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THE BIOSYNTHESIS OF THE STREPTOLIDINE MOIETY IN STREPTOTHRICIN F[1]

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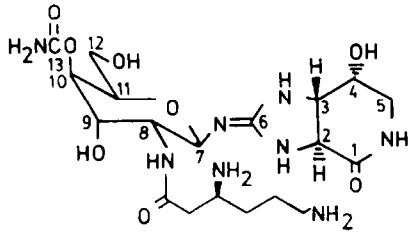
Abstract—A series of arginines specifically labeled either with ^{13}C and ^{15}N or with ^{2}H were synthesized and fed to *Streptomyces* L-1689-23. The streptothricin F isolated in each case was analyzed by either ^{13}C or ^{2}H NMR, respectively, in order to determine the labeling pattern obtained. From these results, it appears that arginine is metabolized to a β-ketoarginine, possibly via a pyridoxal phosphate adduct, and then via cyclization, reduction, rearrangement, and hydroxylation to the streptolidine moiety. The pathway described can also account for the formation of other known antibiotics, and for β-hydroxy-γ-amino acids, generally.

Since there are no long-lived radioactive isotopes of N, the study of N metabolism in the biosynthesis of natural products has lagged far behind those of C and H. Until recently, mass spectrometry had been the only practical tool for studying the fate of metabolic precursors labeled with the stable isotope ^{15}N. While ^{15}N NMR has recently allowed the direct observation of ^{15}N labels,[3,4] we have approached this problem with the use of $^{13}C/^{15}N$ double labeling, whereby the presence and location of ^{15}N labels are detected through heteronuclear spin-couplings in ^{13}C NMR spectroscopy.[5,6] In this manner we have been able to observe bond-making, bond-breaking, and bond-conserving processes.

Streptothricin F (**1**) is the simplest constitutent of a complex of broad-spectrum antibiotics first isolated by Waksman in 1942.[7] Each member contains a central carbamoylated-D-gulosamine unit bearing the lactam form of the novel heterocyclic amino acid streptolidine (**2**). The variation amongst the constituents is due to the number of β-lysine (**3**) units present (1–7 units). The structure of **1** was deduced by van Tamelen in 1961,[8] although the precise location of the carbamate was in doubt until recently, when it was established at C-10 by a total synthesis,[9] and from D induced ^{13}C NMR resonance shifts of D-exchanged **1**.[10] Over the years streptothricin has been repeatedly rediscovered, collecting numerous names along the way,[11] and a substantial number of related antibiotics where the β-lysine has been replaced by other amino acids have also been described.[12]



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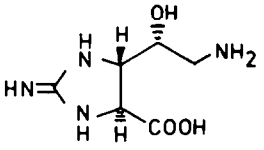

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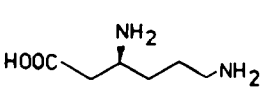

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This ubiquitous occurrence of a rather unusual structure added to our attraction to understanding the biosynthetic chemistry involved. In 1972 Bycroft and King[13] proposed a chemically rational biogenesis of **2** from arginine (**4**) via a dehydroamino acid **5** and rearrangement of a 6-membered cyclic guanidine **6**, as shown in Scheme 1. Results on the biosynthesis of norseothricin[14] and racemomycin A[15]—in each case actually **1**—lent support to this idea. However, in each case the use of [U-^{14}C]arginine precluded determination of the specificity of incorporation, much less of the pathway involved. Work by one of these groups with a third *Streptomyces*[16] resulted in apparently specific incorporations of sodium [1-^{13}C]- and [2-^{13}C]acetates. Unable to explain the observed patterns with a rational pathway, these authors concluded that there existed a second, at that time undefined, pathway to **2**.

The putative intermediate **6** (capreomycidine) occurs in a number of antibiotics,[17] while its C-3 epimer occurs in yet others.[18] Tuberactinomycin A[17] contains 5*S*-hydroxycapreomycidine and viomycin (tuberactinomycin B)[19] contains the 5*R*-isomer; N-methylcapreomycidine occurs in the antibiotic stendomycine.[20] This abundance of similar structures argues strongly for the direct biosynthesis of all of these heterocyclic amino acids from L-arginine (Scheme 2). We report here the extensive use of $^{13}C/^{15}N$ double labeling in studying the biosynthesis of **2**, which has proven to be an excellent vehicle for

