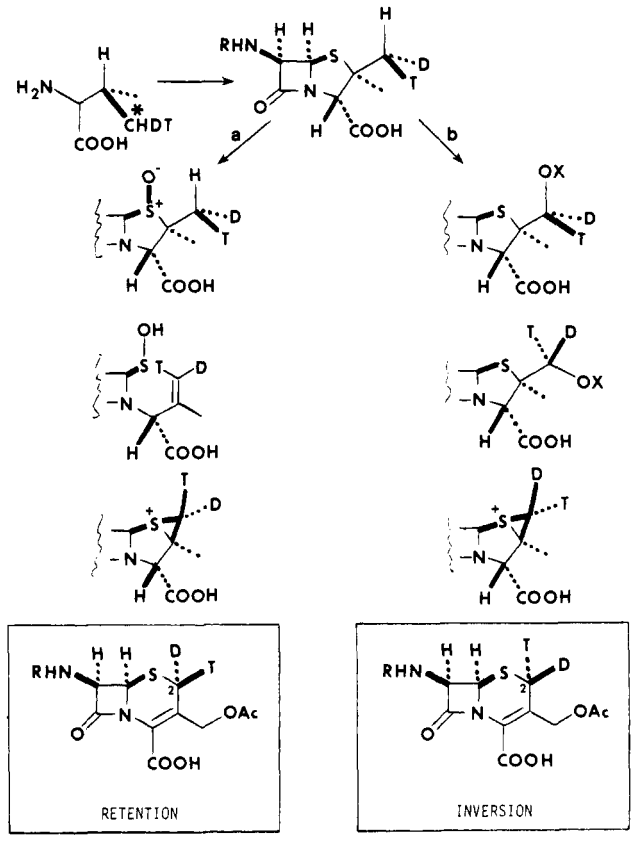
Abstract: D,L-(3*R*,4*R*)- and (3*R*,4*S*)-[4-^{2}H,^{3}H]valine have been incorporated into cephalosporin C in growing cultures of *Acremonium strictum* (ATCC 36225). The distribution of tritium radioactivity at C-2α and C-2β in the latter has been determined in a kinetic assay involving conversion of cephalosporin C to *N*-(*tert*-butoxycarbonyl)deacetylcephalosporin *C*-1β-oxide (**7**) and monitoring the loss of tritium from C-2 at constant pH, ionic strength, and temperature. Control experiments are described which demonstrate the validity of treating the tritium loss data as parallel pseudo-first-order processes for equal and unequal distributions of radioisotope at the diastereotopic C-2 methylene positions. Double label experiments have shown a relatively large, normal isotope effect for the biochemical transformation. To the detection limits of the assay, the penicillin N β-methyl group labeled from chiral-methyl valine suffers complete epimerization in the oxidative ring expansion to deacetoxycephalosporin C.

Compelling evidence has been gathered in recent years that the biosynthesis of the classical penicillin and cephalosporin antibiotics proceeds through formation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (**1**).[1] Oxidative cyclization of **1** with loss of four hydrogen equivalents, but utilizing remarkably a single molecule of dioxygen,[2] takes place without generation of detectable intermediates[3] to afford isopenicillin N [**3**, R = δ-(L-α-aminoadipoyl)].[4] In *Cephalosporia* and *Streptomycetes*, epimerization[5] to penicillin N [**3**, R = δ-(D-α-aminoadipoyl)] provides the substrate for a third oxidative transformation, the ring expansion of **3** to deacetoxycephalosporin C [**4**, R = δ-(D-α-aminoadipoyl), X = H].[6] Allylic hydroxylation of the latter then gives deacetylcephalosporin C (**4**, X = OH)[7] which is acylated to form cephalosporin C (**4**, X = OAc).[8] In this paper we examine the stereochemical fate of valine (**2**) bearing chiral methyl groups exclusively at its 3-*pro*-*R* position (*)[9] through the penam/cephem ring expansion in which the β-methyl group (*) of **3** becomes the C-2 methylene (*) of **4**.[10,11] In contrast to the tritium NMR results of Crout,[12] suggesting partial enzymic selection from a locally homotopic or epimerized intermediate, we record below that this transformation occurs with complete epimerization to the detection limits of the assay employed.[13]

Initial formulation of the problem evolved from consideration of the probable stereochemical course of reactions in precedented in vitro chemistry to convert penicillins to cephalosporins. Scheme I illustrates the outcome of this analysis for the case of an (*R*)-methyl group (a) through the well-studied Morin reaction[14] (net retention of configuration) and (b) assuming direct hydroxylation with retention[15,16] at the penicillin β-methyl group and displacement[17] to form the reactive thiiranium intermediate with inversion (net inversion of configuration). For simplicity,

Scheme I



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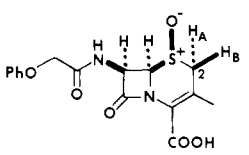
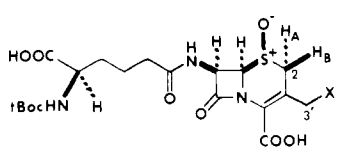
only loss of the hydrogen isotope from the methyl group is shown. While the intrinsic isotope effect for either ring expansion process would, of course, not be infinite, a normal isotope effect for both the electrocyclic process (path a)[18] and the enzymic hydroxylation of an unactivated methyl group (path b)[16,19] might well be anticipated.

The key experimental, indeed conceptual, problem to be resolved in this investigation was the development of a means to determine the orientation of tritium at C-2 suitable for small amounts of cephalosporin C (**4**, X = OAc). Conventional practice[20] would dictate that stereochemical analysis at the C-2 methylene could be achieved by its reconversion to a chiral methyl group by a reaction sequence of defined stereochemistry and assay as chiral acetic acid, e.g., after Kuhn–Roth oxidation, by established means.[21] This approach carries the imprimatur of an established method but was likely to be thwarted by the highly functionalized and labile nature of the cephalosporin nucleus where severe experimental problems would be confronted in maintaining stereochemical integrity at C-2. Alternatively, provided chiral-methyl valine of sufficiently high specific radioactivity could be prepared, stereochemical analysis by tritium NMR[12,22] would in principle be trivial. Wary of the technical difficulties posed by each of these approaches and in particular of the stereochemical uncertainties promised by degradation, we sought to exploit in a controlled fashion the propensity for base-catalyzed exchange to occur adjacent to sulfur bearing a formal positive charge. Oxidation of cephalosporins having a C-7 amide hydrogen takes place selectively to form the more hindered β-sulfoxide.[23] While sulfoxide oxygen appears to direct metalation by strong bases in aprotic solvents,[24] experiments with conformationally constrained systems[25] reveal that deprotonation by hydroxide and alkoxide bases in protic solvents occurs with marked stereoelectronic preference for sulfinyl anion formation anti to the S–O bond.[26] Indeed when 7-(phenoxyacetyl)deacetoxycephalosporin *C*-1β-oxide (**5**) and *N*-(*tert*-butoxycarbonyl)cephalosporin *C*-1β-oxide (**6**) in deuterium oxide



5



6 X = OAc

7 X = OH

(pH 8.11 and 7.60,[27] respectively, 25 mM phosphate buffer, 32 ± 1 °C) were examined by ^{1}H NMR spectroscopy and the disappearance of the C-2 hydrogens was treated as parallel pseudo-first-order processes,[28] it was found that H_A exchanged roughly 10 times faster than H_B.[29] Analogous experiments with the α-sulfoxide corresponding to **5** gave only a slight difference in the relative rates of exchange, favoring now H_B over H_A. The sulfone from **5** exchanged with poor selectivity and far too rapidly to be of use for a kinetic assay.

