Biosynthesis of Blasticidin S from L-α-Arginine. Stereochemistry in the Arginine-2,3-aminomutase Reaction

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Abstract: A series of labeled α-arginines have been fed to fermentations of *Streptomyces griseochromogenes* in order to examine the mechanism of L-β-arginine formation in the biosynthesis of the antibiotic blasticidin S. [3-^{13}C,2-^{15}N]Arginine was synthesized and fed; analysis of the derived antibiotic by ^{13}C NMR spectroscopy revealed the retention of the original α-nitrogen and its intramolecular migration to the β-position, revealing the presence of an arginine-2,3-aminomutase. Feedings of [2,3,3-$^{2}H_3$]-, [3,3-$^{2}H_2$]-, and [2-^{2}H]arginines revealed the complete retention of the original β-hydrogens with migration of one to the α-position, as well as partial loss of the original α-hydrogen presumably due to arginine racemase activity. (3*R*)-[3-^{2}H]- and (3*S*)-[3-^{2}H]arginines were synthesized unambiguously and used to determine that the *pro*-3*R* hydrogen of α-arginine migrates to the α-position (C-2). δ-*N*-[$^{13}CH_3$]Methylarginine was synthesized, mixed with [*guanidino*-^{14}C]arginine, and fed to *S. griseochromogenes*. A 42% incorporation of radioactivity from arginine was obtained, but no ^{13}C enrichment was observed in the blasticidin S sample, indicating that arginine, itself, is the aminomutase substrate.

Blasticidin S, **1**, an antifungal antibiotic produced by *Streptomyces griseochromogenes*, was first isolated by Takeuchi et al. in 1958.[2] Its structure was elucidated by chemical degradation,[3] and the absolute configuration was assigned on the basis of the work of Fox[4] and Yonehara.[5] X-ray diffraction studies[6] have supported these results. Biosynthetic studies on this antibiotic were originally carried out by Seto et al.,[7] and it was found that the primary precursors were cytosine, D-glucose, L-arginine, and L-methionine (Scheme I). Their work also led to the isolation of a number of related metabolites such as leucylblasticidin S,[8] the pentopyranines,[9] pentopyranic acid,[10] blasticidin H,[11] and demethylblasticidin S.[12] Some of these may be intermediates in the biosynthesis of blasticidin S. We have recently reported preliminary results concerning the conversion of L-α-arginine to the L-β-arginine moiety of **1**.[13]

In addition to blasticidin, β-arginine is also found in the side chain of the cyclic peptide antibiotic LL-BM547β.[14] Naturally occurring β-amino acids are relatively rare and include β-lysine,[15] β-alanine, β-tyrosine,[16] β-leucine,[17] (dimethylamino)-β-phenylalanine,[18] and *N*-methyl-β-glutamic acid.[19] β-Alanine is known to be formed from degradation of uracil[20] or from the decarboxylation of aspartic acid.[21] Detailed studies on two others, β-tyrosine, **2**,[22] and β-lysine, **3**,[23] have appeared in the literature, and in each case the results indicated fundamentally different mechanisms for their formation.

β-Tyrosine (found in the antibiotic edeine) is formed from tyrosine by the loss of the original α-nitrogen and the *pro*-3*S* hydrogen (indicative of an ammonialyase type of process), as shown in Scheme II,[22] whereas β-lysine is formed from L-α-lysine

Table I. ^{1}H NMR Spectral Data

proton position	chemical shift multiplicity (*J*, Hz)	proton position	chemical shift multiplicity (*J*, Hz)
H-3	6.02, d (7.5)	H-12a	2.74, dd (16.2, 4.8)
H-4	7.59, d (7.5)	H-12b	2.62, dd (16.2, 8.1)
H-5	6.46, d (0.9)	H-13	3.64, m
H-6	6.09, ddd (10.2, 1.8, 0.9)	H-14	2.03, m
H-7	5.85, dd (10.2, 2.4)	H-15	3.46, t (7.8)
H-8	4.73, ddd (9.3, 2.4, 1.8)	H-16	3.02, s
H-9	4.10, d (9.3)		

Table II. ^{13}C NMR Spectral Data

carbon position	chemical shift	carbon position	chemical shift	carbon position	chemical shift
1	158.5	7	128.2	13	48.7
2	159.6	8	47.6	14	31.3
3	98.7	9	79.7	15	48.6
4	144.9	10	177.1	16	37.8
5	81.8	11	172.9	17	168.3
6	135.0	12	39.1		

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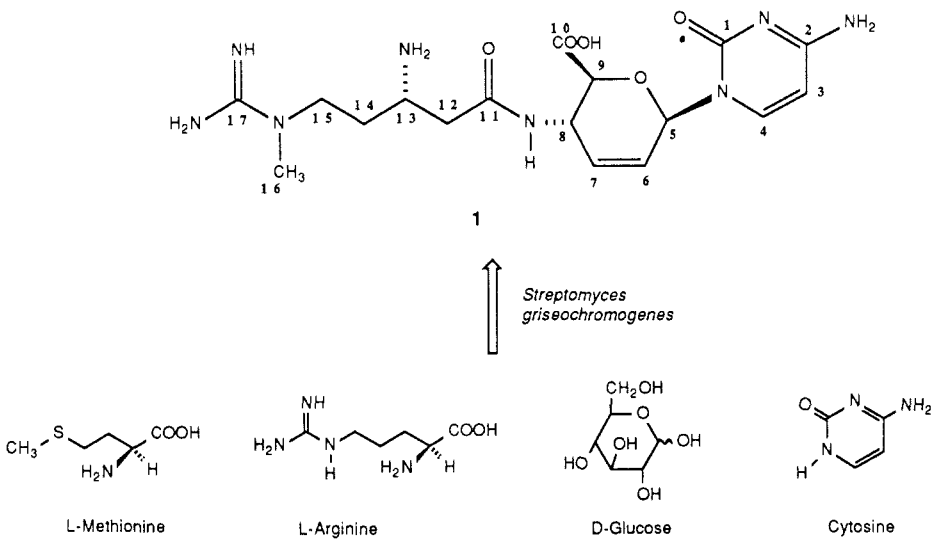
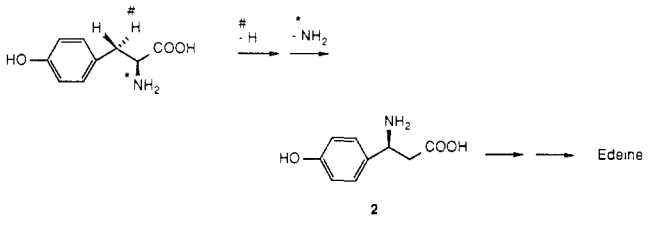
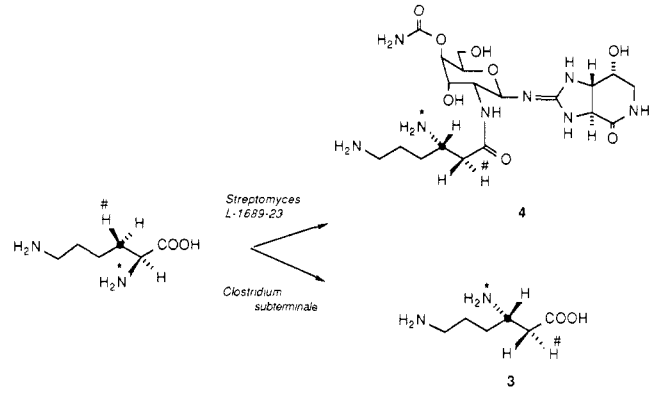
Scheme I



