analogously to $(CH_3O)_3P^{17}O$ except that phenol was used instead of methanol. $Ph_3P^{17}O$ (49 atom % ^{17}O) was prepared by oxidizing triphenylphosphine with the mixture $Et_3N/CCl_4/H_2{}^{17}O$ (5 equiv) in dry dimethoxyethane[26] followed by silica gel chromatography. $(PhO)_2P^{17}OO$ was a byproduct of the coupling reaction of [α-$^{17}O_2$]AMPS to cyanoethyl phosphate, the second step in the synthesis of chiral [α-^{17}O]ADP. $H_4P^{17}O_4{}^+ClO_4{}^-$ was obtained by dissolving $H_3P^{17}O_4$ (1 mmol) in 5 mL of D_2O followed by addition of 631 μL of 70% $HClO_4$. The final solution contained 1.4 M $HClO_4$ and 0.2 M $H_3P^{17}O_4$.

Spectral Methods. ^{17}O NMR spectra were obtained from a Bruker WM-300 spectrometer and ^{31}P NMR spectra from both WP-200 and WM-300 spectrometers. A deuterium lock was used in all cases. The ^{17}O chemical shifts reported are relative to external $H_2{}^{17}O$ (at 25 °C), and the ^{31}P chemical shifts are referenced to external 1 M H_3PO_4. The positive sign represents a downfield shift in both ^{17}O and ^{31}P NMR. Spectral simulations were performed with a program written by Drs. C. Cottrell and A. G. Marshall.

Most of the NMR work described in this paper dealt with ^{17}O-labeled compounds that were also enriched with ^{18}O. There are two different types of ^{31}P NMR work: in the so-called $^{31}P(^{17}O)$ NMR[7,8] a large spectral width and a large line broadening were used such that the broad signal due to ^{31}P–^{17}O species can be observed; in the determination of ^{18}O isotope shift,[4] a small spectral width and a small line broadening (or Gaussian multiplication) were used to obtain high resolution. In the latter case, the broad ^{31}P–^{17}O signal was not detectable.

MgADP was prepared from free ADP and puratronic-grade $Mg(NO_3)_2$ as previously described.[11] Sample sizes were 1.5 mL in most NMR experiments. The preparation of arginine kinase·ADP complexes for ^{31}P NMR studies followed essentially the procedure of Rao and Cohn.[24]

The estimated error in the measurements of "broad $^{31}P(^{17}O)$ NMR signals" is ±10%.

Acknowledgment. This work was supported by National Institutes of Health Grants GM 29041 (M.-D.T.) and GM 30480 (P.A.F.). The NMR spectrometers used (at The Ohio State University) were supported by National Institutes of Health Grant GM 27431 and National Science Foundation Grant CHE 7910019. We thank Drs. A. G. Marshall and C. Cottrell at The Ohio State University for providing computer programs for curve fitting, Ru-Tai Jiang and Yeun-Jung Shyy (also at The Ohio State University) for assistance in the synthesis of stereoisomers of $Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP, and Judy Hart for isolating arginine kinase.

Registry No. Λ-$Co(NH_3)_4{}^-$(S_p)-[α-^{17}O]ADP, 86119-73-5; Δ-$Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP, 86119-74-6; $Co(NH_3)_4$ADP, 63937-09-7; $Co(NH_3)_4$-[α-$^{17}O_2$]ADP, 80539-98-6; Mg[α-^{17}O]ADP, 86119-85-9; MgADP, 7384-99-8; [^{17}O]DPPC, 86119-75-7; DPPC, 2644-64-6; [α-^{17}O]ADP, 81246-59-5; (R_p)-[α-^{17}O]ADP, 83541-22-4; (S_+)-[α-^{17}O]-ADP, 85550-14-7; [α-$^{17}O_2$]ADP, 80547-13-3; [β-$^{17}O_3$,$\alpha\beta$-^{17}O]ADP, 80547-17-7; [α-$^{17}O_2$]AMPS, 80547-08-6; [α-^{17}O]-β-CNEt-ADPαS, 86119-83-7; $H_4P^{17}O_4{}^+ClO_4{}^-$, 86119-77-9; $KH_2P^{17}O_4$, 86119-78-0; $K_2HP^{17}O_4$, 86119-79-1; $(CH_3O)_3P^{17}O$, 80777-98-6; $Ph_3P^{17}O$, 86119-80-4; $(PhO)_3P^{17}O$, 86119-81-5; $(PhO)_2P^{17}OO$, 86119-82-6; $P^{17}OCl_3$, 66943-75-7; $H_3P^{17}OO_3$, 86119-84-8; P, 7723-14-0; ^{17}O, 13968-48-4; ^{18}O, 14797-71-8.

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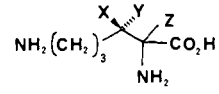
Stereochemistry of Lysine 2,3-Aminomutase Isolated from *Clostridium subterminale* Strain SB4

D. John Aberhart,*† Steven J. Gould,*‡[1] Horng-Jau Lin,† T. K. Thiruvengadam,‡ and Bruce H. Weiller†

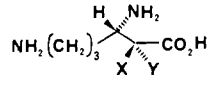
Contribution from the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, and School of Pharmacy, Section of Medicinal Chemistry and Pharmacognosy, The University of Connecticut, Storrs, Connecticut 06268.
Received December 2, 1982

Abstract: The stereochemistry of lysine 2,3-aminomutase in *Clostridium subterminale* strain SB4 has been elucidated. Deuterium NMR has been used to show that the transformation of (2*S*)-α-lysine to (3*S*)-β-lysine proceeds with transfer of the 3-*pro*-*R* hydrogen of α-lysine to the 2-*pro*-*R* position of β-lysine. The 3-*pro*-*S* hydrogen of α-lysine is retained at C-3 of β-lysine. Also the C-2 hydrogen of α-lysine is retained at the 2-*pro*-*S* position of β-lysine. Thus, the reaction proceeds with inversion of configuration at C-2 and C-3. Experiments with [2-^{15}N,3-^{13}C]-α-lysine have shown that the amino group transfer takes place completely intramolecularly. However, conversion of α-lysine-3,3-d_2 led to the formation of mainly β-lysine-d_1 indicating substantially or completely intermolecular hydrogen transfer in the reaction.

The transformation of α-L-lysine, **1a**, into β-L-lysine, **2a**, by the



1 a X = Y = Z = H (2S)
b X = Y = D, Z = H
c X = Y = H, Z = D
d X = D, Y = Z = H
e X = Z = H, Y = D



2 a X, Y = H_2
b X = H, Y = D
c X = D, Y = H

enzyme lysine 2,3-aminomutase constitutes the first step of a major metabolic pathway of lysine in *Clostridia* and other bacteria.[2] The transformation also takes place in several species of *Nocardia* or *Streptomyces*, in which the metabolic product, β-L-lysine, occurs as a constituent of several antibiotics, including myomycin[3] and related compounds,[4] viomycin,[5] roseothricin,[6] geomycin,[7] tuberactinomycin (containing γ-hydroxy-β-lysine),[8] and the strepto-

†Worcester Foundation for Experimental Biology.
‡University of Connecticut.

(1) Career Development Awardee of the National Cancer Institute (CA00627), 1979–1984. Present address: Department of Chemistry, Oregon State University, Corvallis, OR 97331.

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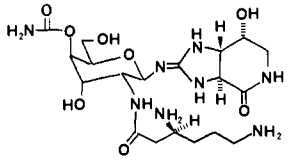
thricins (racemomycins)[9] as exemplified by streptothricin F (**3**).[10]



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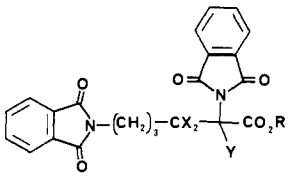


4 a R = Et, X = Y = H
b R = Me, X = Y = H
c R = Me, X = H, Y = D
d R = Me, X = D, Y = H

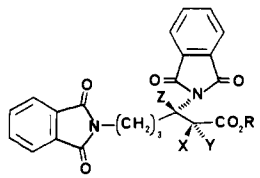
Although the lysine 2,3-aminomutase of *Streptomyces* has not been subjected to detailed study until recently,[10] Barker et al.[11] have purified the lysine 2,3-aminomutase from *Clostridium subterminale* strain SB4, ATCC 29748 (*C.* SB4), to near homogeneity. This air-sensitive enzyme has an equilibrium constant $K = (\beta\text{-L-Lys})/(\alpha\text{-L-Lys}) = 6.7$ at 37 °C. The interconversion of α-L-lysine and β-L-lysine in D_2O or T_2O was shown to occur with little or no exchange of C–H bonds with solvent hydrogens. The enzyme requires protein-bound ferrous ion, and is stimulated by *S*-adenosylmethionine and pyridoxal phosphate, containing at least 1.7 mol of protein-bound pyridoxal phosphate/mol. There was, however, no evidence for the involvement of coenzyme B_{12} in this reaction. In this regard, the rearrangement contrasts with a variety of apparently similar reactions involving a 1,2-interchange of hydrogen plus a second functional group. This class of reactions includes several aminomutase reactions, particularly β-lysine mutase,[12] α-D-lysine mutase,[13] and D-ornithine mutase.[14] In addition, Poston[15] has shown leucine 2,3-aminomutase to be coenzyme B_{12} dependent in a wide variety of organisms including animals, plants, and microorganisms. However, Overton et al.[16] reported no coenzyme B_{12} dependence for a leucine 2,3-aminomutase in tissue cultures of *Andrographis paniculata*. Other β-amino acids are also known.[17,18]

In view of the unique coenzyme requirements found for lysine 2,3-aminomutase as compared with other aminomutase reactions, it was of interest to study this reaction in more detail. In this paper, we describe our studies on the stereochemistry of the lysine 2,3-aminomutase in *C. subterminale* strain SB4.[20] We also report the results of experiments designed to test the *intra- vs. intermolecularity* of the hydrogen and amino group transfers in this reaction.[21]

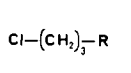
Results

Preliminary Studies: Analytical Methodology. *C. subterminale* SB4 (ATCC 29748) cells were grown on the α-lysine-rich medium described by Barker et al.[11,23] A cell-free extract was prepared by using a French pressure cell, followed by centrifugation. This cell-free extract, supplemented with PLP, Fe(II), DTT, and occasionally *S*-Ad-Met, and made freshly anaerobic by treatment with $Na_2S_2O_4$, as described by Barker et al.,[23] was used for most of the transformations of labeled α-lysines described in this work, except where noted otherwise.

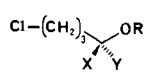
For assaying the lysine 2,3-aminomutase activity of such enzyme preparations, a convenient method of separating and/or detecting α- and β-lysine was required. In previous work[11,12,23-26] this was accomplished by a high-voltage paper electrophoresis technique. Larger scale separations of α- and β-lysine could be carried out by ion-exchange chromatography.[23] In our work, several different techniques were used. In most cases, following the incubation of a labeled lysine with the cell-free extract, the mixture was heated, centrifuged, and filtered, following which the crude amino acid mixture was recovered by absorption on a cation-exchange column and elution with ammonia. After being dried, the crude mixture was treated with phthalic anhydride followed by diazoethane[27] or diazomethane to yield a mixture containing the di-*N*-phthaloyl ethyl or methyl esters of α- and β-lysine, **4a** or **4b** and **5a** or **5b**, respectively. These could be readily separated by



5 a R = Et, X = Y = Z = H
b R = Me, X = Y = Z = H
c R = Et, X = D, Y = Z = H
d R = Et, X = Z = H, Y = D
e R = Et, X = H, Y = Z = D
f R = Et, X = Z = D, Y = H
g R = Me, X = D, Y = Z = H
h R = Et, X = Y = H, Z = D
i R = Me, X = H, Y = Z = D



6 a R = CO_2Et
b R = CDO



7 a R = H, X = Y = D
b R = Ms, X = Y = D
c R = H, X = D, Y = H
d R = H, X = H, Y = D
e R = Ms, X = D, Y = H
f R = Ms, X = H, Y = D

TLC. Radiochromatogram scanning, in runs starting with α-[^{14}C]lysine, gave a rough estimate of the extent of conversion to β-lysine. Typically, conversions of 25% of α-L-[^{14}C]lysine to β-lysine could be obtained with the crude cell-free extract, which was adequate for most of our purposes. In the later stages of our work, however, much better conversions were obtained with purified enzyme preparations.