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To more closely mimic the planned radiochemical assay, sulfoxides **5** and **6** were exhaustively exchanged with deuterium oxide, isolated, dried, and exchanged a second time in deuterium oxide containing a small quantity of high specific activity tritiated water. While the initial rates of radiolabel incorporation would not be the same, at equilibrium the specific tritium activity at positions A and B would be effectively equal (T_A/T_B 50:50), being dependent only on the specific activity of the medium and the solvent isotope effect.[30] After removal of tritium from the acidic OH and NH functions at pH 6.1,[31] sulfoxides **5** and **6** were crystallized to constant specific activity.

If one considers the general case of tritium exchange from C-2α and C-2β of **5** and **6**, the kinetic scheme shown in eq 1 is obtained where T_A and T_B represent the tritium concentrations at C-2α and C-2β, respectively, and k_A and k_B are their first-order rates of loss. A further process, k_i, can be visualized where exchange

$$\text{obsd } T_A \xleftarrow{k_A} T_A \underset{}{\overset{k_i}{\rightleftharpoons}} T_B \xrightarrow{k_B} \text{obsd } T_B \qquad (1)$$

(all exchange assays were conducted in deuterium oxide) of the companion isotope at C-2, in this case deuterium, occurs with stereochemical inversion to carry tritium label from the α-position to the β-locus or vice versa. However, assuming that the relative energies of radioisotope at C-2α and C-2β are the same, then the rate constant for sulfinyl anion inversion, k_i, is the *same* in both directions. While for diastereomers this equality cannot be strictly correct, it may be reasonably taken to be very nearly so.[30]

Two limiting cases may be quickly examined. First, if the barrier to sulfinyl anion inversion were very low ($k_i \gg k_A > k_B$), then the rate of tritium equilibration between T_A and T_B would be rapid relative to the respective rate of loss. If the disappearance of tritium were plotted (semilog) as a function of time, a straight line would result whose slope would afford a rate constant equal to the sum of k_A and k_B. The rapid interchange of T_A and T_B would defeat the kinetic assay, and the ratio T_A/T_B at $t = 0$ would not be accessible despite the fact that the loss of label from T_A and T_B would partition as $k_A/(k_A + k_B)$ and $k_B/(k_A + k_B)$ or k_A/k_B, respectively. This situation is contrary to one's chemical intuition given that sulfinyl anion inversion through deuterium exchange and tritium loss both involve the same set of ionizations. However, such an outcome could conceivably become important were sulfinyl anion formation associated with a very large kinetic isotope effect (k_D/k_T). It will be seen that these factors do not affect the assay in an important way.

Second, the anion inversion barrier is very high ($k_A > k_B \gg k_i$). In this case, tritium at C-2α would neither cross over to C-2β nor the reverse. If one were to plot the exponential loss of tritium from **5** or **6** on exchange as a function of time, parallel first-order processes[28] would be obtained, yielding intercepts at $t = 0$ corresponding to the initial ratio of T_A/T_B. This circumstance clearly would be favorable to the envisioned assay.

A corollary of this case could be imagined if $k_A > k_i \gg k_B$. Under these circumstances, loss of T_B would effectively never take place directly but would occur only by anion inversion involving deuterium loss from C-2α to carry T_B to T_A and subsequent exchange. Plots of tritium loss would still show parallel first-order decays, but the slow process would reflect sulfinyl anion inversion. However, for assay purposes, the intercepts at $t = 0$ for these processes would still reflect the initial T_A/T_B ratio. Nevertheless, again, as in the first case above, behavior of this sort is contrary to expectation owing to the fact that redeuteration of the inverted anion to give deuterium at C-2β is the reverse of tritium loss from T_B and the small isotope effect (k_D/k_T) involved.

A third possibility is that k_i is of the order of k_A or k_B, or that it is of intermediate magnitude ($k_A > k_i > k_B$). This is a significantly more complex case to treat. However, with regard to developing a useful assay of tritium distribution at C-2 in **5** and **6**, the following derivation is to be considered. From eq 1, expressions for the loss of tritium from T_A and T_B are

$$dT_A/dt = -(k_A + k_i)T_A + k_iT_B$$
$$dT_B/dt = k_iT_A - (k_B + k_i)T_B \qquad (2)$$

The general solution has the form

$$T_A(t) = ae^{-\lambda_1 t} + be^{-\lambda_2 t}$$
$$T_B(t) = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t} \qquad (3)$$

To determine the coefficients a, b, A, and B, recall, as discussed above, that at equilibrium on exchange *in* of radioactivity in deuterium oxide medium, the level of tritium labeling at T_A and T_B is effectively equal. Therefore, at $t = 0$ for the exchange of tritium out

$$T_A = \tfrac{1}{2} \qquad T_B = \tfrac{1}{2} \qquad (4)$$

Substituting (4) into (2) at $t = 0$

$$\left(\frac{dT_A}{dt}\right)_{t=0} = -\tfrac{1}{2}k_A$$
$$\left(\frac{dT_B}{dt}\right)_{t=0} = -\tfrac{1}{2}k_B \qquad (5)$$

When the derivative of (3) is taken at $t = 0$ and the result is equated to (5), it can be shown that

$$b = \tfrac{1}{2}\left[\frac{\lambda_1 - k_A}{\lambda_1 - \lambda_2}\right]$$
$$B = \tfrac{1}{2}\left[\frac{\lambda_1 - k_B}{\lambda_1 - \lambda_2}\right] \qquad (6)$$

It will be seen (eq 12) that the quantity of interest is

$$b + B = \tfrac{1}{2}\left[\frac{2\lambda_1 - k_A - k_B}{\lambda_1 - \lambda_2}\right] \qquad (7)$$

Now an explicit expression for $\lambda_{1,2}$ (λ) is required. From the rate equations in (2), the following secular equation may be obtained:

$$\begin{vmatrix} (k_A + k_i) - \lambda & -k_i \\ -k_i & (k_B + k_i) - \lambda \end{vmatrix} = 0 \qquad (8)$$