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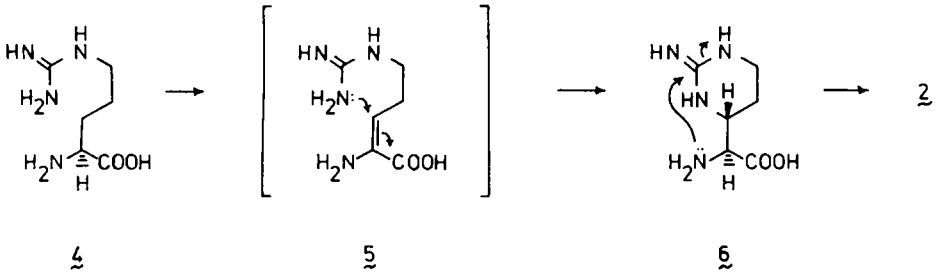
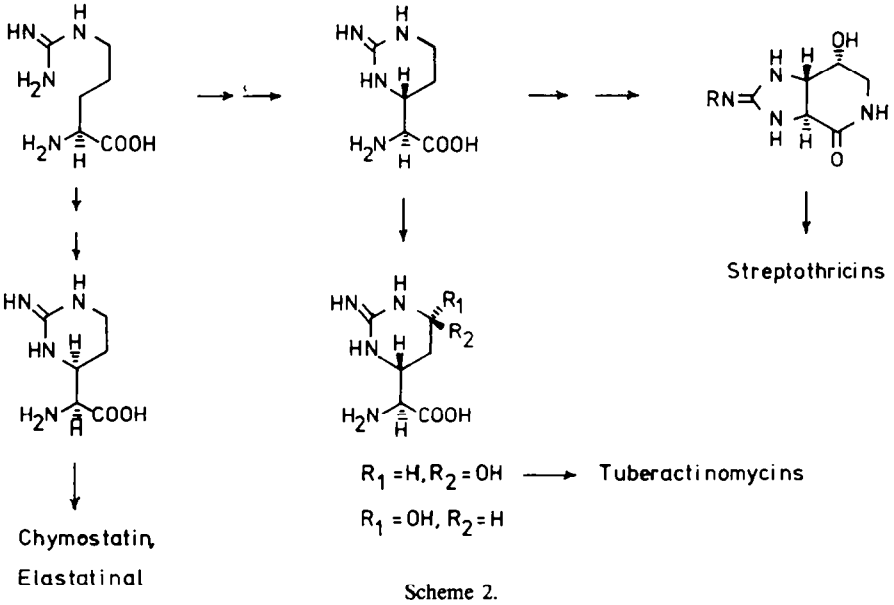


Scheme 1.



Scheme 2.

testing the utility of this heteronuclear spin-coupling approach, as well as the use of ^2H labeling which has provided information on the mechanism of ring formation in streptolidine biosynthesis.

RESULTS AND DISCUSSION

The biosynthesis of streptothricin F was studied in shake cultures of a strain of *Streptomyces* L-1689-23 obtained from Lederle Laboratories, that was grown in a complex medium innoculated with growing mycelium prepared from spores maintained on yeast-malt agar. Titers of 1 were initially quite low (50-80 mg/L), but through a random screening of individual colonies monitored by bioassay with *Bacillus subtilis* ATCC 6633, a stable strain that produced 200–400 mg/L was isolated. Based on time course studies, sterile addition of labeled precursors 12 hr after innoculation of production cultures (the onset of antibiotic production) and harvesting of the cultures 48 hr later were chosen as standard conditions for feeding experiments. The antibiotic was isolated via a series of chromatographic steps (Experimental), and was then converted to the crystalline helianthate salt, which was recrystallized to constant specific radioactivity—a necessary step to insure radiochemical purity.[21] For NMR spectra, the amorphous hydrochloride was obtained by acidification of the helianthate, followed by ion exchange chromatography on a weak anion exchange resin.

[1,2-$^{13}C_2$]*Acetate*. In biosynthetic studies using precursors singly-labeled with ^{13}C, ^{13}C NMR analysis of enrichments in natural products is limited in sensitivity due to the substantial natural abundance (1.1%) of ^{13}C. Furthermore, the use of a singly-labeled precursor can only reveal the final destination(s) of the label, and gives no direct evidence for how it/they had arrived there. This can be particularly difficult with acetate, which as the biologically active coenzyme A thioester is the most central of all primary metabolites and leads directly into numerous interconnected primary pathways. However, the use of [1,2-$^{13}C_2$]acetate (7) presents an exceptional opportunity. The ^{13}C–^{13}C homonuclear spin couplings, which allow the sensitive detection of C_2 units incorporated intact, provides a means whereby alternative pathways can be distinguished, while at the same time a judicious choice of the protocol for feeding 7 has the potential to reveal the interactions of the primary metabolic grids with the specific pathway leading to a secondary metabolite.