A rough estimate of the β-lysine content of the crude amino acid mixture obtained by the ion-exchange procedure described above could also be made by examination of the ^{1}H NMR spectrum. The C-2 protons of β-lysine appear as a broadened doublet at δ 2.8, well separated from other signals of either α- or β-lysine. Although the product of β-lysine mutase, (3*S*,5*S*)-3,5-diaminohexanoic acid[12,28] also has absorption in this

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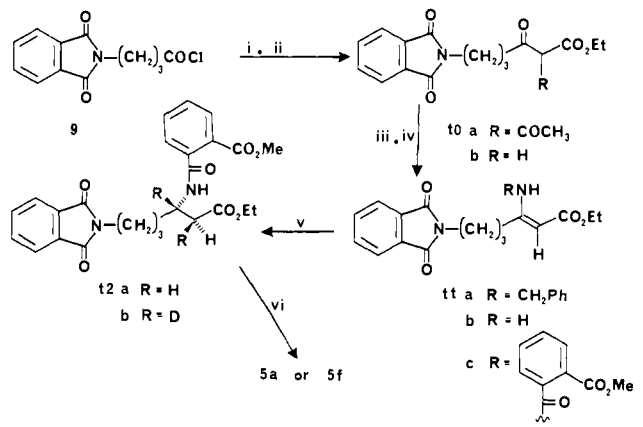
Scheme I. Synthesis of Stereospecifically Labeled (2*R*,3*R* + 2*S*,3*S*)-Di-*N*-phthaloyl-β-lysine-2,3-d_2 Ethyl Ester, 5d[a]



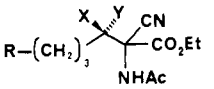
[a] Reagents: (i) ethyl acetoacetate/$Mg(OEt)_2$/ether; (ii) 10% NH_4OH; (iii) NH_3/EtOH; (iv) *o*-carbomethoxybenzoyl chloride/pyridine; (v) H_2 or $D_2/(Ph_3P)_3RhCl$; (vi) heat.

region (δ 3.0–3.1), it also exhibits a doublet at δ 1.64 for the C-6 methyl group. In most cases, with only one exception, no such methyl doublet was detectable in our incubation products (in one run, an unidentified minor doublet at δ 1.73 was observed).

In the later stages of our work a much faster and more sensitive method of assaying incubation mixtures for α- and β-lysine was developed. The method was an adaptation of a procedure reported by Schuster[29] for the separation of a wide variety of amino acids by HPLC, using an amino column with direct detection of the amino acids at ca. 200 nm without derivatization. The method was directly applicable to crude incubation mixtures containing α- and/or β-lysine (ca. 0.1–10 mg/mL), after preliminary filtration through a C_{18} Sep-PAK[30] cartridge. Typical chromatograms are shown in Figure 1.[31]

HPLC analysis of the cell-free system described above revealed the presence of moderate amounts (ca. 0.1–1 mg/mL) of both α- and β-lysine, even before incubations were carried out. Thus, some dilution of labeled materials was to be expected, and we therefore considered the use of deuterated precursors in conjunction with deuterium NMR to be the method of choice for stereochemical studies. In this regard, it was of particular value that the β-lysine could be isolated as the di-*N*-phthaloyl ethyl or methyl ester derivative **5a** or **5b** since the ^1H NMR spectra of these compounds[32] both exhibited well-separated signals for the C-2 and C-3 protons: C-2 H, δ_1 2.75, δ_2 3.19; C-3 H, δ_3 4.70; J_{12} = 16 Hz, J_{13} = 6 Hz, J_{23} = 9 Hz. However, since deuterium resonances, especially for higher molecular weight compounds, are notoriously broad,[33] we wished to confirm that the C-2 resonances of a deuterium-labeled sample could be resolved. For this purpose, (2*RS*)-β-lysine-2-d_1 (**2b** + **2c**) was prepared by refluxing unlabeled β-lysine[34] with concentrated HCl containing 20% DCl. The product was converted to the (2*RS*)-di-*N*-phthaloyllysine-2-d_1 ethyl ester derivative, **5c** + **5d**, whose ^2H NMR spectrum (Figure 2A) showed adequate resolution of the two C-2 signals at δ 2.74 and 3.17.

Incubation of Lysine-3,3-d_2 with Lysine 2,3-Aminomutase: Stereochemistry of Hydrogen Transfer at C-2. We first examined the question of whether a transfer of hydrogen(s) from C-3 of α-lysine to C-2 of β-lysine accompanies the amino group transfer from C-2 to C-3. Although such a transfer was expected from the lack of exchange with solvent hydrogens,[11] the stereochemistry of this *stereochemically cryptic*[35] process was unknown. For this purpose (2*RS*)-lysine-3,3-d_2 (**1b**) was synthesized.[36,37] Ethyl 4-chlorobutyrate (**6a**) was reduced with $LiAlD_4$ to 4-chloro-1-butanol-1,1-d_2 (**7a**). This was converted to the mesylate (**7b**),[38] which was treated with the sodium salt of ethyl acetamidocyanoacetate to yield the condensation product **8b**. This was



8 a X = Y = H, R = Cl
b X = Y = D, R = Cl
c X = Y = H, R = I
d X = Y = D, R = I
e X = Y = H, R = Phth
f X = Y = D, R = Phth
g X = D, Y = H, R = Cl
h X = H, Y = D, R = Cl
i X = D, Y = H, R = I
j X = H, Y = D, R = I
k X = D, Y = H, R = Phth
l X = H, Y = D, R = Phth

converted with NaI/acetone into the 6-iodo analogue **8d**,[39] which upon treatment with potassium phthalimide gave the 6-phthalimido derivative **8f**.[39] The mass spectrum of this intermediate showed only traces (2–3%) of d_1 or d_0 contaminants. Finally, acidic hydrolysis gave the required (2*RS*)-lysine-3,3-d_2 (**1b**).

The lysine-3,3-d_2 was incubated with the cell-free extract of *C.* SB4, giving after workup the β-lysine derivative **5e** (admixed with **5a**). The ^2H NMR spectrum of this product (Figure 2B) clearly showed the presence of equal amounts of deuterium at δ 4.69 (C-3 ^2H) and δ 3.18 (C-2 ^2H),[40] with only traces of absorption at other positions. The results show that one C-3 hydrogen (deuterium) atom is transferred to C-2 of β-lysine and the other C-3 hydrogen (deuterium) atom is retained at C-3.

In order to assign the absolute configuration at C-2 of β-lysine produced by deuterium transfer from C-3 in this process, it was necessary to assign the C-2 proton (deuteron) resonances of **5**. For this purpose, stereospecifically labeled **5f** was synthesized (Scheme I). 4-Phthalimidobutyryl chloride[41] (**9**) was converted, by treatment with the magnesium salt of ethyl acetoacetate,[42] into **10a**, which was then treated with dilute NH_4OH[43] to yield **10b**. Treatment of **10b** with benzylamine[44] resulted in the formation of **11a**. It was our intention to carry out catalytic reduction of **11a** to the saturated analogue, preferably with concurrent or

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(30) Sep-PAK is a trademark of Waters Associates, Inc.
(31) The method is analogous to a method published by Barker et al. for the separation of several diamino acids using an amino acid analyzer: Herbst, M. M.; Baltimore, B. G.; Bozler, G.; Barker, H. A. *Anal. Biochem.* **1974**, *58*, 322–330. The present method for detecting α- and β-lysine appears to be much faster but less sensitive than the method of Barker et al.
(32) For the ^1H NMR spectrum of **5a**, see ref 20a.
(33) Battersby, A. R.; Edington, C.; Fookes, C. J. R.; Hook, J. H. *J. Chem. Soc., Chem. Commun.* **1982**, 181–182.
(34) Pelizzoni, F.; Jommi, G. *Ann. Chim. (Rome)* **1959**, *49*, 1461–1463.
(35) Hanson, K. R.; Rose, I. A. *Acc. Chem. Res.* **1975**, *8*, 1–10.
(36) An alternative synthesis of **1b**, reported in our preliminary communication,[20] led to the formation of (2*RS*)-lysine-3,3-d_2 which (subsequent to publication) we found contained substantial d_1 content. Since the d_1 component, introduced as a result of some D–H exchange in the reduction of methyl 4-acetamidobutyrate with $NaBD_4$ in ethanol, would not have been stereospecifically labeled, no conclusions regarding the stereochemistry of the biosynthetic reaction reported in that paper are invalidated.
(37) For an alternative synthesis of lysine-3,3-d_2, see: Roessler, F.; Hesse, M. *Org. Mass Spectrom.* **1977**, *12*, 83–92.
(38) Pattison, F. L. M.; Millington, J. E. *Can. J. Chem.* **1956**, *34*, 757–768.
(39) Fields, M.; Walz, D. E.; Rothchild, S. *J. Am. Chem. Soc.* **1951**, *73*, 1000–1002.
(40) A minor amount of absorption at δ 2.75 may result from a little racemization in the rather vigorous derivatization step using phthalic anhydride at 180 °C. Also, a minor unidentified impurity appears at δ 4.03; this peak was not present in the products from three other runs with lysine-3,3-d_2.
(41) Kukalenko, S. S.; Gracheva, N. A.; Chilikin, L. G. *Zh. Org. Khim.* **1973**, *9*, 1401–1404.
(42) Lund, H.; Voigt, A. "Organic Syntheses"; Wiley: New York, 1943; Collect. *Vol.* II, pp 594–596.
(43) Viscontini, M.; Merckling, N. *Helv. Chim. Acta* **1952**, *35*, 2280–2282.
(44) Steck, E. A.; Brundage, R. P.; Fletcher, L. T. *J. Org. Chem.* **1959**, *24*, 1750–1752.