Scheme II



Scheme III



by the intramolecular migration of the original α-nitrogen to the β-position accompanied by the intermolecular migration of the *pro*-3*R* hydrogen to the *pro*-2*S* position.[23] Formation of β-lysine in streptothricin F, **4**, was studied in whole cells (*Streptomyces* L-1689-23),[23b] and formation of free **3** was studied[23a] with a purified aminomutase from *Clostridium subterminale*, as shown in Scheme III.[24] We have now extended our studies of the formation of the β-arginine portion of blasticidin S for comparison with these other pathways.

Results and Discussion

Initially, it was necessary to optimize the fermentation conditions for adequate production of antibiotic. It was found that the composition of the fermentation medium as well as the type of flask used was critical in this regard. The use of baffled flasks[25] in place of flat-bottomed Erlenmeyer flasks increased the production from ≈200 mg/L to ≈1200 mg/L. The bioassay procedure for determining blasticidin S production was also modified, and *Bacillus circulans* spores are now used. With the production of **1** optimized, as much as 45% of the material indicated by bioassay could be recovered. Finally, by using ^{1}H–^{1}H COSY, standard DEPT, ^{1}H–^{13}C HETCOR, and LR HETCOSY experiments,[26] the earlier NMR assignments[3,11,12] were extended to cover all proton and carbon resonances. The complete proton and carbon NMR assignments are given in Tables I and II, respectively.

DL-[3-^{13}C,2-^{15}N]**Arginine.** An initial experiment was designed to find out if the β-arginine moiety was formed from α-arginine by the migration of the α-nitrogen and, if so, whether the migration was intramolecular or intermolecular. DL-[3-^{13}C,2-^{15}N]Arginine was synthesized and fed, and the derived antibiotic was analyzed by ^{13}C NMR spectroscopy to determine if there was a new ^{13}C–^{15}N bond revealed by the heteronuclear spin coupling.[27] The synthesis design was based on our earlier synthesis of DL-[3-^{13}C,2-^{15}N]lysine[28] and is shown in Scheme IV.

Thermal rearrangement[29] of the *N*-nitroso sulfonamide **5** to tosylate **6** was achieved in >50% yield, and the tosylate was carried on to arginine, **7**. Labels were introduced by using Na[^{13}C]CN and potassium [^{15}N]phthalimide to give **7a**.

Labeled arginine **7a** (37 mg) was fed, along with DL-[1-^{14}C]-arginine (16.46 μCi), to *S. griseochromogenes* in two portions at 24 and 36 h after inoculation of the production broth. Standard workup yielded 412 mg of **1a**, and a 30% incorporation of ^{14}C into blasticidin S was determined. This would predict a 5.2% enrichment in ^{13}C on the basis of incorporation of both enantiomers. The ^{13}C NMR spectrum of **1a** showed a doublet (J_{CN} = 3.2 Hz) overlapping the natural abundance singlet (1.6 Hz upfield isotope shift, subtraction of a natural abundance spectrum easily revealed the doublet) for C-13 (46.51 ppm), while normalizing the integrals against the resonance for C-15 gave an apparent enrichment of 3.8%. With the evidence for arginine racemase activity described in the next section, it is clear that only one enantiomer was used directly, and this was apparently the L-enantiomer.[7b] These data reveal that the nitrogen originally at C-2 of arginine had, indeed, migrated intramolecularly to C-3, forming a new ^{13}C–^{15}N bond. A doublet resulting from an intermolecular process would have occurred in only 0.2% of the **1a** molecules; this value was obtained

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(25) These flasks (Bellco Glass Inc., Vineland, NJ, Stock No. 2547) have projections from the bottom that increase aeration during incubation in the gyrotory shaker.
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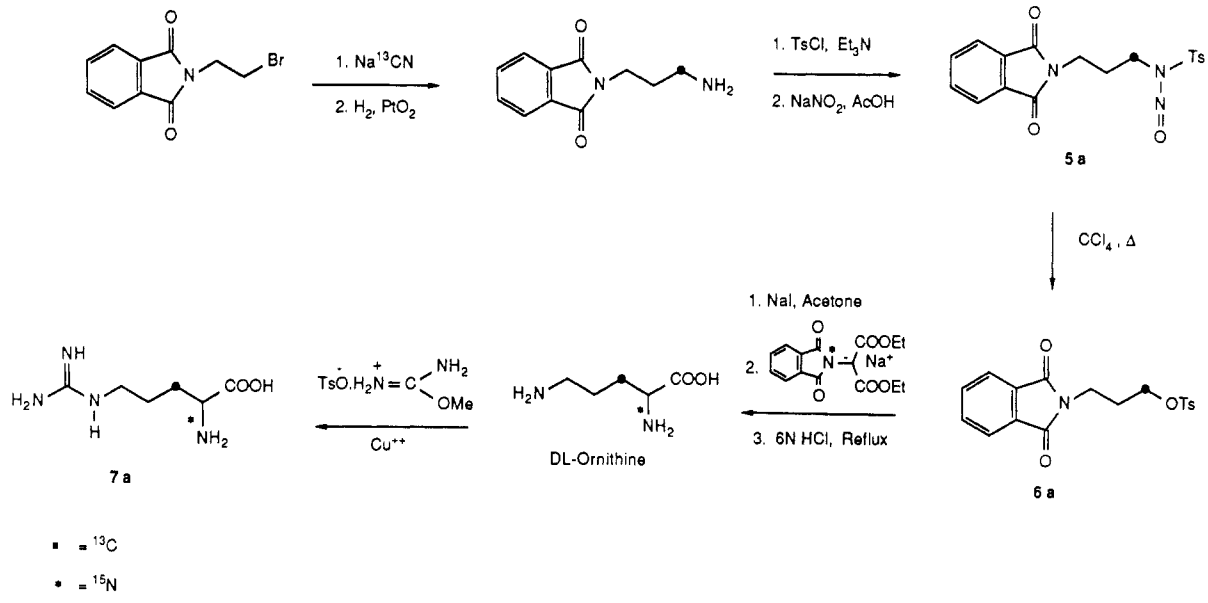
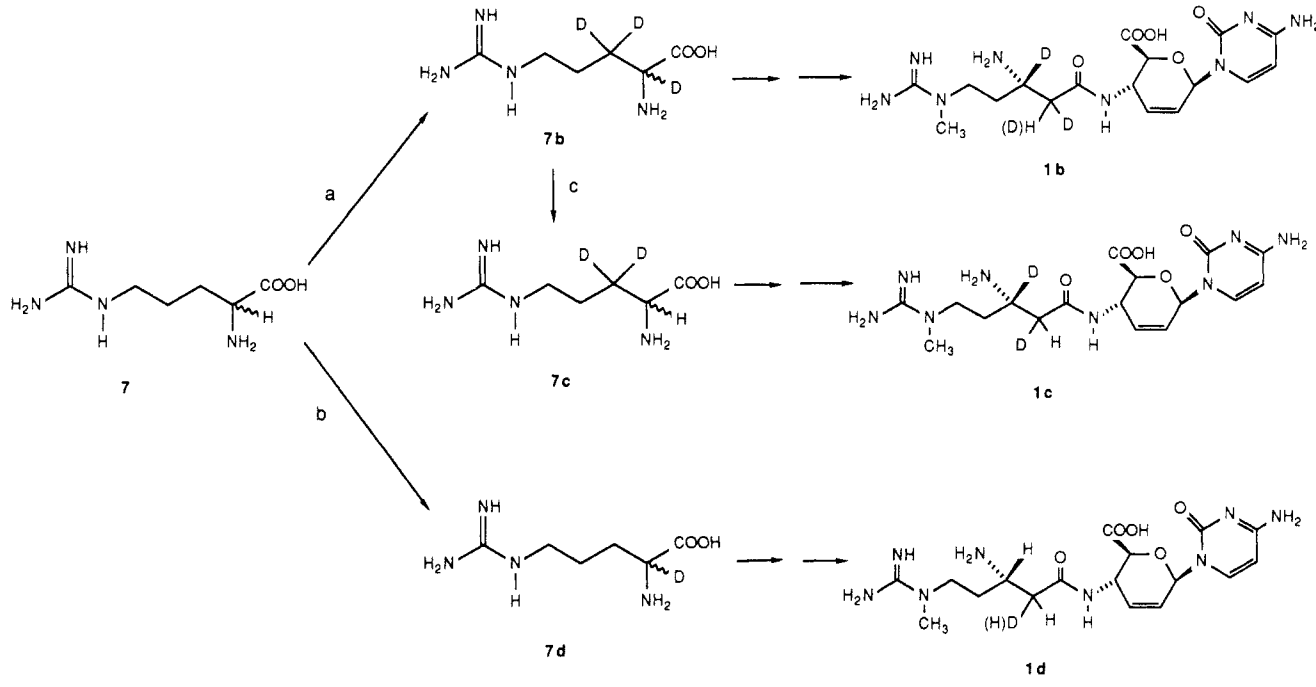
(29) (a) White, E. H. *J. Am. Chem. Soc.* **1955**, *77*, 6008–6010. (b) White, E. H. *Ibid.* **1955**, *77*, 6011–6014.