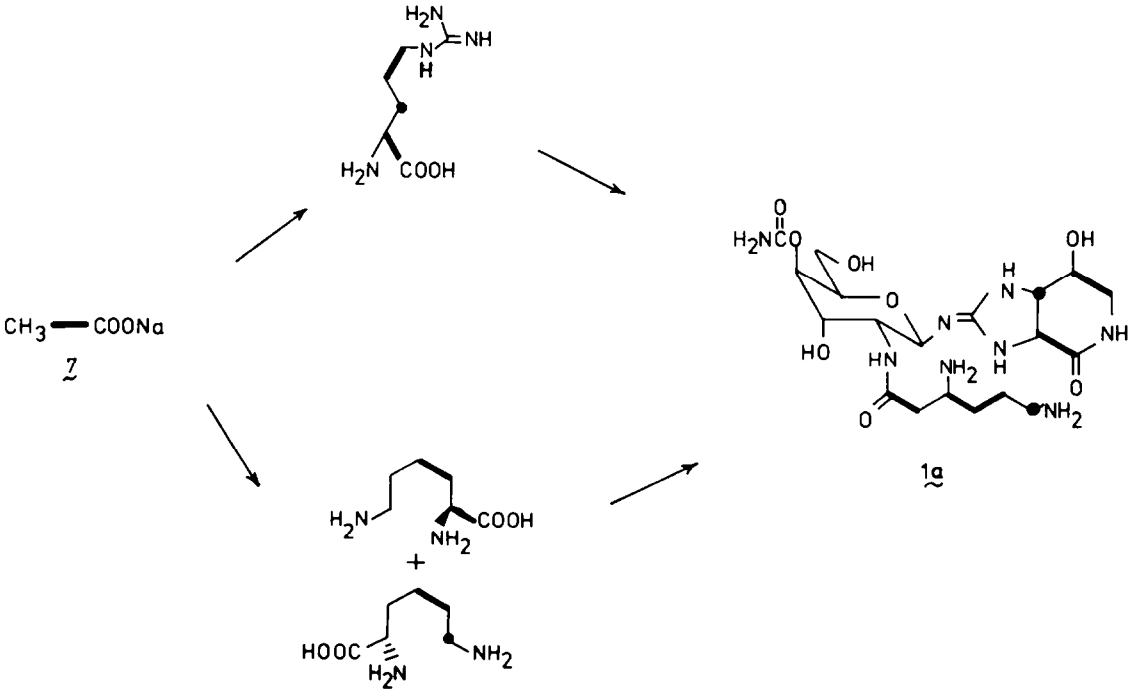
To study the biosynthesis of streptothricin F, preliminary feedings with [1-^{14}C]acetate diluted with varying amounts of unlabeled acetate were carried out and yielded overall incorporations of 0.9–1.5%. Next, by feeding at the beginning of a fermentation half of a quantity of **7** mixed with [1-^{14}C]acetate, strong evidence was obtained for the primary precursors to the streptolidyl and β-lysine portions of **1**, as well as the pathways generating these precursors. It was then possible to clarify the results of the previous workers.[22] By feeding the second half of **7** at the onset of antibiotic production we were able to obtain an enhanced—and more specific—labeling of **2**. This labeling pattern is summarized in Scheme 3 and Table 1.

An explanation of the β-lysine labeling has been described.[22] The labeling pattern observed for the streptolidyl moiety is precisely that predicted for the incorporation of **7** into **2** via **4** formed by the Tri-Carboxylic Acid Cycle,[23] followed by metabolism according to Scheme 1. The second pulse of **7** understandably led to a more specific labeling at C-4 and C-5. Thus it appeared that there was only one pathway to **2**, with arginine the direct primary precursor.

Arginine syntheses. A series of arginines doubly-labeled with ^{13}C and ^{15}N were next chosen to demonstrate the direct labeling of **2** by arginine and to test the biogenetic proposal in Scheme 1. Two basic routes were used to prepare these arginines, with isotopes introduced at the necessary steps for each target.

The synthesis shown in Scheme 4 is based on literature reactions; however, modifications were introduced that afford significantly higher yields. Phase transfer catalysis was particularly effective. Potassium phthalimide reacted with 1,3-dibromopropane in the presence of hexadecyltributyl phosphonium bromide to give **8** in 71% yield, and reacted with diethylbromomalonate to give **9** in quantitative yield with the same catalyst. The most difficult step in the synthesis was coupling **9** and the iodide **10** to yield the bisphthalimide **11**. The use of highly polar, nonprotic solvents such as DMF failed to give any reaction, neither did phase transfer catalysts. While the literature[24] called for simply mixing and fusing the two solids at 160°, this gave unreproducible yields of 0–20%. However, when intimate mixing was achieved by heating at reflux a toluene solution of **10** with the powdered, freshly-generated Na-salt of **9** followed by removing the toluene by distillation, fusing the residue at 160° did give **11** reproducibly in 75–80% yields. The ornithine hydrochloride (**12**) obtained by acid hydrolysis of **11** was then treated with O-methylisourea tosylate[25] in the presence of Cu^{2+} to give arginine (**4**) that was purified via the flavianate salt.

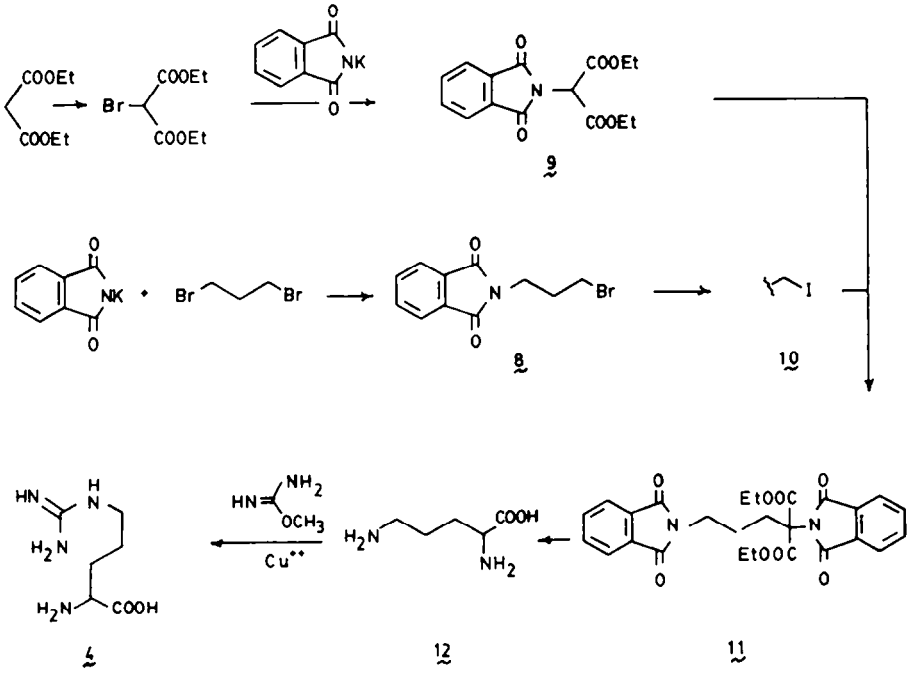
The second route is shown in Scheme 5. This was used specifically to introduce labels at C-3 of arginine, and is based on our recently published lysine synthesis.[26] 2-Bromoethylphthalimide (**13**) was prepared in standard fashion and reacted in DMSO with one equivalent of sodium cyanide to given the nitrile **14** in 95% yield. The nitrile was reduced in ethanol containing a trace of concentrated HCl using a Pt catalyst, and the unstable amine hydrochloride **15** in methylene chloride was immediately converted to the sulfonamide **16**[27]; the overall yield was typically 60%. The N-nitroso sulfonamide **17** was easily generated,[28] and rearranged[29] by heating in carbon tetrachloride in the presence of one equivalent of anhydrous sodium carbonate. The tosylate **18**, obtained in 74%