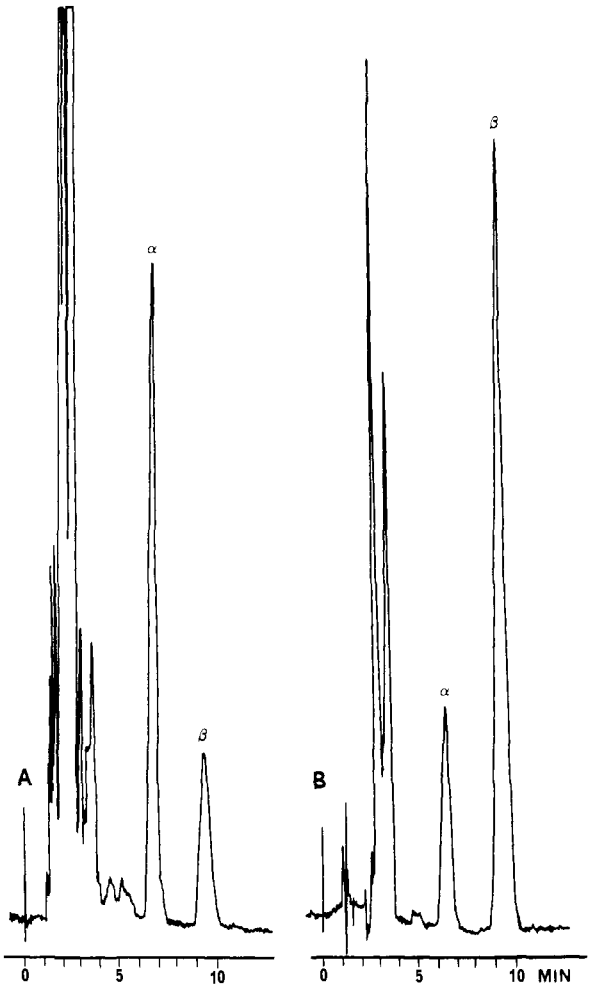


Figure 1. HPLC chromatograms of incubation products obtained after incubation of α-lysine with lysine 2,3-aminomutase. (a) Typical product from incubation of (2*RS*)-α-lysine with the crude cell-free extract of *C. subterminale* strain SB4; (b) Product from incubation of (2*S*)-α-lysine with purified lysine 2,3-aminomutase. Conditions: see Experimental Section.

subsequent elimination of the *N*-benzyl group. However, **11a** was very resistant to hydrogenation using Wilkinson's catalyst. As an alternative, **10b** was treated with dry ammonia in ethanol,[45] giving **11b**, which was acylated with *o*-carbomethoxybenzoyl chloride[46] in pyridine/$CHCl_3$ at 60 °C to form the (*Z*)-*o*-carbomethoxybenzamide (**11c**). The *Z* stereochemistry of the intermediate is assigned on the basis of the chemical shift of the vinyl proton, δ 5.10, which is highly characteristic of this stereochemistry in such compounds.[47,48] Reduction of **11c** with hydrogen or deuterium using Wilkinson's catalyst[49,50] gave racemic **12a** or **12b**, respectively, which on heating at 200 °C was converted to the desired, racemic **5a** or **5f**, respectively.[51] The latter showed in the ^1H NMR spectrum a slightly broadened singlet at δ 3.16, with only traces of absorption in the δ 2.8 region. It follows that, for such a compound having a 3*S* configuration, as in β-lysine formed by lysine 2,3-aminomutase, the 2-*pro-R* hydrogen appears

(45) Glickman, S. A.; Cope, A. C. *J. Am. Chem. Soc.* **1945**, *67*, 1017–1020.
(46) Burgstahler, A. W.; Eliel, E. L. *J. Am. Chem. Soc.* **1949**, *71*, 2251–2252.
(47) Schmidt, R. R.; Schwille, D.; Sommer, U. *Justus Liebigs Ann. Chem.* **1969**, *723*, 111–118.
(48) Aberhart, D. J.; Lin, H.-J. *J. Org. Chem.* **1981**, *46*, 3749–3751.
(49) Young, J. F.; Osborn, J. A.; Hardin, F. H.; Wilkinson, G. *J. Chem. Soc., Chem. Commun.* **1965**, 131–132.
(50) Achiwa, K.; Soga, T. *Tetrahedron Lett.* **1978**, 1119–1120.
(51) For similar two-step preparations of *N*-phthaloyl derivatives, see: (a) Balenovic, K.; Gaspert, B. *Chem. Ind. (London)* **1957**, 115. (b) Adriaens, P.; Meesschaert, B.; Janssen, G.; Dumon, L.; Eyssen, H. *Recl. Trav. Chim. Pays-Bas* **1978**, *97*, 260–262.

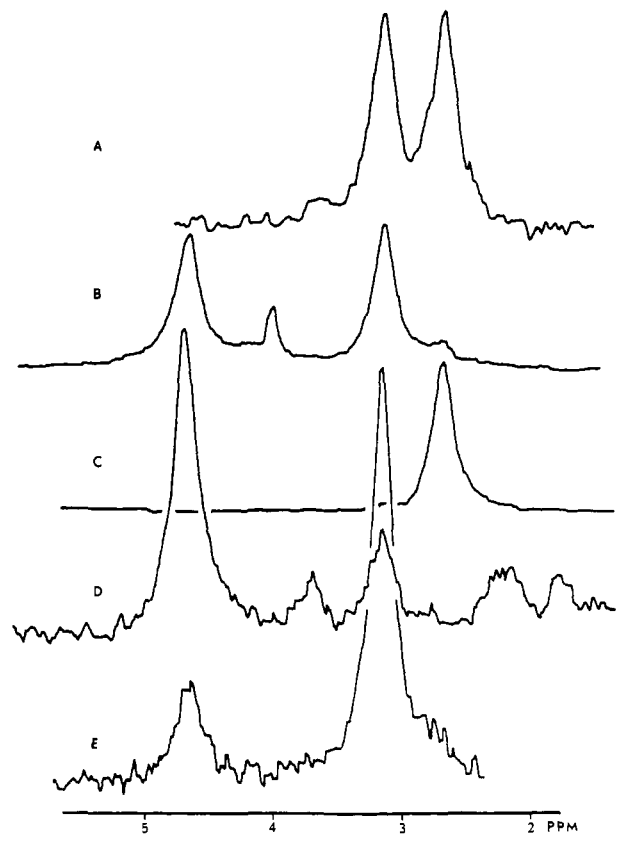


Figure 2. 41.44-MHz ^2H NMR spectra, 4000 data points; 1000 Hz sweep width (SW), 85° pulse, 0.5-Hz line broadening (LB), except (C) (2000-Hz SW, 50° pulse, 0-Hz LB): (A) synthetic (3*RS*)-(**5c** + **5d**), 394 transients; (B) biosynthetic product **5e** from **1b**, 1675 transients; (C) biosynthetic product **5g** from **1c**, 739 transients; (D) biosynthetic product **5h** from **1e**, 4682 transients; (E) biosynthetic product **5d** from **1d**, 1250 transients.

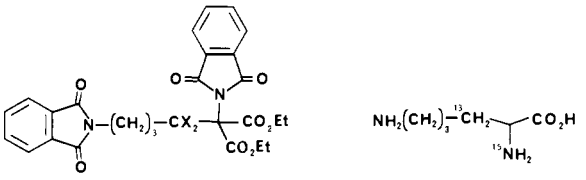
at δ 3.16 and the 2-*pro-S* hydrogen at δ 2.75. It therefore follows that the biosynthetic β-lysine derivative produced from (2*RS*)-lysine-3,3-d_2 bears deuterium in the 2-*pro-R* position as in **5e** and that the amino group of L-α-lysine is replaced in the migration by deuterium with *inversion* of configuration at C-2.

Incubation of Lysine-2-d_1 with Lysine 2,3-Aminomutase. The foregoing conclusion implies that the original C-2 hydrogen of α-lysine remains at the 2-*pro-S* position of the biosynthetic β-lysine. This would be anticipated from the apparent lack of exchange of hydrogens with the medium using *purified* lysine 2,3-aminomutase.[11] However, supporting evidence for this expectation was sought and found in the results of two experiments, the first using the crude cell-free enzyme preparation and the second using a partially purified enzyme.

Since the crude extract of *C.* SB4 contains an α-lysine racemase (but not a β-lysine racemase), it would be expected that α-lysine-2-d_1 would undergo exchange with the medium. Conversely, unlabeled lysine would incorporate a C-2 deuterium in a D_2O-containing medium. Thus, unlabeled lysine was incubated with a cell-free extract of *C.* SB4 cells prepared as previously described except that D_2O was used in place of H_2O for all solutions used in the extract preparation. After working up in the usual manner, di-*N*-phthaloyl-β-lysine methyl ester (**5g**) was obtained. Its ^2H NMR spectrum showed that deuterium labeling was primarily located, as expected, in the 2-*pro-S* position (δ 2.74, 76%). However significant labeling was also present at the 2-*pro-R* position (δ 3.14, 10%) and at C-3 (14%), probably as a result of de novo biosynthesis of lysine in the D_2O-rich medium.

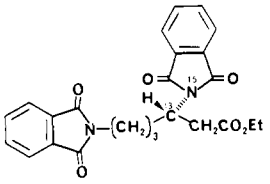
In a second approach to this problem, a cell-free homogenate of *C.* SB4 cells (in H_2O) was subjected to the heat step as described by Barker et al.[23] in their large-scale preparation of β-lysine. This heated enzyme preparation converted α-lysine to β-lysine in considerably better yield than did the crude enzyme preparation,

as shown by HPLC analysis, Figure 1B. Since it was anticipated that most, if not all, of the lysine racemase activity would be lost in the heat step, we required lysine-*2*-d_1 (**1c**). This was synthesized by hydrolysis of **13a**[52] in a mixture of DCl, D_2O, and CD_3CO_2D.

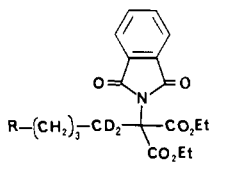


t3 a X = H
b X = D

t4



t5



t6 a R = Cl
b R = I

The product showed only a trace of absorption in the ^1H NMR for a C-2 proton. A portion of the product was converted to the di-*N*-phthaloyl methyl ester derivative **4c**, whose ^2H NMR showed a single peak at δ 4.82. Also the mass spectrum indicated a deuterium content of 91% d_1 and 9% d_0.[53,54] The (2*RS*)-lysine-*2*-d_1 (**1c**) was then converted by the heated enzyme preparation to β-lysine (**2c**), which was isolated by the reported ion-exchange technique.[23] A portion of the product was converted to **5g**, which showed a single ^2H signal at δ 2.77 (Figure 2c). The mass spectrum of this product indicated less than the expected deuterium content (70% d_1, 30% d_0), but the results strongly support the conclusion that the C-2 hydrogen (deuteron) of α-lysine is retained at the α-position in the conversion by lysine 2,3-aminomutase to β-lysine, in which it occupies the 2-*pro-S* position, as in **2c**.

Stereochemistry of Hydrogen Transfer from C-3. The foregoing results establish that one of the diastereotopic C-3 hydrogens of α-L-lysine is transferred to C-2 *pro-R* in the lysine 2,3-aminomutase reaction in *Clostridia*. For the identification of the transferred hydrogen, (2*RS*,3*R*)-lysine-*3*-d_1 (**1d**) and (2*RS*,3*S*)-lysine-*3*-d_1 (**1e**) were synthesized. 4-Chloro-1-butanol-*1,1*-d_2 (**7a**) was oxidized with pyridinium chlorochromate[55] to 4-chlorobutyraldehyde-*1*-d_1 (**6b**), which was reduced with either (+)- or (−)-pinanyl-9-BBN[56,57] to yield (1*S*)-4-chloro-1-butanol-*1*-d_1 (**7c**) or (1*R*)-4-chloro-1-butanol-*1*-d_1 (**7d**), respectively. The absolute configurations of these products were expected from literature precedents and were supported by NMR analyses of the corresponding (−)-camphanate esters,[58] which showed the expected relative shifts of the C-1 protons (*pro-S* hydrogen more strongly shifted) and which also showed that their configurational purities were ca. 90–95%. Furthermore, a sample of **7c** was converted, by prolonged refluxing with $LiAlH_4$ in THF, into (1*S*)-1-butanol-*1*-d_1, whose (−)-camphanate ester also showed the expected shifting behavior and high configurational purity. The latter sequence eliminates any possibility that the chlorine atom of **6b** or **7** could cause a reversal of both the expected steric course of reduction and the shifting behavior of the (−)-camphanate esters. The alcohols **7c** and **7d** were then converted into the corresponding mesylates **7e** and **7f**, which were converted, as before, to the condensation products **8g** and **8h**, respectively. These were then converted, via the 6-iodo intermedates **8i** and **8j**, and the 6-phthalimido intermediates **8k** and **8l**, into the required lysines **1d** and **1e**. These were then incubated with the crude lysine 2,3-aminomutase from *C.* SB4, and the resultant β-lysines isolated as the di-*N*-phthaloyl ethyl ester derivatives. Their ^2H NMR spectra showed that they are primarily labeled as shown in **5d** [from (3*R*)-lysine-d_1 (**1d**)] and **5h** [from (3*S*)-lysine-d_1 (**1e**)]. The deuterium spectra (parts e and d of Figure 2, respectively) show some nonstereospecific labeling, probably as a result of some incomplete stereospecific labeling in the precursors and/or extraneous biochemical process occurring concurrently. However, it is clear from the spectra that, for the most part, the 3-*pro-R* hydrogen of α-lysine is transferred to C-2 *pro-R*, and the 3-*pro-S* hydrogen retained at C-3. Thus, the aminomutase reaction proceeds with *inversion* of configuration at both migration termini.