Expanding the determinant and solving for λ, two roots are found (λ_1 corresponding to the faster and λ_2 the slower process):

$$\lambda_1 = \tfrac{1}{2}[(2k_i + k_A + k_B) + ((k_A - k_B)^2 + 4k_i^2)^{1/2}]$$
$$\lambda_2 = \tfrac{1}{2}[(2k_i + k_A + k_A) - ((k_A - k_B)^2 + 4k_i^2)^{1/2}] \qquad (9)$$

Substituting (8) into (7) and rearranging terms

$$b + B = \tfrac{1}{2}\left[1 + \frac{\dfrac{2k_i}{|k_A - k_B|}}{1 + \dfrac{4k_i^2}{(k_A - k_B)^2}}\right] \qquad (10)$$

Let $p = 2k_i/[k_A - k_B]$, then (10) becomes

$$b + B = \tfrac{1}{2}\left[1 + \frac{p}{(1 + p^2)^{1/2}}\right] \qquad (11)$$

It can be seen that at the limits $p \to 0$, $b + B = {}^1/_2$ and $p \to \infty$, $b + B = 1$. Recalling (3) and noting that $\lambda_1 > \lambda_2$, for $t \gg \lambda_1$, the following approximation may be made:

$$T_A + T_B \simeq (b + B)e^{-\lambda_2 t} \qquad (12)$$

Taking the logarithm of each side of eq 12 defines a line whose slope is λ_2 and whose y intercept is ln $(b + B)$. If it were found experimentally that this intercept corresponds to one-half of the total amount of tritium at C-2 of the cephalosporin in question, then $(b + B) = {}^1/_2$. But remembering (11), for $b + B$ to be experimentally equal to ${}^1/_2$, p and, therefore, k_i must be effectively

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(31) Control experiments showed no exchange at C-2 in 24 h at this pH.

Table I. Tritium Distributions in Positions A and B for Sulfoxides **5** and **7** as Determined in the Exchange Assay

expt	substrate (source)	theoret T_A/T_B	obsd T_A/T_B
1	**5** (chemical exch.)	50:50	54:46 ± 2
2	**5** (10-min exch.)	89:11	85:15 ± 2
3	**5** (120-min exch.)	86:14	76:24 ± 2
4	**7** (chemical exch.)	50:50	50:50 ± 2
5	**7** (from (*R*)-valine)		50:50 ± 2
6	**7** (from (*S*)-valine)		48:52 ± 2

0. That is, if a sample of cephalosporin sulfoxide bearing equal amounts of tritium at T_A and T_B (in principle from exchange in of tritium to equilibrium) shows a 50:50 ratio of y intercepts at $t = 0$ upon graphical treatment of the tritium loss data, then the sulfinyl anion inversion barrier must be comparatively high. In this circumstance, the terms involving k_i may be ignored and the equations in (2) simplify to

$$dT_A/dt = -k_A T_A$$
$$dT_B/dt = -k_B T_B \quad (13)$$

These equations, of course, define precisely the second case discussed qualitatively above for parallel first-order losses of tritium from T_A and T_B.

Observed deviations from a 50:50 result, therefore, reflect the intervention of sulfinyl anion inversion in the absence of tritium loss. Returning for a moment to the boundary case discussed earlier, in the limit where the inversion barrier is low, loss of tritium from the diastereotopic C-2 methylene positions will partition as k_A/k_B. Therefore, as the inversion barrier is lowered, one would expect the experimentally determined ratio obsd T_A/obsd T_B to vary from 50:50 toward k_A/k_B. That is, fractions of total observed tritium activity greater than 50% at T_A would qualitatively reflect increasing contributions of the inversion process.

As exchange incubations were carried out over approximately 6000 min (greater than two half-lives for the slower exchanging site), immediately prior to assay, the cephalosporin C derivative **6** was treated at pH 6.4–6.6[31] with citrus acetylesterase[32] to produce the more hydrolytically stable[33] deacetyl compound **7**.[34] In separate experiments under carefully controlled conditions of pH, ionic strength, and temperature, sulfoxides **5** (experiment 1) and **7** (experiment 4) gave tritium loss data as a function of time in deuterium oxide medium which, when treated as parallel pseudo-first-order processes, gave $t = 0$ intercepts corresponding to the experimentally determined distributions of tritium initially at positions A and B (Table I). For the deacetoxycephalosporin C derivative **5**, this deviation is small, and for **7** it is experimentally undetectable. This key finding establishes the general validity of the assay to measure the distribution of tritium between loci A and B by monitoring exchange from each as parallel but essentially first-order processes.[35,36]

Having secured an assay for the distribution of tritium at the C-2 methylene of cephalosporin, attention was turned to the incorporation of D,L-(3*R*,4*R*)- and (3*R*,4*S*)-[4-^{2}H,^{3}H]valine **2**, whose syntheses have been previously described.[9] *Acremonium strictum* (ATCC 36225) was maintained on agar plates of modified LePage and Campbell medium.[37] Colonies were propagated in the seed medium of Caltrider and Niss[38] for 4 days to inoculate each of four flasks containing Demain's medium (40 mL/250-mL Erlenmeyer flask).[39] Chiral-methyl valine (2 mg/flask) was administered 5 times between 92 and 140 h of growth. After 168 h, the mycelia were harvested and the cephalosporin C (**4**, X = OAc) produced (ca. 200 μg/mL) was purified by chromatography on carbon and finally isolated as a well-separated peak by preparative liquid chromatography.[40] Lyophilization yielded 17.2 and 22.4 mg of **4** (X = OAc) from the (*R*)- and (*S*)-methyl valine experiments, respectively, which were separately diluted to 100 mg with radioinactive material, converted to *N*-(*tert*-butoxycarbonyl)cephalosporin *C*-1β-oxide (**6**) and crystallized to constant specific activity.

In duplicate trials, the radiolabeled samples of **6** so obtained were treated with citrus acetylesterase[32] and submitted to the stereochemical assay. The averaged distributions of tritium are shown in Table I (experiments 5 and 6). Within the limits of the assay, it was clear that the distribution of tritium activity at the C-2 methylene of the biosynthetically generated cephalosporin was *equal* from both (*R*)- and (*S*)-methyl valine. This wholly unexpected outcome led to one final test of the exchange assay; that is, an unequivocal demonstration that a sample of cephalosporin having an *unequal* distribution of tritium activity at the C-2 methylene of known ratio would assay correctly.