Scheme IV

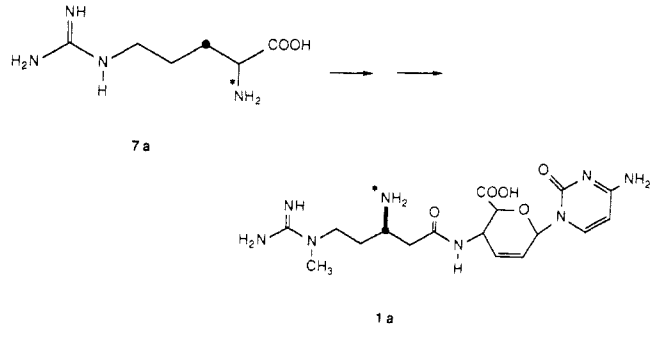


Scheme V[a]



[a] Pyridoxal HCl, $Al_2(SO_4)_3$, D_2O, 125 °C; (b) D_2O, DCl, 180 °C; (c) H_2O, HCl, 180 °C.

by multiplying the effective ^{13}C concentration (4.9%) with the effective ^{15}N concentration (4.1%) that accounts for both natural abundance and enrichment contributions.

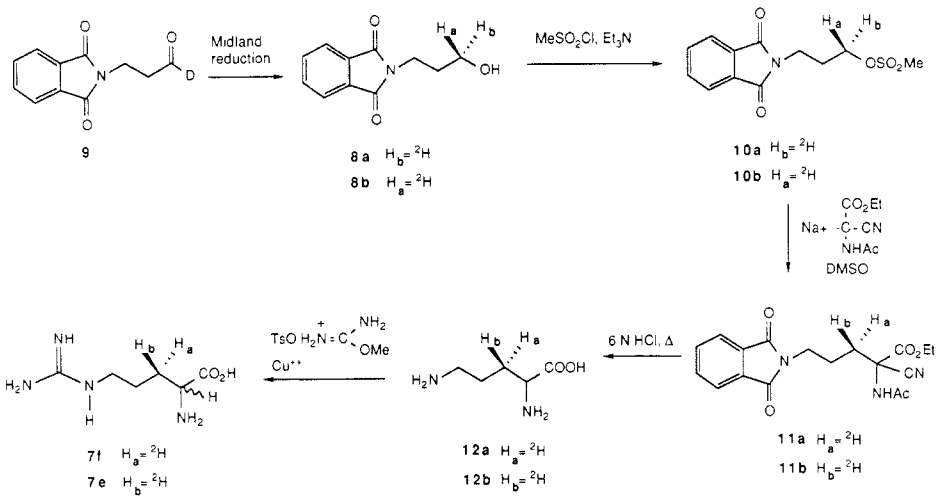


Deuteriated Arginines. In order to investigate the fate of hydrogens at C-3 of arginine during the migration of the α-amino group to the β-position, a series of feedings with deuteriated arginines were carried out.

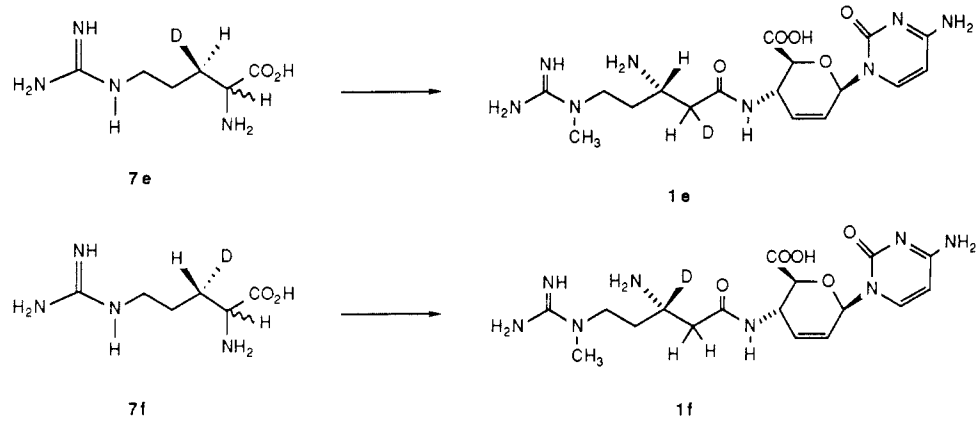
DL-**[2,3,3-$^2H_3$]Arginine (7b).** This trideuterioarginine, **7b**, was readily prepared from **7** by using the procedure of LeMaster and Richards (Scheme V), resulting in >98% enrichment at each of the three positions.[30] It was then fed, and the resulting blasticidin S, **1b**, was analyzed by ^2H NMR spectroscopy. This indicated the presence of ^2H at C-13 and C-12 of blasticidin S in a ratio of 1:1.2 (Scheme V). A ratio of 1:1 would be expected if there were a loss rather than a migration of hydrogen from the β- to the α-position, while a ratio of 1:2 would be expected if there were complete hydrogen migration. The fractional ratio could have been due to a partial migration or due to partial loss of the original α-hydrogen of arginine itself. This was clarified by the following two feedings.