Intramolecularity of Amino Group and Hydrogen Transfers. We wished to determine whether the transfer of the amino group and hydrogen atoms in this rearrangement occurred in a completely intramolecular or intermolecular manner or by a combination of the two. For the study of the amino group transfer, (2*RS*)-[3-^{13}C,2-^{15}N]lysine (**14**) was synthesized.[10c,21] On the basis of the isotopic content of the synthetic reagents, this product was 91 atom % enriched in ^{13}C and 98 atom % enriched in ^{15}N. In order to minimize the possibility of recombination of labeled species in the event that the amino group transfer occurred intermolecularly, the labeled compound (10%) was mixed with unlabeled (2*RS*)-lysine (90%). The mixture was then incubated with a cell-free extract of *C.* SB4, and the β-lysine isolated as the di-*N*-phthaloyl ethyl ester derivative **15**. The ^{13}C NMR spectrum of **15** was practically identical with the ^{13}C NMR of the unlabeled analogue **5a**, except that a singlet at δ 47.69 in **5a** was now flanked by a doublet, J = 8.35 Hz, of ca. 4-fold greater intensity than the residual singlet, δ 47.66 in **15**, which compared with other signals was not significantly changed in intensity. The 4-fold enrichment observed was considerably less than expected (8-fold) and probably results from dilution of the labeled precursor with endogenous lysine in the enzyme preparation. It is clear from the spectrum that virtually all of the β-lysine formed from the ^{13}C-enriched precursor contains a ^{13}C–^{15}N bond. The result, therefore, establishes that, in lysine 2,3-aminomutase from *C.* SB4, the amino group transfer occurs by an *intra*molecular process.

Our approach to the study of the intra-/intermolecularity of the hydrogen transfer from C-3 of α-lysine to C-2 of β-lysine was to incubate (2*RS*)-lysine-*3,3*-d_2 (**1b**), admixed with unlabeled (2*RS*)-lysine, and to determine by mass spectrometry the extent of deuterium enrichment in the product. Thus, a mixture of **1b** (10%) plus **1a** (90%) was incubated with a cell-free extract of *C.* SB4. Examination of the isolated β-lysine derivative **5e** by mass spectrometry revealed an isotopic distribution of d_0, 93%, d_1, 7%, and d_2, indistinguishable from zero. Since the deuterium content of the precursor was rather low, a very small amount of d_2-labeled product might have been missed, but it is certain that practically all of the deuterated product was d_1. It should be recalled that the mass spectrum of the final synthetic intermediate **8f** in the synthesis of **1b** showed a very high d_2 content, and it is extremely unlikely that any C-3 deuterium could be lost in the acidic hydrolysis of this compound.

In view of this unexpected result, we wished to repeat the experiment using a more highly purified preparation of lysine 2,3-aminomutase. Thus, the crude enzyme was subjected to the purification sequence described by Barker et al.,[11] proceeding through the heat and dialysis stage (step 4).[11] The enzyme at this stage cleanly converted L-α-lysine into a mixture of α- and

(52) Fink, R. M.; Enns, T.; Kimball, C. P.; Silberstein, H. E.; Bale, W. F.; Madden, S. C.; Whipple, G. H. *J. Exp. Med.* **1944**, *80*, 455–475.

(53) This deuterium content is regarded as a minimum, since racemization has been reported in the treatment of α-amino acids with phthalic anhydride at >150 °C: (a) Sheehan, J. C.; Chapman, D. W.; Roth, R. W. *J. Am. Chem. Soc.* **1952**, *74*, 3822–3825. (b) Bose, A. K.; Greer, F.; Price, C. C. *J. Org. Chem.* **1958**, *23*, 1335–1338.

(54) For an alternative synthesis of lysine-*2*-d_1, see: Battersby, A. R.; Murphy, R.; Staunton, J. *J. Chem. Soc., Perkin Trans. 1*, **1982**, 449–453.

(55) Corey, E. J.; Suggs, W. J. *Tetrahedron Lett.* **1975**, 2647–2650.

(56) (a) Midland, M. M.; Greer, S.; Tramontano, A.; Zderic, S. A. *J. Am. Chem. Soc.* **1979**, *101*, 2352–2355. (b) Midland, M. M.; Petre, J. E.; Zderic, S. A.; Kazubski, A. *J. Am. Chem. Soc.* **1982**, *104*, 528–531. (c) Brown, H. C.; Pai, G. G. *J. Org. Chem.* **1982**, *47*, 1608–1610.

(57) (a) Parry, R. J. *J. Chem. Soc., Chem. Commun.* **1978**, 294–295. (b) Parry, R. J.; Trainor, D. A. *J. Am. Chem. Soc.* **1978**, *100*, 5243–5244. (c) Parry, R. J.; Naidu, M. V. *J. Am. Chem. Soc.* **1982**, *104*, 3217–3219.

(58) Gerlach, H.; Zagalak, B. *J. Chem. Soc., Chem. Commun.* **1973**, 274–276.

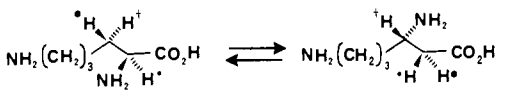
β-lysine having approximately the reported equilibrium ratio,[11] as shown by HPLC. Also, the enzyme was free of any detectable α-lysine racemase activity, as measured by the standard manometric method.[59]

Since our supplies of (2*RS*)-lysine-*3,3*-d_2 (**1b**) were exhausted, a new synthesis of **1b** was developed by using a route similar to that used for the synthesis of **14**. Thus, 4-chloro-1-butanol-*1,1*-d_2 mesylate (**7b**) was treated with the sodium salt of diethyl phthalimidomalonate[52] to give **16a** (not isolated). The crude product was treated with NaI/acetone to give **16b** (not isolated) which, upon treatment with potassium phthalimide, gave **13b** (not isolated). Finally, acidic hydrolysis of **13b** gave crystalline (2*RS*)-lysine-*3,3*-d_2 (**1b**). Although the overall yield was moderate, the sequence involved no intermediary purification steps and is readily amenable to large-scale work. A portion of the product was converted to the di-*N*-phthaloyl methyl ester derivative **4d**, which showed d_0, 0.3%, d_1, 2.8%, and d_2, 96.9%, by mass spectrometry.

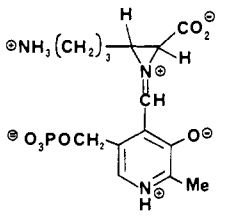
The (2*RS*)-lysine-*3,3*-d_2 (**1b**), mixed with unlabeled (2*RS*)-lysine (**1a**) to a ratio **1b**:**1a** = 1:3, was then incubated with lysine 2,3-aminomutase, resulting in a 1:5 mixture of β-:α-lysine. The β-lysine was purified as the di-*N*-phthaloyl methyl ester derivative **5i**. Its mass spectrum indicated an isotope distribution: d_2, 88%; d_1, 9%; d_2, 3%. These results were therefore consistent with the mass spectral results of the previous conversion of (2*RS*)-lysine-*3,3*-d_2 to β-lysine, which showed essentially only monodeuterated product.[60]

Discussion

The results presented in this paper clearly show that in *C.* SB4, the lysine 2,3-aminomutase reaction proceeds with inversion of configuration at both C-2 and C-3, **17** ⇄ **18**. The reaction thus



17 18



19

has the same stereochemistry at the amino group migration terminus as that reported for the coenzyme B_{12} dependent β-lysine mutase reaction,[61] in which the 6-amino group of β-lysine replaces the 5-*pro-S* hydrogen to form (3*S*,5*S*)-3,5-diaminohexanoic acid with inversion of configuration at C-5. The stereochemistry of hydrogen transfer to C-6 in the β-lysine mutase reaction has not been reported. The only other aminomutase reaction subjected to stereochemical scrutiny[62] is the tyrosine α,β-mutase reaction, in which L-α-tyrosine is converted to (3*S*)-β-tyrosine. Parry has shown that the reaction proceeds with inversion of configuration at C-3 of tyrosine.[17b] The reaction requires ATP, but neither pyridoxal phosphate nor coenzyme B_{12} is required. Also, the reaction proceeds with exchange of nitrogen as well as the 3-*pro-S* hydrogen of α-tyrosine and both C-2 hydrogens of β-tyrosine with ammonium ions and hydrogens, respectively, in the medium. In view of these facts, and especially the markedly differing coenzyme requirements of β-lysine mutase and tyrosine α,β-mutase, as compared with those of lysine 2,3-aminomutase, it may be irrelevant to compare their stereochemistries with that of lysine 2,3-aminomutase.

The studies with (2*RS*)-[3-^{13}C,2-^{15}N]lysine (**14**) revealed that, in *C.* SB4, the amino group transfer occurred completely *intra*molecularly. In contrast, the conversion of (2*RS*)-lysine-*3,3*-d_2 (**1b**) to β-lysine proceeded to yield essentially only monodeuterated β-lysine. Furthermore, in every run, the β-lysine produced from lysine-*3,3*-d_2 contained an equal amount of deuterium at the 2-*pro-R* and 3-positions, as shown by ^{2}H NMR. Thus, unless equal amounts of hydrogen (deuterium) were exchanged from these positions, which seems most unlikely, the results can only be explained by concluding that hydrogen (deuterium) transfer occurs (at least in part) intermolecularly. This unexpected result would nonetheless be consistent with reports that lysine 2,3-aminomutase proceeds without exchange of C–H bonds with the medium.

A mechanistic interpretation of these results is unfortunately further complicated by the fact that the total deuterium content of the products, even in runs with purified lysine 2,3-aminomutase, was always less than expected from the deuterium content of the precursor. In the last run, the enzyme was dialyzed and could not have contained endogenous α- or β-lysine. It is conceivable that the rate of this reaction is substantially altered by the deuterium substitution, i.e., $k_H/k_D > 1$, so that unlabeled β-lysine is formed in preference to deuterated β-lysine from a mixture of $d_0 + d_2$ α-lysine. This point requires further study, which we plan to carry out in due course. For the present, it seems impossible to escape the conclusion that a predominately *inter*molecular hydrogen transfer occurs in the conversion of α-lysine to β-lysine.

Despite the considerable amount of work now completed in this and in previous studies[2a,11] of lysine 2,3-aminomutase, the mechanism remains unclear. It remains unknown whether the required pyridoxal phosphate engages in Schiff base formation and, if so, with which nitrogen. We had considered that an aziridine intermediate might be involved in the reaction, as has been suggested for other aminomutase reactions.[63,64] If an intermediate such as **19** is involved in this reaction, it should be noted that this requires the removal, at least temporarily, of one hydrogen from the substrate. No obvious acceptor, such as coenzyme B_{12}, is readily apparent from the known features of the enzyme, but the results obtained with lysine-*3,3*-d_2 suggest that there might be one operating, which is capable of delivering the abstracted hydrogen back to a different substrate molecule (possibly in a different subunit of this hexameric enzyme[2a]). Such a process would be consistent with the observed stereochemistry, the attack of nitrogen at C-3 and the delivery of the transferred hydrogen to C-2 both inverting the configuration at these centers without a requirement for extensive conformational changes in the substrate. However, we prefer not to speculate further about the mechanism until more experimental information about this enzyme becomes available.