Presuming sulfinyl anion inversion to be a minor process, as established above, the exchange in of radioactivity into loci A and B of a cephalosporin sulfoxide derivative could be treated as independent processes and the T_A/T_B ratio estimated at any time t. This was accomplished as follows where the change in tritium concentration at A and B can be expressed as

$$dT_A/dt = -k_A T_A + G_A \quad dT_B/dt = -k_B T_B + G_B \quad (14)$$

where k_A and k_B are the first-order rate constants for loss (determined graphically in the exchange assay) and G_A and G_B are the zero-order rates of gain of tritium activity at A and B from a pool of effectively constant specific activity. At $t = 0$, no tritium resides at A or B, and it can be shown that

$$T_A = \frac{G_A}{k_A}(1 - e^{-k_A t}) \quad T_B = \frac{G_B}{k_B}(1 - e^{-k_B t}) \quad (15)$$

Use of these equations would require evaluation of G_A and G_B. The absolute amounts of tritium at A and B, however, are not of direct interest, but the ratio T_A/T_B is. At equilibrium $dT_A/dt = dT_B/dt = 0$ and $T_A = T_B$. Therefore, $G_A k_B/G_B k_A = 1$ and hence the ratio

$$\frac{T_A}{T_B} = \frac{G_A k_B}{G_B k_A}\frac{(1 - e^{-k_A t})}{(1 - e^{-k_B t})}$$

simplifies to

$$\frac{T_A}{T_B} = \frac{(1 - e^{-k_A t})}{(1 - e^{-k_B t})} \quad (16)$$

This is a well-behaved function which, when taken at its limits, $t \to 0$, $T_A/T_B = k_A/k_B$ and at $t \to \infty$, $T_A/T_B = 1$, the case which has been demonstrated in Table I (experiments 1 and 4). Using the more easily handled sulfoxide **5**, exhaustive deuteration and short-term exchange of tritium was carried for 10 and 120 min. After pH 6.1 wash and crystallization to constant specific activity, each unequally labeled specimen of **5** was assayed. Agreement with theory (Table I) was very good for the 10-min sample (ex-

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(34) Over the course of the exchange assay of **7**, concommitant hydrolysis of the β-lactam ring was on the order of 10%.

(35) A number of experimental limitations prevent more accurate determination of the sulfinyl anion inversion barrier for the present experimental systems such as small variations in pH (pD) and inaccuracies in its initial adjustment (the absolute rates of exchange are extremely sensitive to pH), slow hydrolysis of the β-lactam substrates during the course of the exchange assay,[34] and low levels of protium in the substrate and assay medium as well as, of course, the inversion process itself, small though it is by the measures that we have been able to apply. Care was taken to control these factors as much as possible as noted in the Experimental Section.

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Table II. Incorporation of D,L-(3*R*,4*R*)-[4-^{2}H,^{3}H],[4-^{14}C]Valine into Cephalosporin C

expt	valine fed, mg/160 mL	ceph. C isol., day	^{3}H/^{14}C (% ^{3}H retained) valine	**6**
1	40	7	4.53	4.70 (104)
2	20	6.5	4.67	5.25 (112)
	20	8	4.67	4.96 (106)

periment 2) and somewhat less so for the 120-min sample (experiment 3) owing to a small amount of anion inversion during the tritium in and exchange out as well as other minor experimental limitations.[35] In the event, it is clear that the assay is capable of distinguishing both equal and unequal distributions of tritium at positions A and B, slightly underestimating the latter for the case of sulfoxide **5**. The assay would be expected to be more accurate for the cephalosporin derivative **7** where sulfinyl anion inversion is apparently a less significant process.

The validity of the exchange assay was finally, therefore, not found wanting. The equal distribution of tritium at the diastereotopic C-2 methylene positions of cephalosporin from both (*R*)- and (*S*)-methyl valine must be interpreted in two extremes or some combination of the two: (a) the intrinsic isotope effect (k_H/k_D) for functionalization of the penicillin N (**3**) β-methyl group is unity and the assay is blind to any stereochemical information that may in fact have been contained in the experiment[41] or (b) $k_H/k_D \neq 1$ and stereochemical scrambling at the methyl center has occurred. That the isotope effect is normal, and indeed substantial, can be shown in two ways.

First, a kinetic isotope effect from label in the 3-*pro*-*R*-methyl group will only be expressed in the penam/cephem ring expansion itself (primary), apart from a small β-secondary isotope effect possible during sulfur insertion at the adjacent tertiary valinyl center in penicillin formation. If the intrinsic isotope effect for methyl functionalization were unity, a doubly labeled sample of valine would be expected to lose one-third of its tritium activity relative to a ^{14}C-internal standard on incorporation into cephalosporin C. D,L-(3*R*,4*R*)-[4-^{2}H,^{3}H]Valine[9] was mixed with D,L-[4-^{14}C]valine. Two incorporation experiments were carried out as summarized in Table II. Experiment 1 was conducted according to the procedure of experiments 5 and 6, Table I. A very slight increase in the ^{3}H/^{14}C ratio (4%) was observed in the cephalosporin C isolated as **6**. A conceivable cause for this small apparent increase in tritium level could be catabolism of the cephalosporin C in fermentation involving a step sensitive to hydrogen isotope at C-2. To eliminate this possibility, experiment 2 was performed feeding half the amount of valine in five equal portions between 72 and 120 h. Separate isolations of cephalosporin at 6.5 and 8 days revealed essentially unchanged tritium retentions, variations owing to experimental error in scintillation counting of **7**.

A second, independent line of evidence that the isotope effect is normal and quite large is evident in the ^{3}H NMR analysis of chiral-methyl valine incorporation into cephalosporin C recently reported by Crout.[12] With and without broad-band proton decoupling, the tritium resonances were unaffected, indicating that tritium at both positions A and B is always paired with deuterium and not detectably with protium.

We, therefore, conclude that in the oxidative ring expansion of penicillin N to deacetoxycephalosporin C, reaction of the penicillin β-methyl group takes place with a substantial primary isotope effect but with overall loss of stereochemical integrity in the formation of the cephem S–C-2 bond. In contrast to this result, Crout has reported[12] that the ratio T_A/T_B is ca. 2:1 from *both* (*R*)- and (*S*)-methyl groups at the valine 3-*pro*-*R* position. This observation is based on integrations of NMR resonances and may be an artifact of differential spin–lattice relaxation peculiar to tritium coupled to deuterium in a conformationally rigid and isolated spin system. Moreover, to base such a level of enzymic discrimination for a locally homotopic reactive intermediate or otherwise racemized methyl group on steric grounds[42] or on a dual isotope effect is unprecedented.