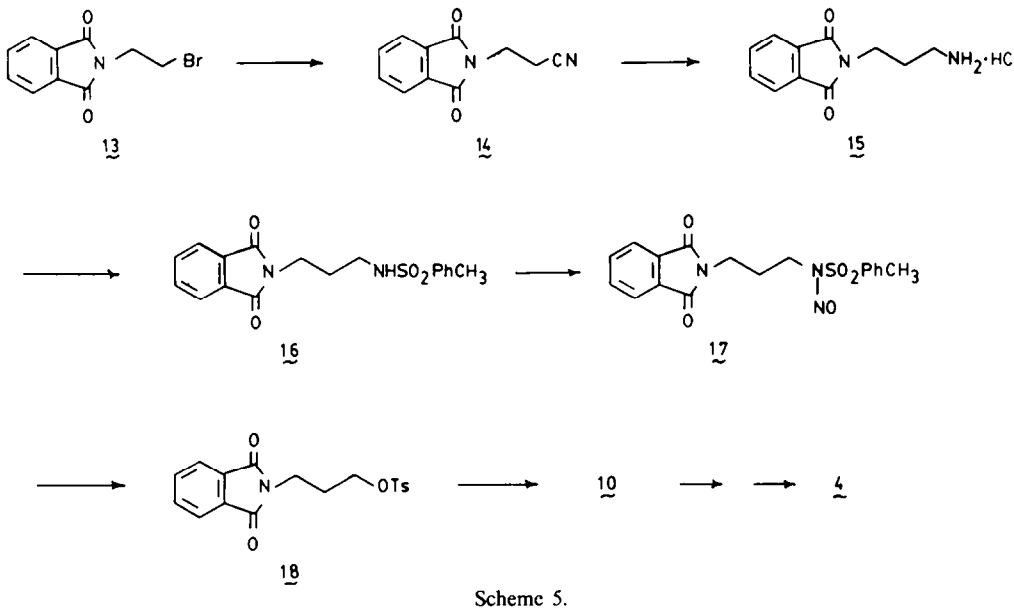
Scheme 3.

Table 1. ^{13}C NMR Data from feeding experiments

		Incorporations									
		[1,2-$^{13}C_2$]Acetate		4a		4b		4c		4d	
Carbon#	Chemical Shift	%enrich.	J_{CC}	%enrich.	J_{CN}	%enrich.	J_{CN}	%enrich.	J_{CN}	%enrich.	J_{CN}
14	169.7	0.3	48.1 Hz								
1	167.7	0.6	55.5					7.4	14.8		
6	160.4			26.4	20.0	2.9	12.0				
13	155.4			0.4	26.0						
7	76.5										
11	71.1										
10	67.6										
9	64.1										
3	58.9	0.8								1.9	7.2
4	58.5	1.5	37.0								
12	57.9										
2	52.1	0.7	55.5								
5	46.8	1.9	37.0								
8	46.6										
16	45.9	0.2	37.0								
19	36.6	0.9									
15	34.2	0.5	48.1								
17	26.9	0.4	37.0 35.1								
18	20.5	0.3	35.1								



Scheme 4.



Scheme 5.

yield, was next converted to the iodide **10** and then to arginine in standard fashion.

In this manner, arginines **4b**, **4c**, and **4d** were prepared. Arginine **4a** was provided by the Los Alamos Scientific Laboratory. Isotope content of the arginines and of the various intermediate compounds were determined by mass spectrometry, and the positions of the isotopes were confirmed by ^{13}C and ^{1}H NMR spectroscopy.

[$^{13}C/^{15}N$]*Arginines*. Tracer amounts of DL[1-^{14}C]arginine diluted with varying amounts of unlabeled L-arginine were fed to different production broths 12 hr after they had been innoculated from the same seed broth. Five to 10% incorporations were obtained and the amount of streptothricin F produced did not seem to be affected. In the subsequent stable isotope feedings a ^{14}C-labeled arginine was included.