(30) LeMaster, D. M.; Richards, F. M. *J. Labelled Compd. Radiopharm.* **1982**, *19*, 639–646.

Scheme VI



Scheme VII



DL-**[3,3-$^2H_2$]Arginine (7c).** This was prepared by removing the α-deuterium of trideuterioarginine (**7b**) by acid-catalyzed exchange in H_2O–HCl at elevated temperature.[31] Feeding of **7c** (>98% ^2H at C-3 and <10% ^2H at C-2) and ^2H NMR analysis of the derived **1c** revealed that ^2H was distributed at C-12 and C-13 in a ratio of 55:45 (approximately 1:1 when normalized for the original deuterium content of **7b**), indicating that deuterium had migrated intact from the β- to the α-position while the nitrogen was migrating in the reverse direction (Scheme V).

DL-**[2-^2H]Arginine (7d).** The fractional deuterium observed in the feeding of **7b** now appeared to have resulted from the loss of the α-hydrogen by some coincidental process. This assumption was confirmed by feeding DL-[2-^2H]arginine, **7d**, available from the acid-catalyzed exchange of arginine in D_2O–DCl (>89% enrichment was obtained). The ^2H NMR spectrum of the derived **1d** showed a signal corresponding to deuterium at C-13; by comparing the deuterium content based on integration of the NMR signal with the deuterium content expected on the basis of concomitant [^{14}C]arginine incorporation, it was found that only 40% of the ^2H had been retained (Scheme V). This loss was probably due to exchange of the α-proton catalyzed by arginine racemase (EC 5.1.1.9).[32]

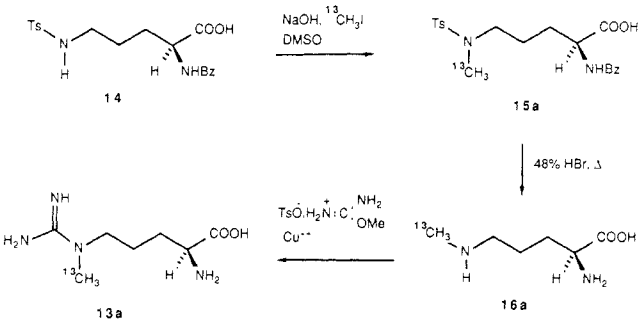
(3*R*)-DL-[3-^2H]Arginine (7e) and (3*S*)-DL-[3-^2H]Arginine (7f). The stereochemistry of hydrogen migration in the arginine aminomutase reaction was next investigated. This required [3*R*-^2H]arginine, **7e**, and [3*S*-^2H]arginine, **7f**. It was envisioned that these could be obtained from chirally deuteriated phthalimidopropanols, **8a** and **8b**, respectively. The syntheses of **8a** and **8b** were based on Midland reductions of the deuteriated aldehyde **9**, and the chirality analysis was based on the ^1H NMR spectra of the (−)-camphanate esters in the presence of Eu(fod)$_3$.[33] While the initial result was misleading, degradation of **8b** to a known configurational standard led to the correct assignments.[34] The chirally deuteriated phthalimidopropanols **8a** and **8b** were then converted to the arginines as shown in Scheme VI.

Phthalimidopropanol was treated with methanesulfonyl chloride to give the mesylate **10**.[34] Nucleophilic displacement of the mesylate by using the sodium salt of ethyl acetamidocyanoacetate was initially difficult; however, the reaction proceeded in reasonable yield when carried out in DMSO.[35] The resulting product **11**, with inverted stereochemistry when chirally deuteriated substrate was used,[36] was hydrolyzed in refluxing 6 N HCl to give ornithine, **12**, which was converted to **7** by treatment with *O*-methylisourea tosylate.[37] By the use of these procedures, (2*RS*,3*R*)-DL-[3-^2H]- and (2*RS*,3*S*)-DL-[3-^2H]arginines, **7e** and **7f**, were obtained.

Chirally deuteriated arginines **7e** and **7f** were then fed separately, and the resulting blasticidins, **1e** and **1f**, respectively, were

(31) Thomas, A. F. *Deuterium Labelling in Organic Chemistry*; Appleton-Century Crofts: New York, 1971; p 209.
(32) Soda, K.; Yorifuji, T.; Ogata, K. *Methods in Enzymology*; Academic: New York, 1970; Vol. XVIIA, p 341.
(33) Gerlach, H.; Zagalak, B. *J. Chem. Soc., Chem. Commun.* **1973**, 274–275.
(34) See the accompanying paper: Prabhakaran, P. C.; Gould, S. J., Orr, G. R.; Coward, J. K. *J. Am. Chem. Soc.*, preceding paper in this issue.
(35) Zaugg, H. E.; Dunnigan, D. A.; Michael, R. J.; Swett, L. R.; Wang, A. H.; Sommers, A. H.; DeNet, R. W. *J. Org. Chem.* **1961**, *26*, 644–651.
(36) On the basis of stereochemical studies of the reaction of primary mesylate **10a** with acetate,[34] we assume that the nucleophilic displacement of the mesylate by sodium ethyl acetamidocyanoacetate would also proceed through an S_N2 process, and hence that the stereochemistry would be inverted in this step.
(37) Greenstein, J. P.; Winitz, M. *Chemistry of the Amino Acids*; Wiley: New York, 1961; p 1849.

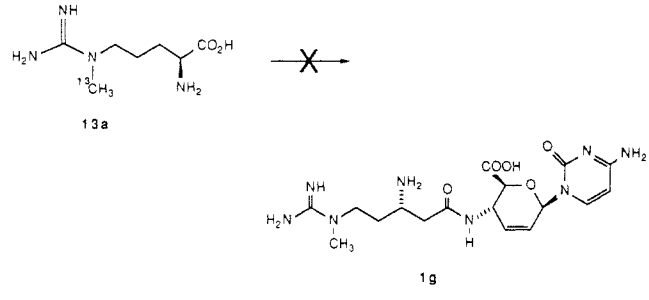
Scheme VIII



analyzed by ^2H NMR spectroscopy. From these results, it was clear that the *pro*-3*R* hydrogen had migrated in the aminomutase process (Scheme VII). These results also indicated that the amino group had migrated with inversion of configuration at what had been C-3 of arginine.