Experimental Section

General. ^{1}H NMR spectra were run on a Varian EM-360 or EM-390 instrument. ^{2}H NMR spectra were run on a Bruker HX 270 instrument. IR spectra were run on a Perkin-Elmer 237 instrument. Mass spectra were measured on an AEI MS-902 or a Varian MAT 731 with EI at 70 eV. Deuterium contents were calculated by published methods.[65] Gas chromatography was performed by using a Varian Model 920 instrument containing a $^1/_4$ in. × 6 ft column packed with 15% SE-30. Liquid chromatography was performed with a Waters instrument equipped with an M-45 pump, a U6K injector, and a Model 450 variable-wavelength detector. Liquid scintillation counting was performed on a Nuclear

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(60) In our earlier experiments with lysine-*3,3*-d_2 synthesized by the alternative route reported in our preliminary communication,[20a] we also observed the formation of mainly β-lysine-d_1 in this conversion. However, this result was considered less reliable, since the precursor was actually a mixture of (d_1 + d_2)-labeled lysine. That is not the case, however, in the two runs discussed in this section.

(61) Rétey, J.; Kunz, F.; Arigoni, D.; Stadtman, T. C. *Helv. Chim. Acta* **1978**, *61*, 2989–2998. *Experientia* **1969**, *25*, 801–802.

(62) We, and others,[16] are investigating the stereochemistry of leucine 2,3-aminomutase.[15]

(63) Walsh, C. "Enzymatic Reaction Mechanisms"; W. H. Freeman: San Francisco, 1979; pp 659–661.

(64) Stadtman, T. C. *Enzymes*, *3rd Ed.* **1972**, *6*, 539–563.

(65) Budzikiewicz, H.; Djerassi, C.; Williams, D. H. "Structure Elucidation of Natural Products by Mass Spectrometry"; Holden-Day: San Francisco, 1964; Vol. 1, pp 34–36.

Chicago Mark III instrument. Samples were dissolved in 0.25 mL of H_2O, and then diluted with 10 mL of New England Nuclear Aquasol. Radiochromatogram scanning was performed on a Nuclear Chicago Actigraph III instrument. Lysine racemase tests were performed by using a Gilson differential respirometer. Pressure hydrogenations were carried out in a Parr 450-mL pressure reactor. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN. French pressure cell extracts were prepared by using an Aminco French pressure cell with a 1-in. diameter piston, at 20 000 psi, in an Aminco pressure cell press. $LiAlD_4$ was obtained from KOR Isotopes. D_2O, 38% DCl/D_2O, and CD_3CO_2D were obtained from Stohler Isotope Co. Deuterium gas was obtained from Matheson Gas Products. [U-^{14}C]Lysine was obtained from New England Nuclear.

Ethyl 2-Acetyl-3-oxo-6-phthalimidobutanoate (10a). A mixture of Mg (0.64 g, 0.026 g-atom), EtOH (2 mL), CCl_4 (0.5 mL), and ether (50 mL) was stirred at 25 °C for 5 h and then treated with a solution of ethyl acetoacetate[42] (3.1 g, 23.8 mmol) in ether (10 mL), added dropwise over 0.5 h. Stirring was continued for 4 h, and then the solution was cooled to 0 °C and treated dropwise over 1 h with 4-phthalimidobutanoyl chloride[41] (6.0 g, 23.8 mmol) in ether (70 mL). The resulting mixture was then stirred at 25 °C for 13 h, then cooled to 0 °C, and neutralized with 1 N H_2SO_4. The ether layer was washed with H_2O, saturated NaCl, dried (Na_2SO_4), and evaporated to yield *ethyl 2-acetyl-3-oxo-6-phthalimidohexanoate* (**10a**): 7.2 g (88%); plates from EtOH; mp 73–75 °C; ν_{max} (KBr) 3450, 1778, 1770, 1710, 1225 cm^{-1}; NMR ($CDCl_3$) δ 1.33 (3 H, t, J = 7 Hz), 2.05 (2 H, m), 2.33 (3 H, s), 2.78 (2 H, m), 3.75 (2 H, m), 4.23 (2 H, q, J = 7 Hz), 7.78 (4 H, m), 17.82 (1 H, s, D_2O exchangeable).

Anal. Calcd for $C_{18}H_{19}NO_6$: C, 62.60; H, 5.55. Found: C, 62.85; H, 5.64.

Ethyl 3-Oxo-6-phthalimidohexanoate (10b).[43] Ethyl 2-acetyl-3-oxo-6-phthalimidohexanoate (5 g, 14.5 mmol) in ether (75 mL) at 0 °C was treated with 10% NH_4OH (10 mL) with stirring. The mixture was allowed to warm to 10 °C and kept at this temperature for 90 min. The ether layer was separated and the aqueous layer acidified with 10% HCl to pH 2 and extracted with ether (4 × 10 mL). The combined ether extracts were dried (Na_2SO_4) and evaporated to give *ethyl 3-oxo-6-phthalimidohexanoate* (**10b**): 3.62 g (82%); prisms from EtOH; mp 50–51 °C; ν_{max} (KBr) 3450, 1778, 1710, 1225 cm^{-1}; NMR ($CDCl_3$) δ 1.26 (3 H, t, J = 7 Hz), 2.08 (2 H, m), 2.66 (2 H, m), 3.46 (2 H, s; C-2 H_2; D_2O exchangeable), 3.73 (2 H, t, J = 7 Hz), 4.18 (2 H, q, J = 7 Hz), 7.83 (4 H, m).

Anal. Calcd for $C_{16}H_{17}NO_5$: C, 63.36; H, 5.65. Found: C, 63.54; H, 5.75.

Ethyl 3-(*N*-Benzylamino)-6-phthalimidohex-2-en-1-oate (11a).[44,45] A mixture of **10b** (575 mg, 1.9 mmol), benzylamine (204 mg, 1.9 mmol), *p*-toluenesulfonic acid (15 mg), and benzene (15 mL) was refluxed for 13 h, with collection of H_2O in a Dean-Stark trap. The mixture was cooled to room temperature and filtered. Evaporation of the solvent under reduced pressure gave *ethyl 3-(N-benzylamino)-6-phthalimidohex-2-en-1-oate* (**11a**): 0.75 g, 97%; prisms from ethyl acetate; mp 129–130 °C; NMR ($CDCl_3$) δ 1.21 (3 H, t, J = 6 Hz), 1.78–2.34 (4 H, m), 3.68 (2 H, t, J = 7 Hz), 4.02 (2 H, q, J = 6 Hz), 4.34 (2 H, d, J = 6 Hz), 4.51 (1 H, s), 7.23 (5 H, m), 7.71 (4 H, m), 8.92 (1 H, br t, J = 6 Hz).

Anal. Calcd for $C_{23}H_{24}N_2O_4$: C, 70.39; H, 6.16. Found: C, 70.59; H, 6.11.

Attempted hydrogenation of **11a** using Wilkinson's catalyst was unsuccessful. Although hydrogenation occurred using a platinum catalyst, this could not be used for the desired stereospecific reduction.

Ethyl 3-Amino-6-phthalimidohex-2-enoate (11b).[45] A solution of ethyl 3-oxo-6-phthalimidohexanoate (**10b**) (2.23 g, 7.3 mmol) in EtOH (5 mL) was treated with dry NH_3 (ca. 50 mL/min) at 25 °C with stirring for 15 h. The mixture was evaporated under reduced pressure to give a residue which was crystallized from EtOH to yield *ethyl 3-amino-6-phthalimidohex-2-enoate*, (**11b**): 1.21 g (54%); prisms; mp 99–101 °C; NMR ($CDCl_3$) δ 1.27 (3 H, t, J = 7 Hz), 1.78–2.33 (4 H, m), 3.73 (2 H, t, J = 7 Hz), 4.05 (2 H, q, J = 7 Hz), 4.53 (1 H, s), 6.53 (2 H, br, $W_{1/2}$ = 10 Hz), 7.78 (4 H, m).

Anal. Calcd for $C_{16}H_{18}N_2O_4$: C, 63.57; H, 6.00. Found: C, 63.69; H, 6.18.

Ethyl (*Z*)-3-(*o*-Carbomethoxybenzamido)-6-phthalimidohex-2-enoate (11c). A solution of ethyl 3-amino-6-phthalimido-hex-2-enoate (3.0 g, 10 mmol) at 0 °C in $CHCl_3$ (30 mL) was treated dropwise with *o*-carbomethoxybenzoyl chloride[46] (3.62 g, 18 mmol) with stirring. Dry pyridine (3 mL) was added, and the mixture was then stirred at 60 °C for 12 h. After being cooled to room temperature the mixture was washed with diluted HCl (4 × 5 mL), H_2O, saturated $NaHCO_3$, and saturated NaCl. The $CHCl_3$ solution was then dried (Na_2SO_4) and evaporated to yield a residue, 6.7 g, which was chromatographed on column of silica gel (100–200 mesh, 50 g), eluting with a hexane–ethyl acetate gradient. The desired product, **11c**, 3.28 g (71%), was eluted with 35% EtOAc/hexane and was crystallized from EtOH: plates; mp 141–142 °C; NMR ($CDCl_3$) δ 1.27 (3 H, t, J = 7 Hz), 2.12 (2 H, m), 2.97 (2 H, m), 3.82 (2 H, m), 3.85 (3 H, s), 4.19 (2 H, q, J = 7 Hz), 5.10 (1 H, s), 7.37–7.88 (8 H, m), 11.40 (1 H, br s, $W_{1/2}$ = 6 Hz; D_2O exchangeable).

Anal. Calcd for $C_{25}H_{24}N_2O_7$: C, 64.65; H, 5.21. Found: C, 64.77; H, 5.32.

Ethyl 3-(*o*-Carbomethoxybenzamido)-6-phthalimidohexanoate (12a and 12b). A solution of ethyl (Z)-3-(*o*-carbomethoxybenzamido)-6-phthalimidohex-2-enoate (**11c**); (0.73 g, 1.57 mmol) in EtOH (35 mL) containing tris(triphenylphosphine)rhodium(III) chloride (0.14 g, 0.15 mmol) was hydrogenated at 420 psi in a Parr 450-mL pressure reactor at 25 °C for 36 h. The solvent was evaporated under reduced pressure to a residue which was recrystallized from EtOH to yield **12a**: 0.58 g, 79%; fine needles; mp 150–152 °C; NMR ($CDCl_3$) δ 1.24 (3 H, t, J = 7 Hz), 1.80 (4 H, m), 2.67 (2 H, d, J = 7 Hz), 3.75 (2 H, m), 3.85 (3 H, s), 4.20 (2 H, q, J = 7 Hz), 6.60 (br d, 1 H, J = 7 Hz), 7.51–7.85 (8 H, m).

Anal. Calcd for $C_{25}H_{26}N_2O_7$: C, 64.37; H, 5.62. Found: C, 64.26; H, 5.70.

In a similar manner, **11c** was reduced by using D_2 in place of H_2, yielding *ethyl (2R,3R + 2S,3S)-3-(o-carbomethoxybenzamido)-6-phthalimidohexanoate-2,3-d_2* (**12b**): NMR ($CDCl_3$) δ 1.25 (3 H, t, J = 7 Hz), 1.78 (4 H, m), 2.63 (1 H, br s, $W_{1/2}$ = 4 Hz), 3.77 (2 H, m), 3.82 (3 H, s), 4.11 (2 H, q, J = 7 Hz), 7.50–7.75 (8 H, m); the NH signal was not visible.

Ethyl 3,6-Bis(phthalimido)hexanoate (5a). Ethyl 3-(*o*-carbomethoxybenzamido)-6-phthalimidohexanoate (**12a**), 150 mg, was heated in an oil bath at 200 °C under light vacuum (ca. 200 mgHg) for 3 h. The mixture was separated by preparative TLC (solvent: 20% ethyl acetate–hexane) to give the product: 90 mg (64%); prisms from EtOAc/hexane; mp 95–97 °C; NMR ($CDCl_3$) δ 1.12 (3 H, t, J = 7 Hz), 1.5–2.2 (4 H, m), 2.75 and 3.23 (2 H, AB, also coupled to C-3 H; J_{AB} = 16 Hz, J_{AX} = 6 Hz, J_{BX} = 9 Hz), 3.69 (2 H, t, J = 7 Hz), 4.03 (2 H, q, J = 7 Hz), 4.72 (1 H, m), 7.76 (8 H, m).