A mixture of 99.6 mg L-[guanido-^{13}C, $^{15}N_2$]arginine (**4a**) (92 atom% ^{13}C, 94 at.% ^{15}N) and 10 μCi DL-[1-^{14}C]arginine were fed to two 250 mL broths, and subsequently worked up to afford 125 mg of pure helianthate of **1**.[30] The L/DL mixture was chosen specifically to determine whether one or both racemates were active in the biosynthetic pathway.

The ^{13}C NMR spectrum of streptothricin F trihydrochloride **1b** (Fig. 1), generated from the helianthate, showed two enriched spin-coupled resonances; the guanidine C appeared as a quintet[31] at δ160.4 and the carbamate carbon appeared as a trio[31] at δ155.4.[32] Coupling constants are given in Table 1. The highly enriched (26.4%) quintet firmly established the intact incorporation of the guanido group, and allowed the assignment of the C-6 and C-13 resonances. Based on the radioactivity that had also been incorporated, utilization of only the L-enantiomer would have led to a 23.8% enrichment, while an 11.9% enrichment would have resulted from the equal utilization of both enantiomers. Thus, the observed value of 26.4% indicated the utilization of only L-arginine. Since the ^{13}C and ^{14}C labels were at opposite ends of the precursor and separated by a potentially labile carbon–nitrogen bond, it appeared that the data supported a pathway involving the intact L-arginine.

The intact incorporation of **4** was confirmed by use of arginine **4b**[30] (co-fed with DL-[5-^{14}C]arginine), which was synthesized according to Scheme 4 using potassium [^{15}N]phthalimide (98 at.% ^{15}N) and [^{13}C]urea (90 at.% ^{13}C). In this compound the stable isotope labels, ^{13}C and ^{15}N, were again separated by the potentially labile bond. If **4b** were first metabolized to ornithine and then back to arginine, it would have been statistically unlikely that the ^{15}N and ^{13}C labels would again reside in the same molecule, and only an enhancement of the natural abundance singlet would be expected. However, the ^{13}C NMR spectrum of the derived **1c** showed a 2.9% enrichment of the guanido carbon spin-coupled to an adjacent ^{15}N. A slight upfield shift (1.05 Hz) due to an isotope effect[33] was also observed for the spin-coupled doublet. The observed spin-coupling clearly demonstrated the formation of a new C–N bond, and confirmed that arginine had been incorporated intact. Thus, all three nitrogens attached to C-6 in **1** were derived from arginine. Most importantly, the results gave clear support for Scheme 1.

Arginine **4c** was synthesized according to Scheme 4, and arginine **4d** according to Scheme 5. These were each fed in order to further confirm the validity of Scheme 1, and to further test the general utility of the $^{13}C/^{15}N$ spin-coupling technique. In each case both isotopes were incorporated and the spin-coupled doublets observed in the ^{13}C NMR spectra (J_{CN} = 14.8 and 7.2 Hz, respectively) demonstrated the formation of the remaining two new C–N bonds predicted according to Scheme 1.

[^{2}H]*Arginines*. While there could be no doubt that the skeleton of **2** is biosynthesized along lines indicated by Scheme 1, 1,4-addition to a dehydroarginine **5** provided only one of the possibilities for the formation of **6**. As outlined in Scheme 6, oxidation of

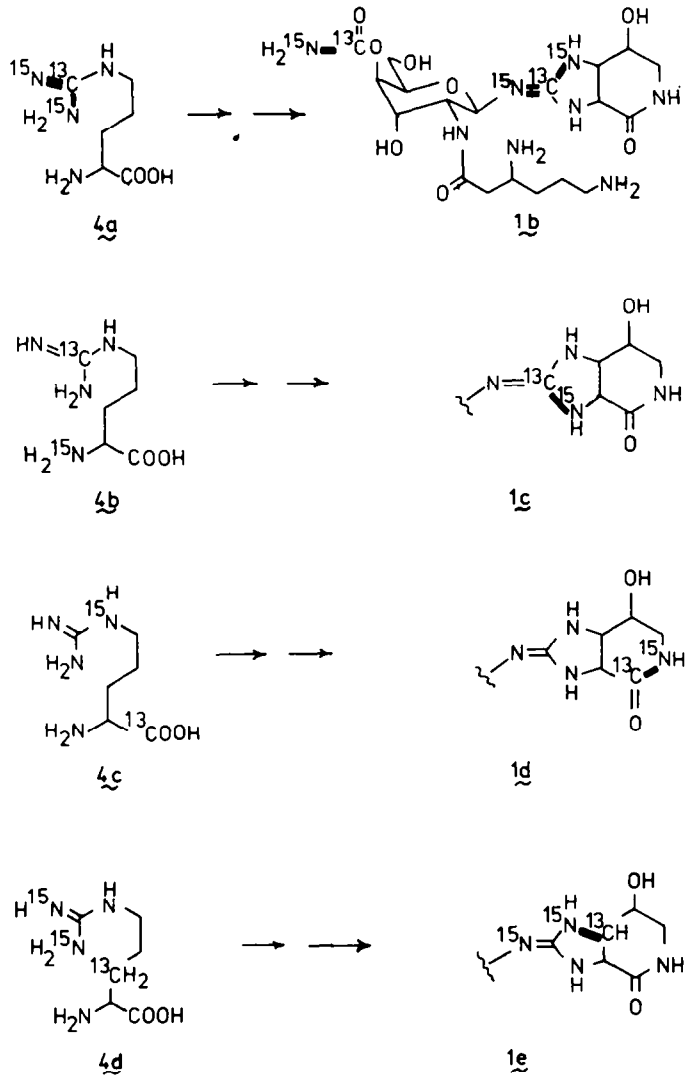


Fig. 1.