δ-*N*-[$^{13}CH_3$]Methyl-L-arginine (13a). The β-arginyl moiety of the blasticidins contains a methyl group at the δ-nitrogen. However, antibiotic LL-BM547β[14] contains the unsubstituted β-arginyl moiety. In order to identify the correct substrate for the 2,3-aminomutase, δ-*N*-methylarginine, **13**, was tested as an intermediate. δ-*N*-[$^{13}CH_3$]methylarginine, **13a**, was prepared (Scheme VIII) by modifying the procedure of Paik et al.[38] in order to maximize the yield based on the [^{13}C]methyl iodide used. Thus, δ-*N*-tosyl-α-*N*-benzoylornithine, **14**,[39] was converted to δ-*N*-[$^{13}CH_3$]methyl-δ-*N*-tosyl-α-*N*-benzoylornithine, **15a**, with $^{13}CH_3I$ in dry DMSO. Hydrolysis of **15a** in 48% HBr at reflux afforded δ-*N*-[$^{13}CH_3$]methylornithine, **16a**, and this was converted to **13a** in standard fashion.[38]

A mixture of [*guanidino*-^{14}C]arginine and **13a** was fed to *S. griseochromogenes*, and the resulting **1g** was analyzed by liquid scintillation for incorporation of arginine and by ^{13}C NMR spectroscopy for incorporation of δ-*N*-methylarginine. While a 42% incorporation of radioactivity was determined, there was no indication of ^{13}C-enrichment at the *N*-methyl resonance of **1g**. Thus, the substrate for the aminomutase is apparently arginine itself, and N-methylation occurs at a later stage in the pathway.



Conclusions

The results reported here for the conversion of L-α-arginine to L-β-arginine are completely consistent with those previously obtained for the conversion of L-α-lysine to L-β-lysine. Although it remains to determine whether L-β-arginine is an intermediate, the probability is clearly very high. Efforts are now underway to isolate the previously unknown arginine-2,3-aminomutase from extracts of *S. griseochromogenes*. The ^{13}C-enrichment in **1a** (3.8%)—less than the expected 5.2% for incorporation of both enantiomers of arginine and more than the 2.6% expected for incorporation of only one—and the levels of deuterium incorporation from C-2 of **7b** and **7d** suggest that the known arginine racemase (EC 5.1.1.9) may also be present in this organism.

The results of isotope labeling studies define the boundaries of acceptable mechanisms for reaction processes. It is, therefore, interesting to note the consistency for β-lysine and β-arginine formation and the distinctly different boundaries for β-tyrosine formation. Thus, these compounds that appeared structurally to be members of a coherent group of metabolites—β-amino acids derived from α-amino acids—in fact are not biogenetically related. The enzymes responsible for the first two probably evolved from a common ancestor while the enzyme responsible for β-tyrosine apparently evolved independently of these.

Experimental Section

Materials. All solvents were reagent grade and used directly as purchased except for tetrahydrofuran (THF), which was distilled over sodium using benzophenone ketyl as indicator.

Sodium [^{13}C]cyanide (90 atom % enriched) was obtained from MSD Isotopes, and potassium [^{15}N]phthalimide (98 atom % enriched) was obtained from the Los Alamos Stable Isotope Resource, Los Alamos, NM. Deuterium-depleted water (natural abundance × 0.0046%) was obtained from the Aldrich Chemical Co. as was D_2O (99.8 atom % enriched).

DL-[1-^{14}C]Arginine and DL-[*guanidino*-^{14}C]arginine were obtained from Research Products International, Mount Prospect, IL.

Syntheses. *N*-(2-Cyanoethyl)phthalimide.[40] Sodium cyanide (0.08 g, 19.7 mmol) was added to a stirred solution of *N*-(2-bromoethyl)-phthalimide (5.0 g, 19.7 mmol) in dry DMSO (15 mL), and the mixture was stirred at 65 °C for 6 h. After cooling, the mixture was poured into ice water (100 mL), and the precipitate was filtered, washed with water, and dried. Recrystallization from 95% EtOH–H_2O yielded 2.53 g (84%) of crystals: mp 153–155 °C (lit.[40] mp 153–154 °C); IR (Nujol) 3010, 2220 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.85 (m, 4 H), 3.96 (t, 2 H, J = 6.8 Hz), 2.83 (t, 2 H, J = 6.8 Hz).

Via the same procedure, (bromoethyl)phthalimide (8.50 g, 33.7 mmol) was converted to ^{13}C-labeled (cyanoethyl)phthalimide (5.43 g, 80%) by using Na^{13}CN (1.69 g, 33.7 mmol, 90 atom % enriched): ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.85 (m, 4 H), 3.96 (dt, 2 H, J = 6.8 Hz, $^3J_{C-H}$ = 3.3 Hz), 2.83 (dt, 2 H, J = 6.8 Hz, $^2J_{C-H}$ = 6.7 Hz).

***N*-[3-(*p*-Tolylsulfonamido)propyl]phthalimide.**[40] A mixture of (cyanoethyl)phthalimide (2.0 g, 10 mmol) and PtO_2 (50 mg, 0.22 mmol) in absolute ethanol (75 mL) and concentrated HCl (1 mL) was stirred under H_2 at 1 atm of pressure until the theoretical amount of H_2 (448 mL) was taken up. The catalyst was removed by filtration, and the filtrate was concentrated to dryness in vacuo. The resulting solid was stirred at room temperature with *p*-toluenesulfonyl chloride (1.98 g, 1.04 mmol) in CH_2Cl_2 (100 mL) in the presence of Et_3N (3 mL) for 24 h. Solvent removal under reduced pressure yielded a crude product, which was recrystallized from EtOH–H_2O to give 1.78 g (59% from (cyanoethyl)phthalimide) of pure product: mp 158–160 °C (lit.[40] mp 154–156 °C); ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.2–7.7 (m, 8 H), 5.25 (t, 1 H, J = 7.5 Hz), 3.7 (t, 2 H, J = 7.2 Hz), 2.9 (dt, 2 H, J = 7 Hz), 2.41 (s, 3 H), 1.82 (tt, 2 H, J = 6.5 Hz); ^{13}C NMR (20 MHz, $CDCl_3$) δ 21.5, 28.3, 34.7, 40.1, 123.4, 127.0, 127.1, 129.7, 131.1, 134.2, 137.2, 143.3, 168.6.

Via the above procedure, (cyano[1-^{13}C]ethyl)phthalimide (2.0 g, 10 mmol) was converted to 1.78 g of labeled sulfonamide (59% from (cyanoethyl)phthalimide).

***N*-[3-(*N*-Nitroso-*p*-tolylsulfonamido)propyl]phthalimide (5).**[40] The sulfonamide (1.36 g, 3.46 mmol) in Ac_2O (16 mL) and glacial acetic acid (4 mL) was cooled to 0 °C, and finely powdered sodium nitrite (5.25 g, 76.2 mmol) was added over a period of 8 h with constant stirring. The stirring was continued for an additional 10 h at 0 °C, and the mixture then poured into ice-cold water (50 mL) and stirred for 30 min. This was extracted with ether, and the ether layer was washed with H_2O, 5% $NaHCO_3$, H_2O, and aqueous NaCl. After drying (Na_2SO_4), the solvent was removed under reduced pressure to give crude product, which was recrystallized from CH_2Cl_2–petroleum ether. This yielded 1.16 g (87%) of yellowish crystals: mp 117–119 °C (lit.[40] mp 118–119 °C); IR (1% KBr) 3490, 2940, 1740, 1465 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.2–7.7 (m, 8 H), 3.7 (t, 2 H, J = 7 Hz), 2.85 (t, 2 H, J = 7 Hz), 2.35 (s, 3 H), 1.8 (tt, 2 H, J = 7 Hz).