Anal. Calcd for $C_{24}H_{22}N_2O_6$: C, 66.35; H, 5.10. Found: C, 66.34; H, 5.19.

Similarly, **12b** was heated at 200 °C, and the product, *ethyl (2R,3R + 2S,3S)-3,6-bis(phthalimido)hexanoate-2,3-d_2* (**5f**), was isolated by preparative TLC: NMR ($CDCl_3$) δ 1.12 (3 H, t, J = 7 Hz), 1.5–2.2 (4 H, m), 3.15 (1 H, br s, $W_{1/2}$ = 3 Hz), 3.67 (2 H, t, J = 7 Hz), 4.02 (2 H, q, J = 7 Hz), 7.7 (8 H, m).

4-Chlorobutanal-*1-d* **(6b).** To a mechanically stirred suspension of $LiAlD_4$ (20 g, 99% d) in absolute ether (800 mL) at −15 °C under N_2 was added *ethyl 4-chlorobutyrate* (**6a**) (110 g, 0.73 mol, neat) dropwise over 30 min while the temperature was kept below −10 °C with a dry ice–acetone bath. After the addition, the temperature was raised to +15 °C, and the mixture was stirred 30 min and then cooled to +5 °C. Then saturated Na_2SO_4 solution (84 mL) was added in 1-mL portions, maintaining the temperature at ca. 10–15 °C. The resultant white suspension was vacuum filtered and the residue thoroughly washed with ether. The combined filtrates were evaporated under reduced pressure and the product distilled to yield *4-chloro-1-butanol-1,1-d_2* (**7a**): 75.0 g (93%); bp 78–80 °C (10 mmHg); GLC retention time identical with authentic 4-chloro-1-butanol; NMR ($CDCl_3$) δ 1.2–2.3 (4 H, m), 3.4–3.7 (2 H, m, $-CH_2Cl$), 4.45 (1 H, –OH). A portion of this product, 40.0 g (0.36 mol), was added all at once to a stirred suspension of pyridinium chlorochromate[55] (118.6 g, 0.55 mol) in dichloromethane (800 mL; filtered through Al_2O_3, activity grade I). The mixture was magnetically stirred without cooling for 4 h. Absolute ether (800 mL) was added, and the mixture was stirred for 15 min. The resultant solution was decanted and filtered through a 5 × 10 cm column of Florisil (100–200 mesh). The residue in the reaction vessel was washed with ether/CH_2Cl_2 (1/1, 400 mL) followed by ether only (400 mL), and the decanted solvents were also filtered through the column. The filtrate was evaporated under reduced pressure to a light green oil, which was distilled to yield *4-chlorobutanal-1-d_1* (**6b**): 13.1 g, 33%; bp 44–48 °C (10 mmHg); NMR ($CDCl_3$) δ 1.7–2.3 (2 H, m), 2.5–2.9 (2 H, m), 3.5–3.8 (2 H, m); no detectable aldehyde signal was present.

(1*S*)- and (1*R*)-4-Chloro-1-butanol-*1-d_1* **(7c and 7d).**[56,57] To a solution of 9-BBN in THF (0.50 M, 220 mL; Aldrich) under N_2 at 25 °C was added (+)-α-pinene (Aldrich; $[\alpha]_D$ +46.5°, 14.98 g, 0.11 mol) and the solution was refluxed for 2 h. After the solution was cooled to +10 °C, *4-chlorobutanal-1-d_1* (**6b**) (11.7 g, 0.11 mol) was added all at once with magnetic stirring. The temperature rose rapidly to 30 °C. The solution was stirred without cooling for 2 h, then decanted into a N_2-filled flask, and evaporated under reduced pressure (ca. 10–20 mmHg, at 35–40 °C). The flask was filled with N_2 and cooled in ice–H_2O. Then

ether (180 mL) was added, and the solution was cooled in ice–H_2O and treated with ethanolamine (6.7 g, 0.11 mol) while stirring 10 min. The resultant white suspension was vacuum filtered and the residue was washed with ether. The filtrate was evaporated under reduced pressure to an oil. This was dissolved in THF (50 mL) and cooled in ice–H_2O to +10 °C. Then NaOH solution (3 M, 25 mL) was added. After the mixture was recooled to 10 °C, 30% H_2O_2 (15 mL) was added in 1-mL portions while the temperature was kept below 20 °C. The exothermic reaction ended after ca. 8–10 mL of 30% H_2O_2 had been added. Stirring was continued at 20 °C for 10 min, then H_2O (200 mL) was added, and the solution was extracted with ether. The ether extract was washed with saturated NaCl, dried (Na_2SO_4), and evaporated to an oil which was distilled to yield *(1S)-4-chloro-1-butanol-1-d_1* (**7c**): 8.26 g (69%); bp 78–85 °C (10 mmHg); analysis by GLC showed ca. 90–95% purity with some higher boiling impurities. The product was adequately pure for subsequent steps.

The stereoisomer, *(1R)-4-chloro-1-butanol-1-d_1* (**7d**), was prepared in exactly the manner except that (−)-α-pinene (Aldrich; $[\alpha]_D$ −42°) was used in place of (+)-pinene.

Samples (272 mg, 2.45 mmol) of the products were treated with (−)-camphanyl chloride[58] (1.0 g, 4.63 mmol; Fluka) while stirring in dry pyridine (4 ml) at 70 °C for 3 h. After the usual workup procedure, the corresponding *(−)-camphanate esters*, ca. 600 mg, were obtained as oils: NMR ($CDCl_3$) δ 0.98 (3 H, s), 1.09 (3 H, s), 1.15 (3 H, s), 1.4–2.9 (8 H, m), 3.58 (2 H, m), 4.24 (1 H, br s, $W_{1/2}$ = 12 Hz). A sample of *(1RS)-4-chloro-1-butanol-1-d_1* (**7c + 7d**), prepared from 4-chlorobutanal by reduction with $LiAlD_4$, as described above for the reduction of ethyl 4-chlorobutyrate, was similarly converted to the (−)-camphanate ester.

A portion (1.1 g) of *(1S)-4-chloro-1-butanol-1-d_1* (**7c**) in dry THF (25 mL) was refluxed with $LiAlH_4$ (1.0 g) for 72 h. The mixture was cooled and treated with saturated Na_2SO_4 and filtered, and the filtrate was evaporated. The resultant product was distilled in a Kugelrohr apparatus at 110–120 °C to yield *(1S)-1-butanol-1-d_1* (0.4 g) which was converted to the (−)-camphanate ester in the usual way. A sample of *(1RS)-1-butanol-1-d_1*, prepared by reduction of *n*-butyraldehyde with $LiAlD_4$ in ether at 25 °C followed by workup as above, was also converted into the (−)-camphanate ester.

4-Chloro-1-butanol-*1,1-d_2* Mesylate (7b) and (3*S*)- and (3*R*)-4-Chloro-1-butanol-*1-d_1* Mesylate (7e and 7f). The following procedure is typical. A solution of the *4-chloro-1-butanol* (**7a, 7c,** or **7d**) (4.0 g, 36.5 mmol) and methanesulfonyl chloride (4.6 g, 40.1 mmol) in CH_2Cl_2 (25 mL) at −78 °C was treated dropwise with triethylamine (4.1 g, 40.1 mmol). The mixture (paste) was allowed to warm to 25 °C and stirred at 25 °C for 2 h and then poured into cold H_2O (25 mL). The CH_2Cl_2 phase was separated, dried (Na_2SO_4), and evaporated to give an oil which was distilled to give the products **7b, 7e,** or **7f** (ca. 63%): bp 165–167 °C (14 mmHg) [lit.[38] bp 115 °C (0.5 mmHg)]; NMR ($CDCl_3$) δ 1.91 (4 H, m), 3.02 (3 H, s), 3.58 (2 H, m, $-CH_2Cl$), 4.25 (1 H, br s, $W_{1/2}$ = 8 Hz, $-CHDSO_2-$; absent in d_2 analogue).

Ethyl (2*RS*)-2-Acetamido-6-chloro-2-cyanohexanoate (8a) and *3,3-d_2* (8b), (3*R*)-*3-d_1* (8g), and (3*S*)-*3-d_1* (8h) Analogues. The following procedure is typical. Ethyl acetamidocyanoacetate (3.4 g, 20 mmol) was added to a solution of sodium ethoxide (20 mmol) in EtOH (20 mL). The solution was stirred at 60 °C for 10 min, then cooled to 25 °C, and treated dropwise with the unlabeled or labeled *4-chlorobutyl methanesulfonate* (3.73 g, 20 mmol). The resultant mixture was refluxed for 15 h, then cooled, and filtered. The solvent was evaporated under reduced pressure to give a brown syrup (5.5 g). This was chromatographed on a column of 85 g of silica gel, 100–200 mesh, by using a hexane–ethyl acetate gradient (initially pure hexane, increasing to 50% EtOAc/hexane). The product, 1.9 g, eluted by 50% EtOAc/hexane, was then crystallized from H_2O: prisms; mp 92–94 °C; ^1H NMR ($CDCl_3$) of **8a** δ 1.38 (3 H, t, *J* = 7 Hz), 1.60–2.30 (6 H, m), 2.13 (3 H, s), 3.57 (2 H, t, *J* = 7 Hz), 4.33 (2 H, q, *J* = 7 Hz), 6.95 (1 H, br s, $W_{1/2}$ = 4 Hz).

Anal. Calcd for $C_{11}H_{17}ClNO_3$: C, 50.68; H, 6.57. Found: C, 50.60; H, 6.62.

The ^2H NMR of **8g** and **8h** had signals at δ 2.11 and 2.26 (equal intensities); **8h** showed minor impurities (ca. 10%) absorbing at δ 3.54 and 4.32.

Ethyl (2*RS*)-2-Acetamido-2-cyano-6-iodohexanoate (8c) and *3,3-d_2* (8d), (3*R*)-*3-d_1* (8i), and (3*S*)-*3-d_1* (8j) Analogues. The following procedure is typical. *Ethyl 2-acetamido-6-chloro-2-cyanoacetate* (1.0 g, 3.85 mmol), NaI (4.6 g, 31 mmol), and acetone (20 mL) were refluxed for 16 h. The mixture was cooled, filtered, and evaporated under reduced pressure to a residue which was extracted with $CHCl_3$ and H_2O. The $CHCl_3$ phase was dried (Na_2SO_4) and evaporated to an oil, 1.14 g (essentially pure by TLC), which gave prisms from EtOH: mp 94–95 °C; NMR ($CDCl_3$) of **8c** δ 1.34 (3 H, t, *J* = 7 Hz), 1.6–2.4 (6 H, m), 2.08 (3 H, s), 3.16 (2 H, t, *J* = 6 Hz), 4.28 (2 H, q, *J* = 7 Hz), 7.86 (1 H, br s, $W_{1/2}$ = 4 Hz).

Anal. Calcd for $C_{11}H_{16}N_2O_3I$: C, 37.52; H, 4.87. Found: C, 37.71; H, 4.98.

The deuterated products **8d, 8i,** and **8j** were homogeneous on TLC and were used without crystallization for the next step.