Evidence from cell-free studies indicates that the enzymes responsible for both the penam/cephem ring expansion and the 3′-allylic hydroxylation of deacetoxycephalosporin C (**4**, X = H) to deacetylcephalosporin C (**4**, X = OH) are α-ketoglutarate-dependent dioxygenases.[1,6,7] Recently published work of Blanchard[43] on a hydroxylase of this class, γ-butyrobetaine hydroxylase from calf liver, reports that the oxidation to L-carnitine takes place with retention of configuration (methylene center) and that large secondary isotope effects were measured, reflecting hybridization changes consistent with radical or cationic intermediates. These findings were interpreted as supporting the mechanism of Siegel.[44] Work completed in this laboratory[45] has established that the cephem 3′-hydroxylation also occurs with retention of stereochemistry, paralleling results obtained elsewhere with other types of hydroxylases operating at unactivated methyl centers.[16] While an allyl radical or cation derived from **4** (CH_2X = $CH_2\cdot$ or CH_2^+) would experience a higher barrier to rotation[46] than the analogous intermediate generated at the penicillin β-methyl, it is not valid to infer on this basis alone that α-ketoglutarate dioxygenases suffer a mechanistic imperative which leads to racemization/epimerization during hydroxylation at methyl centers. However, the possible intermediacy of a radical (Scheme II) in the oxidative ring expansion of **3** (* = $CH_2\cdot$) provides a rationale for the observed epimerization at this center and, further, a means to rapidly cleave to a thiyl radical[47] **8** and to enter cephem products **10** by precedented chemistry.[48] Similarly, consumation of the enzymic hydroxylation by one-electron transfer, **3** (* = CH_2^+) to **13**, or capture of the thiyl **11** or primary carbon radical **12** to join known ionic routes[14,17] **13** to the cephalosporin nucleus may also be envisioned.[49,50] 3β-Hydroxycepham **14** [R = δ-(D-α-aminoadipoyl)] has been isolated as a minor metabolite of *C. acremonium*,[50] but it has failed to be converted to deacetoxycephalosporin C (**4**, X = H) in a cell-free system from the same organism known to be capable of producing **4** (X = H) from penicillin N (**3**). Derivation of **14** as a shunt product from the reactive intermediates **9** and **13** may be visualized. Enzymic reactions lacking stereoselectivity are rare, but precedented.[51] As a class, sulfur insertion reactions are poorly understood[52] although it is noteworthy with respect to the case at hand that in the

(41) Unlike the enzymic chiral acetate assay,[21] the exchange assay cannot distinguish whether tritium of a particular orientation is paired with hydrogen or deuterium.

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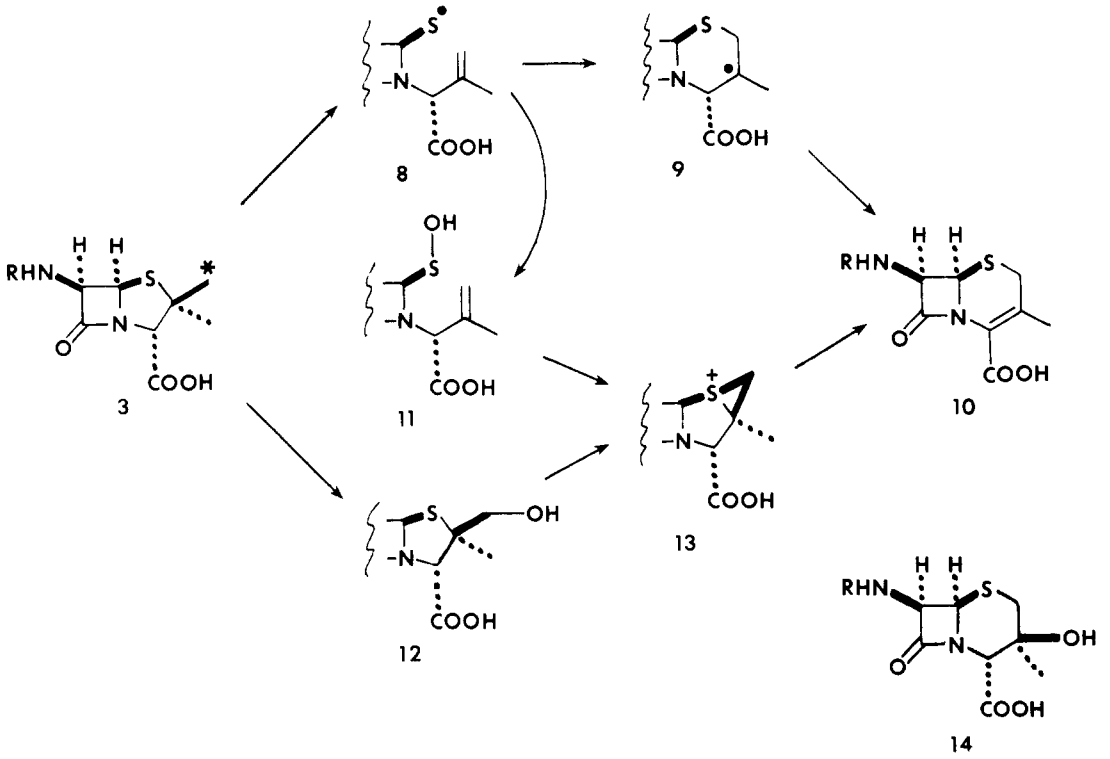
Scheme II



conversion of dethiobiotin to biotin, complete stereochemical scrambling was finally established in the former, bearing a terminal chiral methyl group.[53]

Experimental Section

General. Solvents were reagent grade and, as needed, dried by conventional means. Reactions were performed under a nitrogen atmosphere and monitored by thin-layer chromatography (Analtech, GHLF, 250 μm or Merck-60, F-254, 250 μm). Melting points were taken on a Thomas-Hoover apparatus in open capillaries. Spectroscopic data were obtained with the following instruments: ultraviolet/visible (UV, Cary Model 219), infrared (IR, Perkin-Elmer Model 457A or 599B), proton magnetic resonance [NMR, Jeol MH-100 (100 MHz), Varian CFT-20 (80 MHz), Brüker 360 (Middle Atlantic Regional NMR Facility, Philadelphia, PA)]. Abbreviations used to indicate relative intensities of IR absorptions are: s strong, w weak, and unmarked peaks of medium intensity. For NMR spectra recorded in deuterium oxide, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) was used as the internal standard. A Radiometer Model PHM 62 meter equipped with a GK2321C glass pH electrode was used to monitor the pH of aqueous solutions. Radiometer buffers were used to standardize the electrode and were taken as accurate to ±0.01 pH unit. High-pressure liquid chromatography (HPLC) was performed on a Varian Model 5020 with a fixed wavelength UV detector operating at 254 nm. Analytical separations were achieved on a Whatman Partisil-10 PAC column (4.6 mm i.d. × 250 mm) and preparative runs on a Whatman Magnum 9 column (9.4 mm i.d. × 500 mm). Radioactivity was determined by using a Packard Model 3310 Tri-Carb, a Beckman LS-7000, or an LKB 1020-II Beta liquid scintillation counter for solutions in 1.5 mL of 0.1 M dibasic potassium phosphate buffer and 13.5 mL of Aquasol (New England Nuclear). Specific activities were computed as disintegrations/min/μmol (DPM/μmol) correcting for quenching factors and background counts. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN.