Via the same procedure, ^{13}C-labeled sulfonamide (1.10 g, 3.05 mmol) was converted into 0.94 g (79%) of pure labeled nitroso sulfonamide **5a**.

***N*-(3-Iodopropyl)phthalimide.**[40] The nitroso sulfonamide **5** (0.95 g, 2.45 mmol) with anhydrous Na_2CO_3 (59 mg, 0.56 mmol) was heated at reflux in CCl_4 (50 mL) under N_2 for 24 h. It was then cooled, filtered to remove solid Na_2CO_3, and concentrated under reduced pressure to give 0.78 g of crude tosylate **6**, which was heated at reflux in acetone (100

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mL) with NaI (2.18 g, 14.57 mmol) for 20 h. The mixture was cooled, and the solvent was removed in vacuo. The resulting material was stirred in H_2O (50 mL) for 1 h. After filtration, the solid was washed with cold H_2O and recrystallized from 95% EtOH to give 0.36 g (47% from nitroso sulfonamide **5**) of (iodopropyl)phthalimide: mp 87–88 °C (lit.[40] mp 86–88 °C); ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.78 (m, 4 H), 3.74 (t, 2 H, J = 6.9 Hz), 3.17 (t, 2 H, J = 7.1 Hz), 2.33 (tt, 2 H, J = 6.9 Hz).

Via the above procedure, ^{13}C-labeled nitroso sulfonamide **5a** (1.01 g, 2.6 mmol) was converted into 0.38 g (46% from **5a**) of *N*-(3-iodo[3-^{13}C]propyl)phthalimide.

DL-[3-^{13}C,2-^{15}N]Ornithine Hydrochloride. Diethyl [^{15}N]phthalimidomalonate[40] (648 mg, 2.12 mmol) was heated with fresh sodium ethoxide [prepared by stirring NaH (102 mg of 50% suspension, 2.12 mmol) in absolute EtOH (5 mL) at 60 °C for 30 min], and the resulting yellow solution was subjected to solvent removal in vacuo. The resulting solid was mixed with the labeled iodide (703 mg, 2.2 mmol) in dry toluene at 120 °C and stirred for 30 min. Then the solvent was again removed in vacuo, and the resulting solid mixture was heated at 175 °C for 6 h, cooled to room temperature, slurried with $CHCl_3$ (50 mL), and filtered. The solid was washed with $CHCl_3$, and the combined filtrates were concentrated in vacuo to give 691 mg of crude adduct. This was hydrolyzed with concentrated HCl (10 mL), glacial acetic acid (10 mL), and H_2O (10 mL) at reflux temperature for 12 h. Cooling followed by concentration in vacuo and recrystallization after adjusting to pH 6.0 gave 119 mg (33% from the iodide) of [3-^{13}C,^{15}N]-DL-ornithine hydrochloride: mp 225–230 °C (lit.[41] mp 230 °C dec); ^{1}H NMR (80 MHz, D_2O) δ 3.75 (m, 1 H), 3.00 (m, 2 H), 2.00 (d, 2 H, J_{C-H} = 119.1 Hz), 1.76 (m, 2 H).

DL-[3-^{13}C,2-^{15}N]Arginine Hydrochloride (7a). Via the standard procedure,[37] [3-^{13}C,2-^{15}N]-DL-ornithine hydrochloride (652 mg, 2.95 mmol) was converted into 112 mg (27.2%) of [3-^{13}C,2-^{15}N]-DL-arginine hydrochloride, **7a**: mp 231–235 °C (lit.[42] mp 235 °C dec); ^{1}H NMR (400 MHz, D_2O) δ 3.78 (t, 1 H, J = 7.5 Hz), 3.24 (t, 2 H, J = 7.5 Hz), 1.88 (d, 2 H, J_{C-H} = 156 Hz), 1.75 (m, 2 H); ^{13}C NMR (100 MHz, D_2O) δ 175.2, 157.7, 55.3, 41.4, 28.4 (enriched), 24.8.

DL-[2,3,3-$^2H_3$]Arginine Hydrochloride (7b).[30] L-Arginine (5.10 g, 20 mmol), aluminum sulfate (415 mg, 1.2 mmol), and pyridoxal hydrochloride (0.61 g, 30 mmol) were dissolved in D_2O (30 mL) and lyophilized. Care was taken to minimize the exposure to light by wrapping the flask with aluminum foil. To the resulting solid were added pyridine (1.26 mL, 15 mmol) and D_2O (30 mL), and the solution was evacuated after being frozen under vacuum. The flask was sealed and heated at 125 °C for 48 h. After being cooled to room temperature, the contents were poured into water (200 mL) and adjusted to pH 4.0 with 3 N HCl. The solution was then loaded onto a column of Dowex 50Wx8 (H^+) (100 mesh) resin. After the solution was washed with H_2O, it was eluted with 0.25 N NH_4OH. Ninhydrin-positive fractions were combined, lyophilized, and recrystallized from H_2O–EtOH at ca. pH 6.5 to give 1.6 g (31%) of pure **7b**: mp >230 °C; IR (1% KBr) 3400–3150 (broad), 1670, 1620, 1490 cm^{-1}; ^{1}H NMR (80 MHz, D_2O) δ 3.25 (t, 2 H, J = 8.0 Hz), 1.68 (t, 2 H, J = 8.0 Hz). ^{1}H NMR indicated >98% exchange at both H-2 and H-3.

DL-[3,3-$^2H_2$]Arginine Hydrochloride (7c). DL-[2,3,3-$^2H_3$]Arginine hydrochloride, **7b** (380 mg, 1.8 mmol), was dissolved in 20% HCl (1.1 mL) and H_2O (8 mL) in a heavy-walled tube. This was sealed tightly and heated at 180 °C. Every 48 h, the H_2O–HCl mixture was removed under reduced pressure, fresh H_2O–HCl was added, and the exchange was continued. At the end of eight exchanges, >89% of ^{2}H at C-2 had been removed (estimated by ^{1}H NMR analysis). The product from this reaction was purified by ion-exchange chromatography on Dowex 50Wx8 (H^+) (100 mesh), eluted with 0.24 N NH_4OH to give 120 mg (35%) of pure **7c**: ^{1}H NMR (80 MHz, D_2O) δ 3.82 (s, 0.9 H), 3.35 (t, 2 H), 1.8 (t, 2 H).