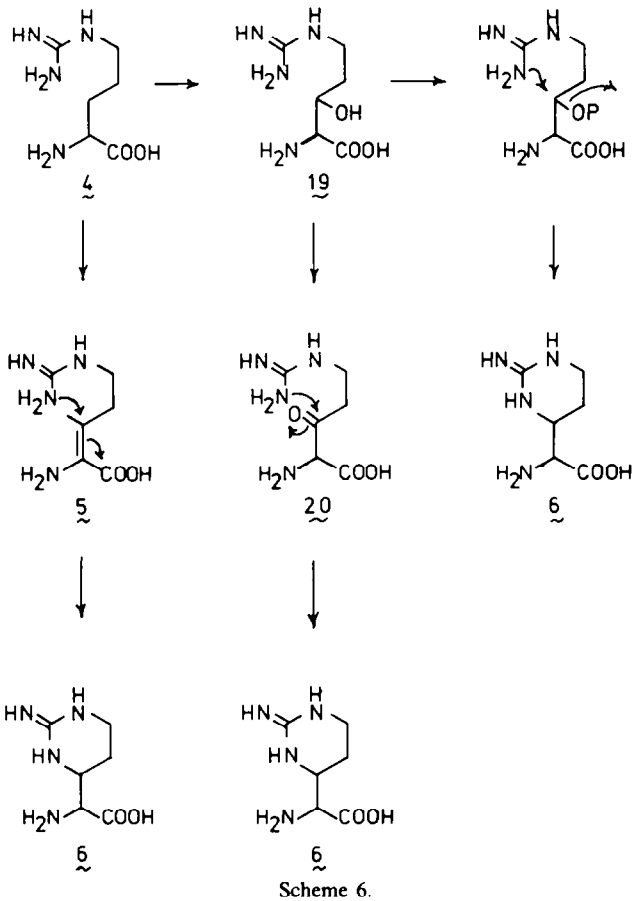
4 to 3-hydroxyarginine (**19**) followed by nucleophilic substitution, or further oxidation to 3-keto-arginine (**20**) followed by reductive amination offered plausible alternatives.

Three deuterated arginines were synthesized. [2-^{2}H]Arginine (**4e**) was obtained by exchange of **4** in D_2O, followed by purification via the flavianate salt, and [3-$^2H_2$]arginine (**4f**) was synthesized by Scheme 5. [2,3,3-$^2H_3$]Arginine (**4g**) was prepared by exchange of arginine in D_2O in the presence of pyridoxal and aluminum sulfate at 100° for 2 days.[34] These arginines were each converted biosynthetically to streptothricin F (Scheme 7). In Scheme 6, route *a* would lead to loss of the D label at the α-C and one of the D's at the β-C. Route *b* would lead to loss of only one D at the β-C. Route *c* would lead to the direct loss of both D's from the β-C, while some or all of the D at the α-C potentially could be lost simply by exchange of the doubly-activated methine.

In order to analyze the antibiotic samples **1f**, **1g** and **1h** obtained from feeding of **4e**, **4f** and **4g**, respectively, it was first necessary to assign the complete proton NMR spectrum of **1**.[35] The 41.44 MHz ^{2}H NMR spectra of **1f–1h** were then obtained at 10° in D depleted water containing *t*-butanol for the chemical shift reference (δ 1.28). Each spectrum was devoid of D resonances at δ4.56 (H-2) and at δ4.02 (H-3), while the ^{14}C incorporations (11.5, 13.7 and 1.6%, respectively) indicated D enrichments of 1.5, 0.4 and 1.8% if one D were specifically retained in each case. With such enrichments, the D content in each of these NMR samples would have been 1.5 (**1f**), 0.8 (**1g**) and 1.6 (**1h**) μmoles, respectively, substantially more than the 0.4 μmoles of D easily detected in the *t*-butanol reference.[36] Within the limits of the sensitivity of ^{2}H NMR, it therefore appears that the D labels were lost in each case, results that indicate the biosynthesis of **2** via 3-ketoarginine (**20**).

CONCLUSIONS

It is clear from the results presented that L-arginine is the direct primary precursor to the streptolidyl moiety of streptothricin F. The metabolism of arginine is now understood in considerable detail, and

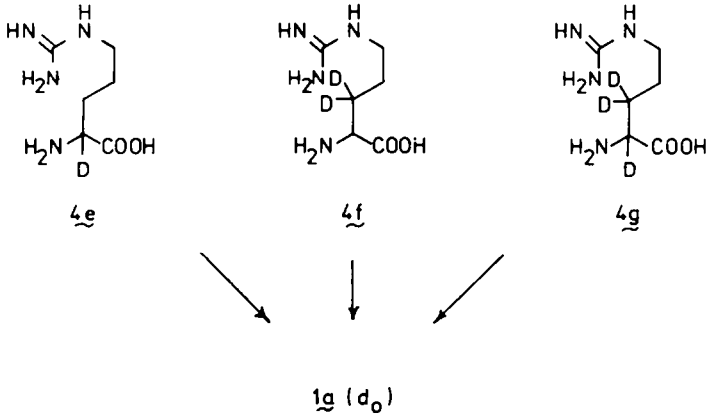


Scheme 6.