7-(Phenoxyacetamido)deacetoxycephalosporanic Acid 1β-Oxide (5). 7-Deacetoxycephalosporanic acid (7-ADCA, 1.00 g, 4.67 mmol) was suspended in a solution of water (20 mL) and acetone (10 mL) containing 980 mg (11.68 mmol) of sodium bicarbonate.[54] The resulting suspension was cooled to 0–5 °C, and phenoxyacetyl chloride (645 μL, 796.7 mg, 4.67 mmol) was added dropwise. After stirring for 2 h at 0–5 °C, the solution was homogeneous and orange–yellow colored. At that time sodium periodate (994 mg, 4.67 mmol) was added, and the resulting solution was stirred at room temperature for 1.5 h.[55] The white precipitate that had gradually formed (10_x salts) was filtered, and the filtrate was acidified to pH 2 with 2 N hydrochloric acid. The resulting precipitate was filtered and dried in vacuo overnight: yield of sulfoxide **5**, 811 mg (2.22 mmol) 48% based on 7-ADCA; mp 204–206 °C dec; IR (KBr) 3600–2700 (br), 3300, 1790, 1770 (s), 1710 (s), 1660 (s), 1600, 1550, 1490, 1440, 1420, 1370, 1300, 1240 (s), 1060, 1010 (s), 900 (w); ^{1}H NMR (D_2O/$NaHCO_3$) δ 2.00 (s, 3 H), 3.64 (br s, 2 H, H-2), 4.90 (d, J = 5 Hz, 1 H, H-6), 6.02 (d, J = 5 Hz, 1 H, H-7), 7.0–7.6 (m, 5 H). Anal. ($C_{16}H_{16}N_2SO_6$): C, H, N.

***N*-(*tert*-Butoxycarbonyl)cephalosporin C.**[56] Cephalosporin C (**4**, X = OAc, 1.0 g, 2.45 mM) was suspended in a solution of *p*-dioxane (10 mL) and water (5 mL) containing sodium bicarbonate (420 mg, 5.0 mmol). After the solution was stirred at room temperature for 10 min, freshly distilled di-*tert*-butyl dicarbonate (635 μL, 600 mg, 2.75 mmol) was added, and the resulting suspension was stirred at room temperature for 3.0 h. At that time, the dioxane/water azeotrope was evaporated. Ethyl acetate (10 mL) was added to the aqueous solution and the aqueous layer was acidified to pH 2 with 6 N hydrochloric acid. The aqueous layer was then extracted with ethyl acetate (4 × 15 mL). Combined ethyl acetate extractions were washed with water (1 × 10 mL), dried (anhydrous $MgSO_4$), and evaporated to yield 1.26 g (2.45 mmol, 100%) of N-protected cephalosporin C as a white, hygroscopic foam: ^{1}H NMR (D_2O/$NaHCO_3$) δ 1.44 (s, 9 H), 1.66 (m, 4 H), 2.16 (s, 3 H), 2.48 (m, 2 H), 3.32 and 3.50 (*AB* q, J = 18 Hz, 1 H, H-2β), 3.66 and 3.84 (*AB* q, J = 18 Hz, 1 H, H-2α), 3.96 (m, 1 H, *t*-Boc-NH-C*H*), 5.23 (d, J = 5 Hz, 1 H, H-6), 5.76 (d, J = 5 Hz, 1 H, H-7).

***N*-(*tert*-Butoxycarbonyl)cephalosporin *C*-1β-Oxide (6).** A 10-mL flask was flamed dry in a stream of argon and charged with 160 mg (0.32 mmol) of *N*-(*tert*-butoxycarbonyl)cephalosporin C. Dry THF (4 mL) was added and the resulting solution stirred at 0–5 °C for 5 min under a positive argon pressure. At that time, 3-chloroperbenzoic acid (60 mg, 0.35 mmol, recrystallized from hexane) was added, and the resulting solution was stirred at 0–5 °C for 5 min. The ice bath was then removed and the solution stirred at 40 °C for 2 h. At that time the product was triturated with a 1:1 diethyl ether/hexane solution. The resulting precipitate was filtered, washed immediately wtih diethyl ether (4 × 5 mL), recrystallized from anhydrous THF/ether/hexane, and dried in vacuo to yield 81 mg (0.15 mmol, 50%) of **6** as a manila-colored powder, mp

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145–150 °C dec; IR (KBr) 3500–2800 (br), 3300, 2960, 1785, 1720 (s), 1515, 1370, 1225, 1160, 1040; ^{1}H NMR (D_2O/$NaHCO_3$) δ 1.37 (s, 9 H), 1.69 (m, 4 H), 2.04 (s, 3 H), 2.35 (m, 2 H), 3.40 and 3.63 (*AB*, J = 18 Hz, 1 H, H-2α), 3.76 and 3.99 (A*B*, J = 18 Hz, 1 H, H-2β), 4.00 (m, 1 H, *t*-Boc-NH–C*H*), 4.85 (d, J = 5 Hz, 1 H, H-6), 5.80 (d, J = 5 Hz, 1 H, H-7). Anal. ($C_{21}H_{29}N_3SO_{11}\cdot H_2O$): C, H, N.

***N*-(*tert*-Butoxycarbonyl)deacetylcephalosporin *C*-1β-Oxide (7).** Fifty milligrams (95.6 μmol) of *N*-(*tert*-butoxycarbonyl)cephalosporin *C*-1β-oxide (**6**) was suspended in 3 mL of water and was dissolved by dropwise addition of 0.02 M sodium hydroxide (final pH 6.5). A solution of acetylesterase[32] (1 mL, 6 units) in 1 mL of water was adjusted to pH 6.5 with 0.02 M sodium hydroxide and was then added, in one portion, to the cephalosporin solution which was stirring at 30 °C. The pH of this mixture was maintained between pH 6.4 and 6.6 by dropwise addition of 0.02 M sodium hydroxide. After 3.5 h, there was no further pH change, and the solution was lyophilized overnight: yield, 110 mg of a light yellow powder; ^{1}H NMR (D_2O/$NaHCO_3$) δ 1.42 (s, 9 H), 1.70 (m, 4 H), 1.90 (s, 3 H, NaOAc), 2.40 (m, 2 H), 3.23 and 3.46 (*AB*, J = 18 Hz, 1 H, H-2α), 3.68 and 3.91 (A*B*, J = 18 Hz, 1 H, H-2β), 3.85 (m, 1 H, *t*-Boc-NH–C*H*), 4.30 (apparent d, J = 5 Hz, 2 H, H-17), 4.87 (d, J = 5 Hz, 1 H, H-6), 5.82 (d, J = 5 Hz, 1 H, H-7).