Ethyl (2*RS*)-2-Acetamido-2-cyano-5-phthalimidopentanoate (11). The following procedure is typical. Ethyl acetamidocyanoacetate (1.95 g, 11.4 mmol) was added to a freshly prepared solution of NaOEt (11.4 mmol) in EtOH (16 mL) and stirred at 65 °C for 30 min. It was cooled, and all EtOH was removed under vacuum. The resulting dry solid was dissolved in dry DMSO (15 mL), mesylate **10**[34] (2.18 g, 7.76 mmol) was then added, and the mixture was stirred at 70 °C for 8 h. After the mixture was cooled to room temperature, the DMSO was removed by rotary evaporation under high vacuum, and the resulting solid was washed with cold H_2O (20 mL). The undissolved material was filtered and recrystallized from 90% EtOH to give 1.75 g (63%) of **11**: mp 212 °C; ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.70–7.90 (m, 4 H), 6.91 (br s, 1 H), 4.32 (q, 2 H, J = 7.1 Hz), 3.77 (t, 2 H, J = 6.3 Hz), 1.9–2.3 (m, 7 H), 1.34 (t, 3 H, J = 7 Hz); ^{13}C NMR (100.6 MHz, $CDCl_3$–DMSO-d_6) δ 170.39, 167.90, 166.47, 134.15, 131.79, 123.09, 116.87, 62.75, 57.14, 36.85, 33.21, 23.42, 21.78, 13.86. Anal. Calcd for $C_{18}H_{19}N_3O_5$: C, 60.50; H, 5.36; N, 11.75. Found: C, 60.17; H, 5.25; N, 11.63.

By following the above procedure, (3*R*)-[3-^{2}H]mesylate **10a** (2.18 g, 7.76 mmol) was converted to 1.75 g (63%) of (3*S*)-[3-^{2}H]pentanoate **11a**, and (3*S*)-[3-^{2}H]mesylate **10b** (2.13 g, 7.58 mmol) was converted into 1.80 g (66%) of (3*R*)-[3-^{2}H]pentanoate **11b**: ^{1}H NMR (80 MHz, $CDCl_3$) δ 7.7–7.9 (m, 4 H), 6.91 (br s, 1 H), 4.32 (q, 2 H, J = 7.2 Hz), 3.77 (t, 2 H, J = 6.3 Hz), 1.7–2.3 (m, 6 H), 1.33 (t, 3 H, J = 7.1 Hz).

(2*RS*)-Ornithine Hydrochloride (12). Ethyl 2-acetamido-2-cyano-5-phthalimidopentanoate (1.75 g, 4.89 mmol) was heated at reflux in 6 N HCl (60 mL) for 15 h. After the mixture was cooled to room temperature and the precipitated solid was removed by filtration, HCl was removed by rotary evaporation. The resulting solid was taken up in 95% EtOH, and concentrated NH_4OH was added dropwise until cloudiness appeared. On leaving the mixture at 4 °C overnight, ornithine hydrochloride crystallized, yielding 735 mg of product (88%).

By following the above procedure, (3*S*)-[3-^{2}H]pentanoate **11a** (1.8 g, 5.03 mmol) was converted into 0.81 g (95%) of (2*RS*,3*S*)-[3-^{2}H]ornithine hydrochloride (**12a**) and (3*R*)-[3-^{2}H]pentanoate **11b** (1.75 g, 4.89 mmol) was converted into 0.73 g (87%) of (2*RS*,3*R*)-[3-^{2}H]ornithine hydrochloride (**12b**) respectively: ^{1}H NMR (80 MHz, D_2O) δ 3.86 (d, 1 H, J = 7.8 Hz), 3.1 (t, 2 H, J = 6.3 Hz), 1.6–2.2 (m, 3 H).

(2*RS*,3*R*)-[3-^{2}H]Arginine Hydrochloride (7e) and (2*RS*,3*S*)-[3-^{2}H]-Arginine Hydrochloride (7g). Via the standard procedure,[37] (2*RS*,3*R*)-[3-^{2}H]ornithine hydrochloride (**12b**) (200 mg, 1.2 mmol) was converted into 120 mg (47%) of (2*RS*,3*R*)-[3-^{2}H]arginine hydrochloride (**7e**), and (2*RS*,3*R*)-[3-^{2}H]ornithine hydrochloride (**12a**) (200 mg, 1.2 mmol) was converted into 130 mg (51%) of (2*RS*,3*R*)-[3-^{2}H]arginine hydrochloride (**7f**): ^{1}H NMR (400 MHz, D_2O) δ 3.82 (d, 1 H, J = 7.6 Hz), 3.31 (t, 2 H, J = 6.8 Hz), 1.95 (m, 1 H), 1.75 (m, 2 H).

δ-*N*-[^{13}CH$_3$]Methyl-L-arginine Hydrochloride (13a). δ-*N*-Tosyl-α-*N*-benzoyl-L-ornithine hydrochloride,[39] **14** (1.0 g, 2.54 mmol), was stirred in aqueous NaOH (2.55 mL of 2 N solution, 5.1 mmol) at 50 °C for 15 min, and the mixture was lyophilized. The resulting solid was dissolved in dry DMSO (5 mL), [^{13}C]methyl iodide (190 μL, 3 mmol) was added dropwise, and the mixture was stirred at room temperature for 24 h. Cold H_2O (25 mL) was added, and the mixture was stirred for 10 min. This was then acidified with 2 N HCl, and the resulting gum was taken up in EtOAc, washed with H_2O, dried (Na_2SO_4), and concentrated in vacuo to give 0.95 g (77% based on MeI) of **15a**: mp 190–192 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 14.9 (br s), 8.9–7.38 (m, 9 H), 4.42 (br m, 1 H), 3.4 (br s, 1 H), 3.0 (m, 2 H), 2.62 (d, 3 H, J_{CH} = 149.2 Hz), 2.37 (s, 3 H), 1.99–1.6 (m, 4 H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 173.55, 166.5, 143.08, 133.95, 133.92, 131.28, 129.71, 128.15, 127.38, 127.06, 52.16, 49.17, 34.3 (enriched ^{13}C), 27.65, 23.72, 20.86.

The above product **15a** (0.85 g, 2.1 mmol) was hydrolyzed at reflux in 48% HBr for 2 h.[38] The precipitate obtained on cooling was removed by filtration, and the supernatant was concentrated and recrystallized from H_2O–EtOH to give 0.35 g (91%) of δ-*N*-[^{13}CH$_3$]methyl-L-ornithine hydrochloride, **16a**: mp 235–236 °C; ^{1}H NMR (400 MHz, D_2O) δ 3.63 (t, 1 H, J = 5.5 Hz), 2.94 (br t, 2 H), 2.58 (d, 3 H, J_{C-H} = 143.4 Hz), 1.8–1.55 (m, 4 H); ^{13}C NMR (100.6 MHz) δ 174.32, 54.47, 48.67, 33.13 (enriched ^{13}C), 27.75, 21.86.