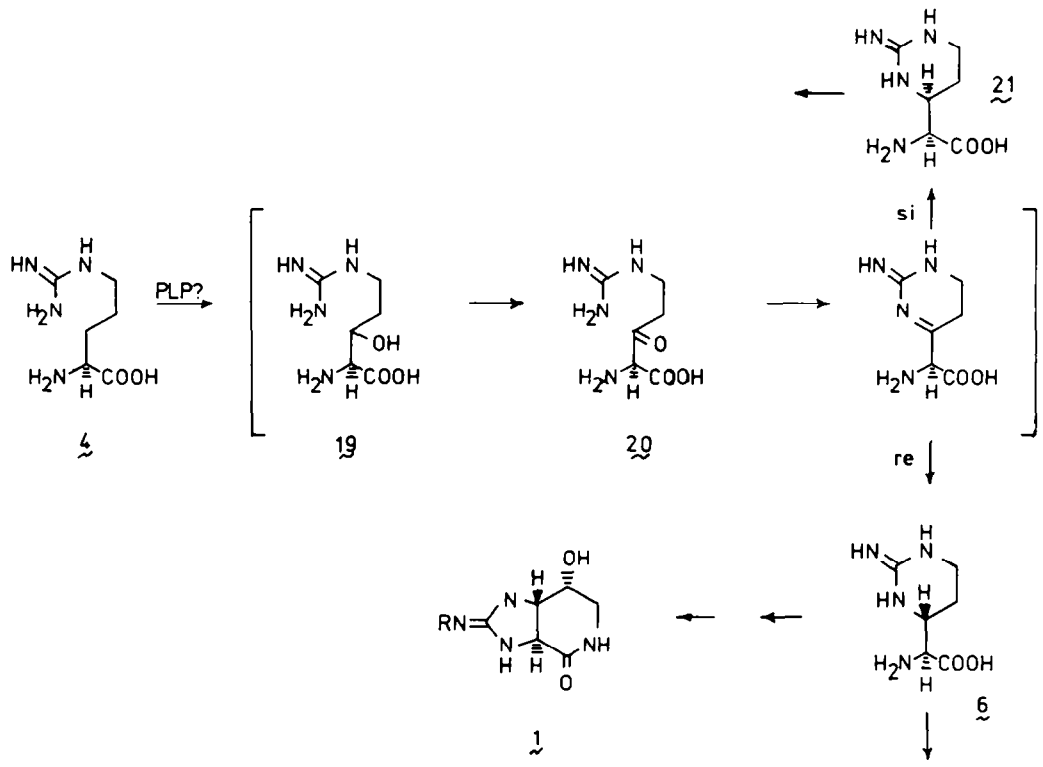
appears to proceed via the β-keto amino acid as deduced from the D labeling studies. The generation of the streptolidine skeleton from arginine has been clearly defined from the observed spin-couplings resulting from the [^{15}N, ^{13}C]arginine feedings. Taken together, it is again apparent that Nature can generate complex structures with remarkably simple pathways.

The present data yield a compellingly attractive picture. As shown in Scheme 8 reduction of the endocyclic imine from the *re* side would lead to capreomycidine and then to streptolidine or to tuberactidine, while reduction from the *si* side would lead to chymostatin[18a] and elastatinal[18b] via **21**.

The loss of both D labels at C-3 of arginine is easily explained by the intermediacy of a ketone, and loss of the D label at C-2 can be explained chemically by enolization of the β-dicarbonyl moiety. However, the repeated loss of all D from C-2 is striking and perhaps provides an unexpected insight to the early