Ethyl 2-Acetamido-2-cyano-6-phthalimidohexanoate (8e) and *3,3-d_2* (8f), (3*R*)-*3-d_1* (8k), and (3*S*)-*3-d_1* (8l) Analogues. The following procedure is representative. A mixture of ethyl 2-acetamido-2-cyano-6-iodohexanoate (1.14 g, 3.2 mmol) and potassium phthalimide (0.57 g, 3.6 mmol) was heated with stirring at 140 °C for 3.5 h. The mixture was cooled, triturated with EtOH (10 mL), and filtered. The filtrate was evaporated under reduced pressure to give 1.5 g of crude product from which pure *ethyl 2-acetamido-2-cyano-6-phthalimidohexanoate*, 0.53 g, was obtained by crystallization from EtOH: prisms; mp 170–172 °C (lit.[39] mp 170–171.5 °C); NMR ($CDCl_3$) of **8e** δ 1.36 (3 H, t, *J* = 7 Hz), 1.5–2.5 (6 H, m), 2.15 (3 H, s), 3.74 (2 H, t, *J* = 6 Hz), 4.34 (2 H, q, *J* = 7 Hz), 7.29 (1 H, br s, $W_{1/2}$ = 4 Hz), 7.82 (4 H, m). The 3,3-d_2 product **8f** had M^+ 373 (only traces, ca. 2–3%, detectable at *m/z* 372).

(2*RS*)-Lysine-*3,3-d_2* (1b) and (2*RS*,3*R*- and -3*S*)-Lysine-*3-d_1* (1d and 1e). The deuterated ethyl 2-acetamido-2-cyano-6-phthalimidohexanoates, **8f, 8k,** and **8e** (323 mg, 0.87 mmol), were refluxed with 6 N HCl (10 mL) for 15 h. After cooling and filtering off the precipitated phthalic acid and decolorization with charcoal, the filtrates were evaporated to give the crystalline lysines (as dihydrochloride) **1b, 1d,** and **1e**, ca. 0.2 g.

Alternative Synthesis of (2*RS*)-Lysine-*3,3-d_2* Monohydrochloride (1b). Diethyl phthalimidomalonate[52,66] (35 g, 0.115 mol) in 85 mL of dry toluene (distilled from Na spheres) was treated with Na spheres (2.62 g, 1 equiv) at reflux under N_2 for 90 min. After being cooled to room temperature, the mixture was filtered through a glass-fiber filter and the yellow residue washed with dry toluene until the washings were colorless. The residue was dried in vacuo, yielding 29.5 g of the sodium salt of diethyl phthalimidomalonate. The entire product was mixed with *4-chloro-1-butanol-1,1-d_2 mesylate* (**7b**) (16.78 g, 88.9 mmol) and xylenes (40 mL) and mechanically stirred at 140 °C (oil bath) for 8 h. TLC (solvent 20% EtOAc/hexane) indicated nearly complete conversion to *ethyl 6-chloro-2-phthalimido-2-carbethoxyhexanoate-3,3-d_2* (**16a**) (not isolated). The mixture was cooled to room temperature and diluted with $CHCl_3$ (500 mL) and H_2O (300 mL). The $CHCl_3$ phase was separated and dried (Na_2SO_4). The aqueous phase was backwashed once with $CHCl_3$, and the $CHCl_3$ extract was combined with the first extract. After evaporation of the $CHCl_3$ and xylene in vacuo, crude **16a**, 21.9 g, viscous red liquid, was obtained.

This product, 20.9 g, was treated with a mixture of acetone (400 mL) and NaI (40 g) at reflux under argon for 23 h. The mixture was cooled to room temperature and filtered, and then the solvent was evaporated under reduced pressure. The residue was triturated with $CHCl_3$ (2 × 250 mL) and filtered. The filtrate was extracted with H_2O (200 mL) and the aqueous extract backwashed with $CHCl_3$ (200 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and evaporated under reduced pressure to yield crude *ethyl 2-carbethoxy-6-iodo-2-phthalimidoehxanoate* (**16b**), 33.6 g (not isolated; ca 90% pure by TLC, solvent 20% EtOAc/hexane), viscous red liquid.

This product, 32.6 g, was mixed with potassium phthalimide (17.0 g) and xylenes (10 mL), and the mixture was mechanically stirred in an open flask at 155 °C (oil bath) for 5.5 h. During the last 2.5 h of reaction, N_2 was passed into the flask to aid evaporation of the xylenes. After being cooled to room temperature, the mixture was triturated with hot $CHCl_3$ (250 mL) and filtered. The residue was washed with additional hot $CHCl_3$ (200 mL). The filtrate was evaporated under reduced pressure and then pumped under high vacuum to yield crude *ethyl 2-carbethoxy-2,6-diphthalimidohexanoate-3,3-d_2*[52] (**13b**) (not isolated) as a red viscous glass, 36.0 g, >95% homogeneous on TLC (solvent 20% EtOAc/hexane).

The product, 35 g, was suspended in a mixture of acetic acid (50 mL), water (150 mL), and concentrated HCl (150 mL), and refluxed for 18 h. Then 100 mL was distilled from the mixture, water (50 mL) was added, and refluxing was continued for 6 h. The mixture was left at 25 °C overnight, then treated with charcoal, and filtered. The straw-colored filtrate was evaporated under reduced pressure to a volume of 150 mL. After being cooled, this was filtered through a glass-fiber filter and then reduced to a 50-mL volume. After a second treatment with charcoal and filtration, the filtrate was evaporated to a syrup (ca. 30 g). This was taken up in a hot mixture of H_2O (10 mL) and EtOH (50 mL) and

(66) Osterberg, E. A. "Organic Syntheses"; Wiley, New York, 1936; Collect. Vol. I, pp 266–267. The preparation of this compound was modified by elimination of the treatment of the crude product with benzene. Instead, the crude product was distributed between an ether/water mixture, and the ether phase was dried and evaporated to give diethyl phthalimidomalonate (82%).

treated with pyridine in 1-mL portions to neutrality. When the mixture was cooled at 4 °C, the crystalline product, 6 g, was obtained. After two recrystallizations from $EtOH/H_2O$, *(2RS)-lysine-3,3-d_2 hydrochloride* (**1b**), 2.1 g, was obtained: NMR (D_2O) δ 1.8 (6 H, br m), 3.23 (2 H, t, J = 7 Hz, C-6 H_2), 3.94 (1 H, slightly br s, C-α H).

A portion of the product (100 mg) was treated with phthalic anhydride (200 mg) in an open flask in an oil bath at 180 °C for 20 min. After being cooled, the product was triturated with a little MeOH, followed by treatment with excess CH_2N_2/ether. The product, *di-N-phthaloyl-lysine-3,3-d_2 methyl ester* (**4d**), was obtained after preparative TLC (solvent 35% EtOAc/hexane, running twice), as a glass, becoming crystalline on standing: NMR ($CDCl_3$) δ 1.0–2.0 (6 H, m), 3.62 (2 H, t, J = 7 Hz, C-6 H_2), 3.70 (3 H, s, OMe), 4.80 (1 H, sl br s, C-2 H), 7.8 (8 H, m); mass spectrum showed d_0, 0.3%, d_1, 2.8%, and d_2, 96.9%.

(2*RS*)-Lysine-2-d_1 Hydrochloride (1c). Ethyl 2-carbethoxy-2,6-diphthalimidohexanoate[52] (**13a**), 60 g, was treated with a mixture of CD_3CO_2D (50 g), D_2O (200 g), and 38% DCl/D_2O (200 g) at reflux for 18 h. Then 100 mL was distilled from the mixture, and refluxing was continued for an additional 16 h. After evaporation of the solvent, and isolation and recrystallization of the product as described above for the synthesis of **1b**, *(2RS)-lysine-2-d_1 hydrochloride* (**1c**), 7.44 g, was obtained; NMR (D_2O) showed only traces <0.05 H for the C-2 proton.

A portion (100 mg) of the product was converted, as described above, to (2*RS*)-di-*N*-phthaloyllysine-2-d_1 methyl ester (**4c**), which also showed in the ^1H NMR ($CDCl_3$) <0.05 H at δ 4.8 for the C-2 hydrogen: ^2H NMR ($CHCl_3$) δ 4.82. The mass spectrum showed 91% d_1 and 9% d_0.

Conversion of Labeled Lysines to β-Lysine by Cell-Free Extract of *Clostridium subterminale* Strain SB4 (*C.* SB4). The original culture of *C.* SB4 (ATCC 29748) was plated on Petri dishes prepared with the medium of Barker et al.,[11] with the addition of methylene blue (2.5 mg/L) and agar (2%). These were incubated anaerobically in a BBL anaerobe jar at 37 °C for periods of several days and then kept at 4 °C. Colonies were later transferred to 100-mL culture media (minus agar) prepared in 125-mL Wheaton bottles (boiled medium sealed under N_2 before sterilizing) and incubated at 37 °C for ca. 24 h. This resulted in dense cultures that remained viable for at least 1 year when kept at 4 °C.

For the production of cells on a large scale, an inoculum (ca. 25–50 mL) from a freshly grown 100-mL culture was transferred to 1500 mL of the same medium in a 2-L bottle and incubated at 37 °C for 12 h. The cells (2–2.5 g/L, wet weight) were collected by centrifugation, resuspended in cold (4 °C) 0.1 M potassium phosphate buffer, pH 7.5, and subjected to rupture in a French pressure cell at 20 000 psi. The resultant mixture was centrifuged at 40000*g* for 30 min, and the supernatant was decanted and readjusted from ca. pH 6.9 to 8.0 with dilute NaOH. Concentrated solutions of dithiothreitol, $FeSO_4$, and pyridoxal phosphate prepared in deaerated H_2O[23,67] were added to give final concentrations of 5, 0.4, and 0.03 mM, respectively.[23] A solution of lysine hydrochloride (100 mg) was prepared in 10 mL of deaerated H_2O, adjusted to pH 8.0 with NaOH, and flushed with N_2 or argon for several hours in a 25-mL volumetric flask. The cell-free extract (ca. 5–10 mL) was then added, and the solution made up to 25 mL with deaerated H_2O. A concentrated solution of $Na_2S_2O_4$ was added to give a final mixed concentration of 12 mM, and then the residual volume of the flask was flushed with N_2. The flask was sealed with a serum cap, and incubated stationary in a water bath at 37 °C for 16–20 h. The mixture was then acidified to pH 2.5 (HCl) and heated in a boiling water bath for 10 min. The mixture was then centrifuged, and the resultant green solution treated with a little charcoal and filtered in an Amicon filtration cell through a PM 10 filter. The amino acids were then recovered from the filtrate by absorption on a column of Dowex 50 W-X8, H^+ form, followed by elution with 2 N NH_4OH and evaporation of the eluate under reduced pressure. Finally, the residue was dissolved in H_2O and freeze-dried, generally yielding 200–300 mg of solids.

In most cases, a portion (100–150 mg) of the product was mixed with 200–300 mg of phthalic anhydride and heated in an open flask at 180 °C for 20 min, then rapidly cooled to room temperature, and triturated with MeOH. The mixture was then treated with excess diazoethane[27] or diazomethane in ether followed by evaporation. The product was separated by preparative TLC (solvent 30% EtOAc/hexane, running twice), giving **5**: prisms from MeOH; ca. 50–100-mg yield.

HPLC Analysis of α-/β-Lysine Mixtures.[29] A 1-mL aliquot of a solution (such as an incubation mixture) containing α- or β-lysine (or both), 0.1–10 mg/mL, was filtered through a C_{18} Sep-PAK[30] cartridge, and the filtrate (ca. 1–10 μL) was directly injected into a Waters μBondapak NH_2 column (3.9 mm i.d. × 30 cm, 10-μm particle size). The solvent was 30% 0.01 M KH_2PO_4/70% CH_3CN/H_2O (100/16),[29] pumped at 2 mL/min. Amino acids were detected by UV at 200 nm, 0.02 AUFS. Although a large number of highly UV absorbing impurities were also detected, the α- and β-lysine peaks usually were well resolved from other unidentified peaks. α- and β-Lysine gave identical detector responses. Retention times were for α-lysine 7.5 min and for β-lysine 10.5 min.