Via the standard procedure,[37] δ-*N*-[^{13}CH$_3$]methylornithine hydrochloride (184 mg, 0.94 mmol) was converted into 90 mg (54% based on recovered ornithine hydrochloride) of δ-*N*-[^{13}CH$_3$]methylarginine hydrochloride, **13a**: mp 260–261 °C; ^{1}H NMR (400 MHz, D_2O) δ 3.63 (t, 1 H, J = 5.9 Hz), 3.25 (m, 2 H), 2.9 (d, 3 H, J_{C-H} = 140 Hz), 1.8–1.5 (m, 4 H); ^{13}C NMR (100.6 MHz) δ 174.62, 156.86, 54.74, 49.98, 36.18 (enriched ^{13}C), 27.62, 22.83.

Feedings. DL-[3-^{13}C,2-^{15}N]Arginine Hydrochloride (7a). Two 200-mL production broths were fed a total of 37 mg (0.17 mmol) of **7a** mixed with 16.46 μCi of DL-[1-^{14}C]arginine. Bioassay after fermentation indicated 412 mg of **1a** were produced, and 270 mg (65% recovery) were obtained after workup, 1.03×10^7 dpm/mmol (30.16% total incorporation). A 3.8% enrichment in both isotopes was determined from the spin-coupled doublet (δ 46.51) in the ^{13}C NMR spectrum.

DL-[2,3,3-$^2H_3$]Arginine Hydrochloride (7b). Two 250-mL production broths were fed a total of 200 mg (0.94 mmol) of **7b** mixed with 10.63 μCi of DL-[1-^{14}C]arginine. Workup yielded 46 mg of **1b** (29% recovery), 1.04×10^7 dpm/mmol (16.2% total incorporation); 22.1 and 18.4% ^{2}H enrichments at C-12 and C-13, respectively were determined by ^{2}H NMR.

DL-[3,3-$^2H_2$]Arginine Hydrochloride (7c). Two 200-mL production broths were fed a total of 97 mg (0.46 mmol) of **7c** and 10.9 μCi of DL-[1-^{14}C]arginine, yielding 240 mg of pure **1c** (51% recovery), 6.44×10^6 dpm/mmol (30.0% total incorporation); 5.6 and 6.8% ^{2}H enrichments

(41) *Dictionary of Organic Compounds*; Oxford University: New York, 1965; Vol. 4, p 2574.

(42) *Dictionary of Organic Compounds*; Oxford University: New York, 1965; Vol. 1, p 276.

at C-12 and C-13, respectively, were determined by ^2H NMR.

DL-**[2-^{2}H]Arginine Hydrochloride (7d).** Feeding a total of 200 mg (0.95 mmol) of **7d** mixed with 13.72 μCi of DL-[1-^{14}C]arginine to two 200-mL production broths yielded 210 mg of pure **1d** (44% recovery), 7.2×10^6 dpm/mmol (27% total incorporation), with 22.7% ^2H enrichment at C-12.

(2*RS*,3*R*)-[3-^{2}H]Arginine Hydrochloride (7e). Feeding a total of 90 mg (0.42 mmol) of **7e** and 2.36 μCi of DL-[1-^{14}C]arginine to two 200-mL production broths afforded 190 mg of pure **1e** (36% recovery), 1.23×10^6 dpm/mmol (31.0% total incorporation), with 11% ^2H enrichment at C-12.

(2*RS*,3*S*)-[3-^{2}H]Arginine Hydrochloride (7f). Two 200-mL production broths were fed a total of 65 mg (0.31 mmol) of **7f** mixed with 4.45 μCi of DL-[1-^{14}C]arginine. Workup yielded 180 mg of pure **1f** (36% recovery), 2.4×10^6 dpm/mmol (32.0% total incorporation), with 8.2% ^2H enrichment at C-13.

δ-*N*-[^{13}CH$_3$]Methyl-L-Arginine Hydrochloride (15a). A 200-mL fermentation was fed 50 mg (0.22 mmol) of **13a** mixed with 1.13 μCi of DL-[*guanidino*-^{14}C]arginine. Workup yielded 260 mg of pure **1g** (53% recovery), 9.95×10^6 dpm/mmol (42% total incorporation). The ^{13}C NMR spectrum was identical with that of natural abundance blasticidin S.

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Supplementary Material Available: General details, culture conditions, isolation of blasticidin S, bioassay of blasticidin S, and spectra of blasticidin S (from DL-[3-^{13}C,2-^{15}N]arginine, DL-[2,3,3-$^2H_3$]arginine, DL-[2-^{2}H]arginine, DL-[3,3-$^2H_2$]arginine, (2*RS*,3*R*)-[3-^{2}H]arginine, and (2*RS*,3*S*)-[3-^{2}H]arginine feedings) (9 pages). Ordering information is given on any current masthead page.

Synthesis of Chirally Deuteriated (*S*-Adenosyl-*S*-methylsulfonio)propylamines and Spermidines. Elucidation of the Stereochemical Course of Putrescine Aminopropyltransferase (Spermidine Synthase)

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Abstract: The stereochemical course of enzyme-catalyzed aminopropyl transfer has been investigated. The stereospecific synthesis of chirally deuteriated (*S*-adenosyl-*S*-methylsulfonio)propylamines and several chirally deuteriated spermidine (−)-camphanamide derivatives has allowed the elucidation of aminopropyltransferase stereochemistry by using ^1H NMR techniques. Putrescine aminopropyltransferase (spermidine synthase) isolated from *Escherichia coli* catalyzed the synthesis of chirally deuteriated spermidines from 1,4-diaminobutane (putrescine) and chirally deuteriated (*S*-adenosyl-*S*-methylsulfonio)propylamines. Derivatization of the biosynthetic spermidines to 1,8-bis(Boc)spermidine (−)-camphanamides and comparison of their ^1H NMR spectra with those of the synthetic standards permits determination of the absolute configuration of the biosynthetic products. The results show that the reaction catalyzed by *E. coli* spermidine synthase proceeds with inversion of configuration at the methylene carbon undergoing nucleophilic attack by putrescine. These data support a single-displacement mechanism proceeding via a ternary complex of enzyme and both substrates.

The polyamines putrescine, spermidine, **2a**, and spermine, **2b**, are an important series of biomolecules and are universally distributed in Nature. In vivo experimentation has shown that an increase in polyamine biosynthesis is closely associated with cell proliferation.[2] The higher polyamines **2a** and **2b** are biosynthesized by enzymatic transfer of an aminopropyl group from (*S*-adenosyl-*S*-methylsulfonio)propylamine, **1**, (decarboxylated *S*-adenosylmethionine, dcAdoMet) to putrescine or to the primary amine on the four-carbon arm of **2a**, respectively[3] (Figure 1). As part of our ongoing investigation of enzymatic alkyl transfer reactions[4,5] we have examined the biosynthesis of **2a**. The alkyl transfer involved in the biosynthesis of spermidine is catalyzed by putrescine aminopropyltransferase (PAPT, EC 2.5.1.16), often referred to as spermidine synthase.[3] As shown in Figure 1, this reaction could occur by either a single-displacement (path A) or by a double-displacement (path B) mechanism.[6-8] In principle these two mechanisms can be distinguished by isotope-labeling experiments[6-8] or by steady-state kinetic investigations.[9]

Several years ago we showed that rat prostate spermidine synthase is inhibited by one of its substrates, (*S*-adenosyl-*S*-

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