7-(Phenoxyacetamido)deacetoxycephalosporanic Acid 1β-Oxide (5) Equally Labeled at the Two Diastereotopic C-2 Positions with Tritium. Sulfoxide **5** (300 mg, 822 μmol) was dissolved in 9 mL of deuterium oxide (99.8%, Aldrich) containing 143 mg (822 μmol) of dibasic potassium phosphate. The pH of the resulting solution was adjusted to 8.05 with solid tribasic potassium phosphate, and the resulting solution was stirred at 32 ● 0.5 °C for 24 h. At that time the pH of the solution was adjusted to pH 2 with 1 N deuterium chloride, and the resulting precipitate was filtered and dried in vacuo to yield 250 mg (683 μmol, 83%) of **5** completely labeled with deuterium at C-2 (>95% by PMR integration). This material (200 mg, 546 μmol) was dissolved in 6 mL of deuterium oxide (99.8%, Aldrich) containing 95 mg (546 μmol) of dibasic potassium phosphate. The pH of this solution was adjusted to pH 8.1 with solid tribasic potassium phosphate at which time 200 μL of tritiated water (25 mCi/g, New England Nuclear) were added. The resulting solution was stirred at 35 °C for 120 h. Workup was as above to yield 172 mg (470 μm, 86%) of **5** equally labeled with tritium at the two diastereotopic C-2 positions. To remove tritium from the acidic positions (amide and carboxyl), the following was performed: The above ^{3}H-labeled material (170 mg, 465 μmol) was dissolved in 5 mL of deuterium oxide (99.8%) containing 80 mg (465 μmol, 1 equiv) of dibasic potassium phosphate. The pH of this solution was 6.1, and the solution was stirred at room temperature for 120 min. After the usual workup (see above), 125 mg (341 μmol, 74%) of 7-(phenoxyacetamido)deacetoxycephalosporanic acid 1β-oxide equally labeled with tritium at the two diastereotopic C-2 positions (and essentially free of tritium at the other acidic positions) was obtained.

N-(*tert*-Butoxycarbonyl)cephalosporin *C*-1β-oxide (**6**) was also equally labeled at C-2 as above except that the workup involved acidification to pH 2 with 1 N deuterium chloride, extraction of the acidified aqueous solution with ethyl acetate (3 × 20 mL), the combined organic extracts were washed with ether (1 × 10 mL) followed by washing with brine (1 × 10 mL), dried anhydrous ($MgSO_4$), and evaporated to yield the equally labeled *N*-(*tert*-butoxycarbonyl)cephalosporin *C*-1β-oxide (**6**).

7-(Phenoxyacetamido)deacetoxycephalosporanic Acid 1β-Oxide (5) Unequally Labeled at the Two Diastereotopic C-2 Positions with Tritium. The entire labeling sequence (both deuterium and tritium) was carried out precisely as described above (for **5** equally labeled at C-2 with tritium) except that after adding the tritiated water, one-half of the solution was quenched with deuterium chloride after stirring at 32 ± 0.5 °C for 10 min, and the remainder was quenched with deuterium chloride after stirring at 32 ± 0.5 °C for 120 min. Subsequent workup and exchange of tritium out of acidic positions (amide and carboxyl) was done as described above.

Tritium Exchange Out of 7-(Phenoxyacetamido)deacetoxycephalosporanic Acid 1β-Oxide (5). 7-(Phenoxyacetamido)deacetoxycephalosporanic acid 1β-oxide (25.5 mg) (70 μmol) containing tritium at the C-2 position was dissolved in 5 mL of deuterium oxide (>99.96%, Aldrich) containing 10 equiv (122 mg, 700 μmol) of dibasic potassium phosphate which had previously been adjusted to pH 8.0 with 1 N deuterium chloride. After adding the cephalosporin sulfoxide, the pH of the resulting solution was quickly adjusted to pH 8.00 (the pH in all exchange *out* experiments was between 8.00 and 8.04) by dropwise addition of a solution of tribasic potassium phosphate in deuterium oxide (140 mM, used to maintain initial phosphate concentration), and the solution was stirred at 32 ± 0.5 °C for the duration of the exchange experiment. At the appropriate times, 100-μL samples were transferred to 25-mL flasks by using an adjustable pipettor (Rainin Pipetman Model P200D, mean error <0.8%, deviation <0.25%), and the sample was then acidified by adding 10 drops of 1 N deuterium chloride. Water (ca. 6 mL) was then added, and the resulting solution was evaporated (rotovap, 40 °C) to dryness. An additional 6 mL of water was added and evaporated in a similar manner. The residue was then dried in vacuo for at least 4 h prior to preparing the counting samples.

Tritium Exchange out of *N*-(*tert*-Butoxycarbonyl)deacetylcephalosporin *C*-1β-Oxide (7). This exchange was conducted in a manner analogous to that described for 7-(phenoxyacetamido)deacetoxycephalosporanic acid 1β-oxide with the exception of using 18.5 mg (38 μL) of radiolabeled (*tert*-butoxycarbonyl)deacetylcephalosporin *C*-1β-oxide.

Graphical Treatment of Exchange Data. The aforementioned dried residues were dissolved in 1.5 mL of 0.1 M dibasic potassium phosphate solution (adjusted to pH 8 with 1 N hydrochloric acid). Flasks were swirled several times and/or sonicated (Bransonic Model 220) to ensure dissolution. Aquasol (13.5 mL) was then added to each aqueous solution. Samples were then counted for 20 min or 2% error (maximum), whichever came first.

All plots were semilog, ln DPM (ordinate) vs. time (abscissa). To obtain an accurate plot of tritium loss due to exchange out of the β-position (slow position), only points taken after five half-lives of the fast position had elapsed were used. At least six points from this time through approximately two half-lives of the slow proton/tritium were used in plotting the data for the slow position. A linear least-squares[57] fit was used to calculate the best straight line through the experimental points.

After obtaining the rate constant and total number of DPM of the slow position, it was necessary to obtain analogous information for the fast position. However, points taken early in the exchange experiment reflect loss of tritium from both the fast position and the slow position. Since the rate constant (k_B) and intercept (B_0) for the slow exchanging position are already known, the amount of exchange from the slow position can be calculated for any time (t) by using the equation[28]

$$\ln B = \ln B_0 - k_B t$$

Thus, points taken early in the exchange experiment could be corrected for loss of tritium from the slow position and a new set of points generated which only reflect the loss of tritium (as a function of time) from the fast position. Eight or more points, from t = 0 through approximately two half-lives of the fast position, were then plotted exactly as done previously for the slow proton, and the rate constant (k_A) and the intercept (A_0, total number of DPM in the fast position) were obtained. A correlation coefficient was then determined to assess how well the data fit the calculated line[57] (all correlation coefficients were >0.995).

Incorporation of Chiral-Methyl Valine into Cephalosporin C. (a) Agar Plates. Spores of *Acremonium strictum* (ATCC 36225, previously known as *Cephalosporium acremonium* CW19) were used to inoculate 15 × 100 mm Petri dishes containing ca. 25 mL of a modified LePage and Campbell[37] medium: 100 mg of glucose, 100 mg of yeast extract (Difco), 50 mg of sodium chloride, 0.1 mL of salt mixture A, 1.0 g of calcium chloride dihydrate, 2.0 g of Bacto-agar (Difco), and water to 100 mL. The above medium minus the agar was adjusted to pH 6.8 before autoclaving. Salt mixture A contained 100 mg of ferrous sulfate heptahydrate, 2.5 g of magnesium sulfate heptahydrate, and water to 100 mL. Plates were grown at 25 °C for 14 days prior to inoculating seed tubes.