Preparation of (2*S*)-β-Lysine-2-d_1. *C.* SB4 cells (10 g, wet weight) were suspended in 0.05 M potassium phosphate buffer prepared by using D_2O, pH 8.0 (meter reading; not corrected for isotope effect), and a cell-free extract was prepared as described above. This was added to a solution of (2*RS*)-lysine (200 mg) in deaerated D_2O (20 mL) in a 50-mL volumetric flask under N_2. After addition of DTT, $FeSO_4$, PLP, and $Na_2S_2O_4$ as before, the flask was filled with D_2O, sealed, and incubated at 37 °C for 20 h. The crude amino acid product mixture was isolated as previously described, giving 462 mg after lyophilization. A portion of the product (143 mg) was treated with phthalic anhydride (300 mg) followed by diazomethane in the usual way to yield *(2S)-di-N-phthaloyl-β-lysine-2-d_1 methyl ester* (**5g**) (91 mg) (ca. 50% d, as estimated from ^1H NMR): ^1H NMR ($CDCl_3$) identical with that of the unlabeled analogue except δ 3.16 (0.5 H, J = 7 Hz) and overlapping signals for unlabeled carrier (ca. 0.5 H each) at δ 3.16 and 2.78; ^2H NMR ($CHCl_3$) δ 2.74 (rel area 62), 3.14 (7.0), 4.78 (11.3).

In a second run, *C.* SB4 cells (13 g, wet weight) were suspended in potassium phosphate buffer (in H_2O), pH 8.0 (60 mL), and ruptured by a French press at 20000 psi. The 40000*g* supernatant, readjusted to pH 8.0, was treated with DTT, $FeSO_4$, and PLP as usual. The mixture was then subjected to the heat step as described by Barker et al.[23] and then cooled and centrifuged at 40000*g*. The supernatant (40 mL) was added to a solution of (2*RS*)-lysine-2-d_1 (**1c**), 2.0 g, in deaerated H_2O (50 mL) containing (2*S*)-[U-^{14}C]lysine (50 μCi, 338 mCi/mmol) at pH 8.0 in a 100-mL volumetric flask under N_2. $Na_2S_2O_4$ was added to 12 mM (final concentration), and the flask was filled with deaerated H_2O and capped. The flask was then incubated at 37 °C for 20 h. HPLC analysis indicated an ca. 25% conversion to β-lysine [presumably 50% of (2*S*)-lysine converted]. The crude amino acid product mixture was isolated by the ion-exchange procedure described above (giving 2.15 g after lyophilization). This was dissolved in 20 mL of citrate/NaCl buffer,[23] and the pH was readjusted to 3.0. The solution was added to a 2.5 × 30 cm column of AG 50 W-X8 resin, 200–400 mesh (resin prewashed with 1 N HCl, H_2O, 1 N NaOH, and H_2O and finally equilibrated with citrate–NaCl buffer). The column was eluted with citrate–NaCl buffer at ca. 40 mL/h, and 20-min fractions were collected. The α-lysine was eluted in fractions 15–40. After 45 fractions were collected, the column was washed with distilled H_2O, followed by 2 N NH_4OH, and the first 300 mL of alkaline effluent was evaporated under reduced pressure. The residue was dissolved in a little H_2O and adjusted to pH 7.0 with dilute H_2SO_4 and dried under reduced pressure, giving *(2S)-β-lysine-2-d_1-sulfuric acid salt* (**2c**), 850 mg, 6 × 10^7 dpm.

A portion of the product was converted in the usual way to *(2S)-di-N-phthaloyl-β-lysine-2-d_1 methyl ester* (**5g**), which had 70% d_1 and 30% d_0 (mass spectrum): ^2H NMR ($CHCl_3$) δ 2.77 only.

Conversion of (2*RS*)-Lysine-3,3-d_2 to β-Lysine Using Purified Lysine 2,3-Aminomutase. A cell-free extract of *C.* SB4 cells was prepared in the usual manner. This was then subjected to the streptomycin treatment, ammonium sulfate cut, and heat–dialysis purification steps, exactly as described by Barker et al.[11] Although the specific activity of the resulting preparation was not accurately measured, it cleanly converted (2*S*)-lysine to β-lysine, resulting in an ca. 4:1 β:α ratio as measured by HPLC.[68] Also, no α-lysine racemase activity could be detected by the standard manometric assay procedure.[59]

The enzyme solution (1.0 mL) was added to a solution containing (2*RS*)-lysine-3,3-d_2 (**1b**) (125 mg) plus unlabeled (2*RS*)-lysine (375 mg) in 10 mL of deaerated H_2O at pH 8.0 in a volumetric flask under N_2. The flask was nearly filled with deaerated potassium phosphate buffer, pH 8.0 and DTT, $FeSO_4$, PLP, and $Na_2S_2O_4$ were added as usual. In addition, *S*-adenosylmethionine (1.14 mM, 0.1 mL) was added (final concentration 4.5 μM). The residual air was flushed with argon, and the flask was sealed and incubated at 37 °C for 20 h. HPLC showed an unexpectedly low β:α-lysine ratio (ca. 1:5). The amino acid product mixture was recovered in the usual way by Dowex 50 W-X8 ion exchange (recovery 550 mg), and a portion of the product converted to the *di-N-phthaloyl-β-lysine methyl ester* derivative (**5i**); the mass spectrum indicated d_0, 88%, d_1, 9%, and d_2, 3%.

Acknowledgment. We thank Dr. Stuart Shapiro for assistance with the techniques of handling anaerobic bacteria. We also thank

(67) Distilled H_2O was boiled, then cooled under N_2, and kept under N_2 until used.

(68) The presence of a contaminant overlapping the α-lysine peak made this analysis less accurate than usual.

Cliff McDonald for some of the ^1H NMR spectra, Peter Demou for the ^2H NMR spectra, recorded at the Northeast Regional NSF-NMR facility, Department of Chemistry, Yale University, supported in part by the National Science Foundation, Grant CHE-7916210, from the Chemistry Division, and Dr. Thomas Leipert, Department of Chemistry, University of Connecticut for the ^{13}C NMR spectra, recorded on an instrument purchased in part with Grant CHE 76-05739 from the National Science Foundation. We thank Dr. Catherine Costello for some of the mass spectra, provided by the facility at M.I.T., supported by National Institutes of Health Grant RR 00317 from the Biotechnology Resources Branch, Division of Research Resources. We thank Prof. H. A. Barker for some helpful information and a sample of β-lysine and (3*S*,5*S*)-diaminohexanoic acid. This work was supported by Grants GM 25919 (D.J.A.) and GM 25996 (S.J.G.) from the National Institutes of Health.

Registry No. **1b**·2HCl, 86146-77-2; **1b**·HCl, 86146-81-8; **1c**, 86146-83-0; **1d**·2HCl, 86195-10-0; **1e**·2HCl, 86195-11-1; **2c**, 86146-87-4; **4c**, 86146-85-2; **4d**, 86146-82-9; **5a**, 86195-07-5; **5f**, 86195-08-6; **5g**, 86146-84-1; **5i**, 86146-88-5; **6a**, 3153-36-4; **6b**, 79595-64-5; **7a**, 66502-79-2; **7b**, 86146-71-6; **7c**, 79595-65-6; **7d**, 79595-66-7; **7e**, 79595-67-8; **7f**, 79595-68-9; **8a**, 86146-72-7; **8b**, 86146-73-8; **8c**, 86146-74-9; **8d**, 86146-75-0; **8e**, 86162-04-1; **8f**, 86146-76-1; **10a**, 79595-56-5; **10b**, 79595-57-6; **11a**, 86146-68-1; **11b**, 79595-58-7; **11c**, 86146-69-2; **12a**, 86195-06-4; **12b**, 86146-70-5; **13a**, 86088-67-7; **13b**, 86146-80-7; **16a**, 86146-78-3; **16b**, 86146-79-4; lysine 2,3-aminomutase, 9075-20-1; 4-phthalimidobutanoyl chloride, 10314-06-4; (1*RS*)-4-chloro-1-butanol-*1*-d_1, 86195-09-7; ethyl 2-acetamido-6-chloro-2-cyanohexanoate-*3*-d_1, 79595-69-0; ethyl 2-acetamido-2-cyano-6-iodohexanoate-*3*-d_1, 79595-70-3; ethyl 2-acetamido-2-cyano-6-phthalimido hexanoate-*3*-d_1, 79791-12-1.

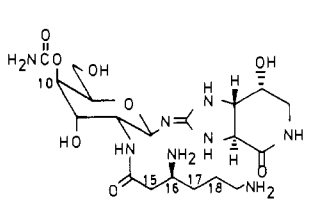
Biosynthesis of Streptothricin F. 5. Formation of β-Lysine by *Streptomyces* L-1689-23

T. K. Thiruvengadam,[†] Steven J. Gould,*[†1] D. John Aberhart,*[‡] and Horng-Jau Lin[‡]

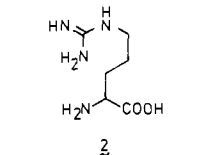
Contribution from the School of Pharmacy, Section of Medicinal Chemistry and Pharmacognosy, The University of Connecticut, Storrs, Connecticut 06268, and Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. *Received December 2, 1982*

Abstract: The formation of the β-lysine moiety of streptothricin F has been studied by feeding to *Streptomyces* L-1689-23 α-[3-^{13}C,^{15}N]-, α-[(3*RS*)-$^2H_2$]-, α-[(3*R*)-^2H]-, and α-[(3*S*)-^2H]lysine and β-[(2*S*)-^2H]lysine. From the analysis of either the ^{13}C NMR or ^2H NMR spectrum of the derived antibiotics, it has been determined that the α-nitrogen migrates to C-3 with inversion of configuration by an intramolecular process, and the 3-*pro-R* hydrogen migrates to C-2 with inversion of configuration by a process that is substantially or completely intermolecular. The very high degree of incorporation of labeled β-lysine indicates it is probably an intermediate in the biosynthesis of streptothricin F.

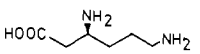
Streptothricin F (**1**),[2] representative of a large family of ubi-



1



2



3

quitous antibiotics, has been a major target in our studies on the biosynthesis of nitrogen-containing antibiotics.[3-6] Its structure was elucidated by chemical degradations[7] and, in part, by X-ray crystallography.[8] Recently, the location of the carbamate moiety was reassigned to C-10 on the basis of model studies,[9] and this was confirmed by the same authors by a total synthesis of **1**.[10] We have also assigned the carbamate to C-10 by a different approach, using deuterium-induced isotope shifts in ^{13}C NMR spectroscopy.[11] Thus, the antibiotic contains a gulosamine unit with three groups appended and is logically formed by a convergent biosynthetic pathway.

We have provided evidence for the specific incorporation of L-arginine (**2**) into the bicyclic moiety (streptolidine),[12] and through feedings of **2** labeled with ^{13}C and ^{15}N or with ^2H we have subsequently revealed the chemistry of this branch of the biosynthesis of **1**.[6] While all members of the streptothricin family contain D-gulosamine and the streptolidine moiety, the amino acid side chain at C-8 is variable.[13] However, L-β-lysine (**3**) is the most frequently encountered side chain, and all homologues up to seven β-lysine units (as a polyamide side chain) are known. The formation of β-lysine has been a particularly intriguing problem.

Naturally occurring β-amino acids are relatively rare and include β-lysine, β-alanine (**4**), β-tyrosine (**5**),[14] N^6-methyl-β-arginine (**6**),[15] β-leucine (**7**),[16] (dimethylamino)-β-phenylalanine (**8**),[17] and

[†] University of Connecticut.
[‡] Worcester Foundation for Experimental Biology.

(1) Career Development Awardee of the National Cancer Institute (CA00627), 1979-1984. Present address: Department of Chemistry, Oregon State University, Corvallis, OR 97331.
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