(b) Seed Tubes. Two or three colonies were removed from 14-day-old agar plates and were transferred to 25 × 150 mm culture tubes containing 10 mL of tryptic soy broth: 1.5 g of tryptone (Difco), 0.5 g of soytone (Difco), 0.5 g of sodium chloride, and water to 100 mL. No pH adjustment was made prior to autoclaving. Seed tubes were grown vertically without agitation at 25 °C for 5 days prior to inoculating seed flasks.

(c) Seed Flasks. The entire contents (10 mL) of one seed tube were used to inoculate each seed flask. Seed cultures were shaken (New Brunswick Scientific Co. gyrorotary Model G25 incubator shaker) at 25 °C and 300 rpm in 250-mL Erlenemeyer flasks containing 40 mL of the seed medium of Caltrider and Niss.[38] Seed flasks were grown for 96 h.

(d) Fermentation Flasks. Four milliliters of a 4 day old seed flask were used to inoculate each fermentation flask. Fermentations were carried out at 25 °C and 300 rpm in 250-mL Erlenmeyer flasks containing 40 mL of Demain's[39] medium. Each fermentation flask consisted of 2 mL of solution A and 20 mL of solution B which were sterilized separately and mixed prior to inoculation: solution A, 18.0 g of sucrose, 13.5 g of glucose, and water to 250 mL; solution B, 2.5 g of D-methionine, 3.75 g of ammonium sulfate, 0.75 g of methyl oleate, 3.75 mL of salts mixture 1, 67.5 mL of salts mixture 2, and water to 250 mL. Solution B minus the methyl oleate was adjusted to pH 7.4 prior to autoclaving. Salts mixture 1 contained 2.0 g of ferrous ammonium sulfate hexahydrate and

(57) "CRC Standard Mathematical Tables"; Selby, S. M., Ed.; CRC Press: Cleveland, 1975; pp 576–577.

100 mL of water. Salts mixture 2 contained 204 g of monobasic potassium phosphate, 208 g of dibasic potassium phosphate, 22.7 g of sodium sulfate decahydrate, 4.9 g of magnesium sulfate heptahydrate, 1.0 g of calcium chloride dihydrate, 400 mg of zinc sulfate heptahydrate, 400 mg of manganese sulfate monohydrate, and 100 mg of cupric sulfate pentahydrate in 1800 mL of water. Fermentation flasks were grown for 168 h.

(e) Incorporation of Chiral-Methyl Valine. Four fermentation flasks, prepared as described above, were used in each feeding experiment. A stock solution of the appropriate chiral-methyl valine was prepared by dissolving 40 mg of (*R*)- or (*S*)-methylvaline in 2.0 mL water. Each of the four flasks was fed (freshly autoclaved pipets and valine solution) with 0.1 mL of the stock valine solution after 92, 104, 116, 128, and 140 h of incubation. The mycelia were harvested after 168 h.

(f) Isolation Radiolabeled Cephalosporin C. After 168 h, the contents of the four fermentation flasks were centrifuged at 20000*g* for 30 min at 5–15 °C. The supernatant (150 mL) was adjusted to pH 5 with 6 N HCl and then assayed for cephalosporin C by HPLC (solvent,[40] 80:11.3:6:3, water/acetonitrile/methanol/acetic acid; flow rate, 2.0 mL/min; 10-μL injection). The broth was then applied to a 2 × 17 cm activated carbon column (25 g, Calgon Activated Carbon Type CAL 12 × 40, Calgon Corp., Pittsburgh, PA). The column was washed with 200 mL of water and then eluted with a 1:1 solution of acetone/water until HPLC indicated that no additional cephalosporin C was being eluted (~200 mL). The combined acetone/water eluants were then evaporated (rotovap) at 38 °C to a total volume of 6.0 mL. This viscous solution was then filtered through a 0.2 μm Millipore filter (Nalge Co.). This material was then applied in 1.0 mL fractions to a Whatman Magnum 9 HPLC column (solvent as above, flow rate 5.0 mL/min), and the fraction corresponding to cephalosporin C (retention time = 45 min) was collected (ca. 300 mL total volume was collected from the six injections). The solution containing the cephalosporin C was adjusted to pH 5 by bubbling ammonia gas through the stirred solution at 0–5 °C. This solution was concentrated to a volume of 30 mL on a rotovap at 38 °C and assayed for cephalosporin C. Analytical HPLC indicated that the cephalosporin C was >95% pure. The ultraviolet absorption maximum at 260 nm (ϵ = 8900)[58] indicated that the solution derived from (*R*)-methyl valine contained 17.2 mg of cephalosporin C, while that from (*S*)-methyl valine contained 17.2 mg of cephalosporin C, while that from (*S*)-methyl valine contained 22.4 mg of cephalosporin C. The cephalosporin C content of each of these solutions was then diluted to a total of 100 mg with cold cephalosporin C (86% by weight according to UV assay) and reduced to a volume of 10 mL (rotovap, 38 °C). The resulting yellow oil was lyophilized overnight (Virtis Model 10-020) to a yellow paste and then placed under high vacuum at 40 °C to sublime ammonium acetate. The material that remained after sublimation of the ammonium acetate was dissolved in 5 mL of water and relyophilized to yield 135 mg of a manila powder which was converted to its *N*-(*tert*-butoxycarbonyl)cephalosporin *C*-1β-oxide (**6**) and crystallized to constant specific radioactivity.

(g) Incorporation of D,L-(3*R*,4*R*)-[4-^{2}H,^{3}H]$_3$[4-^{14}C]Valine into Cephalosporin C. Experiment 1 in Table II was carried out as described in (a)–(f) above. Experiment 2 was analogous except that in (e), 20 mg of total valine were fed in five equal portions at 72, 84, 96, 108, and 120 h after inoculation. Mycelia from parallel sets of fermentations were harvested after 6.5 and 8 days. The cephalosporin C produced was isolated as in (f). D,L-[4-^{14}C]Valine was obtained from Research Products International Corp. (Elk Grove Village, IL). Specific activities and ^{3}H/^{14}C ratios for the triply labeled valines were accurately determined for the *N*-acetyl derivative crystallized to constant specific radioactivity.

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Note Added in Proof. We are pleased to note that since submission of this manuscript Prof. Crout (cf. ref 12) has published revised results more in keeping with the findings presented here.[59]

Registry No. 1, 32467-88-2; **2**, 516-06-3; **3** [R = δ-(L-α-aminoadipoyl)], 58678-43-6; **3** [R = δ-(D-α-aminoadipoyl)], 525-94-0; **4** (R = δ-aminoadipoyl; X = H), 26924-74-3; **4** (R = δ-aminoadipoyl; X = OH), 1476-46-6; **4** (R = δ-aminoadipoyl; X = OAc), 61-24-5; **5**, 96244-43-8; **5** labeled (isomer I), 96194-51-3; **5** labeled (isomer II), 96194-52-4; **6**, 96194-49-9; **7**, 96194-50-2; *N*-(*tert*-butoxycarbonyl)cephalosporin C, 51813-40-2.

(58) Lemke, P. A.; Brannon, D. R., ref 22, p 370–437.

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