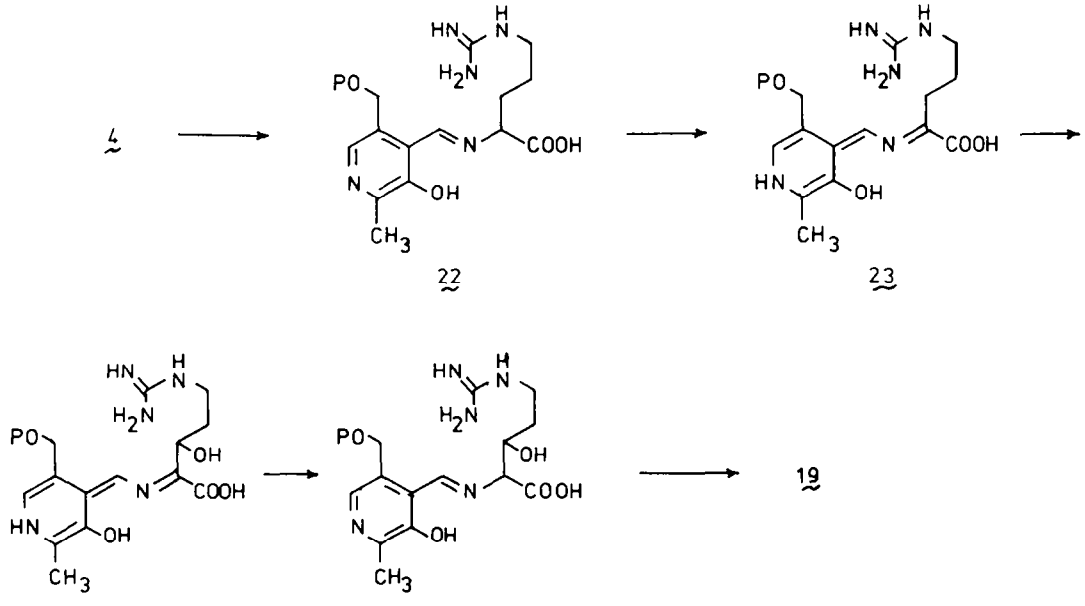


Scheme 7.



Scheme 8.

steps in the pathway. Loss cannot be due to a transaminase reaction because the α-N is retained (**1c**). Loss due to an epimerase reaction is extremely unlikely since the 2*S*-configuration of the starting arginine is retained in the product, and this holds for *all* of the biogenetically related iminohexahydropyrimidylglycines (viomycin, tuberactinomycin, chymostatin, elastatinal, etc.). In fact, considering the feeding protocol used—repeated addition of the deuterated arginine at 12, 20 and 30 hr—it would be remarkable if simple chemical exchange or a coincidental enzymatic reaction, not obligatory for the formation of **2**, resulted in complete loss of the α-D in all three experiments.



Scheme 9.

Hydroxylation of isolated aliphatic carbons is well documented in biological systems, and such a reaction (e.g. at C-3 of **4**) could be involved in the formation of **2**. However, condensation of arginine with a carbonyl compound, such as pyridoxal phosphate, to yield Schiff base **22**, followed by tautomerization to **23** would yield a more reactive allylic β-carbon, as shown in Scheme 9.[37] This would, at the same time, necessarily remove a D label located at C-2. If correct, its generality would possibly extend to the formation of erythro-β-hydroxyhistidine, erythro-β-hydroxyleucine,[38] threo-β-hydroxyleucine,[39] β-hydroxyvaline,[40] and β,γ-dihydroxyglutamic acid.[41] Furthermore, antibiotic AT-125[42] would be most easily understood as biosynthesized from erythro-β-hydroxyglutamic acid, and the hydroxypiperazine unit of antibiotic BBM-928A[43] is most likely derived from erythro-β-hydroxyornithine. All of these are 2*S*-amino acids and the near-exclusivity of erythro geometry again argues for a common biosynthetic mechanism.

EXPERIMENTAL

General. ^{1}H NMR spectra of synthetic intermediates were taken on a Hitachi Perkin–Elmer R-24 spectrometer; ^{13}C NMR spectra were taken at 67.88 MHz and 125 MHz on Bruker HX 270 and WM 500 spectrometers, respectively; ^{2}H NMR spectra were taken at 41.44 MHz on a Bruker HX 270 spectrometer. All ^{13}C NMR spectra were broad band decoupled and ^{2}H NMR spectra were proton decoupled and run unlocked. Samples run on the HX 270 were contained in 10 mm tubes containing a cylindrical 0.50-mL capacity insert (Wilmad Glass Co.), and samples run on the WM 500 were contained in 5 mm tubes. Samples for ^{13}C NMR spectra were dissolved in 2% pyridine/D_2O with the middle pyridine resonance at 135.5 ppm used for reference, while those for ^{2}H NMR spectra were dissolved in D depleted water (Aldrich Chemical Co.) and spiked with *t*-BuOH to provide a chemical shift reference at 1.28 ppm. ^{2}H NMR spectra were obtained at 10°; the pH of samples was between 6.5–7.0 in most cases.

IR spectra were obtained with a Beckman Microlab 620MX Computing Spectrometer, and mass spectra were measured on an AEI MS-902 using EI. All radioactive measurements were carried out in Packard Tri-Carb 3375 or Beckman LS 8000 liquid scintillation counters. Samples were prepared either by combustion to CO_2 in a Packard Model 306 Sample Oxidizer and trapped in Carbosorb/Permafluor V, or by dissolving in 0.2 mL MeOH, adding 0.2 mL sat aq dithionite to decolorize the orange soln, and then adding 10 mL Aquascint scintillation cocktail. Microsamples were weighed on a Cahn Model 4400 electrobalance. All measurements were done in duplicate to ±1% standard deviation. Counting efficiencies were determined by spiking with [^{14}C]-n-hexadecane standard purchased from Amersham/Searle. M.ps were determined in a Hoover capillary m.p. apparatus.

Linear TLC was done using Baker-flex ^{R} Silica Gel IB-F sheets and circular TLC was done using 3.5″ × 3.5″ squares of Merck Silica Gel 60 F-254 Al-backed sheets. Biorad IRC-50, and AG3-X4 resins were purchased from Biorad; Sephadex LH-20 was purchased from Sigma Chemical Co. Sodium [^{13}C]cyanide and [^{13}C]urea were purchased from Prochem Isotopes, Inc. Potassium [^{15}N]phthalimide, diethyl [1,3-$^{13}C_2$]malonate, and L-[guanido-^{13}C, $^{15}N_2$]arginine were provided by the Los Alamos Scientific Laboratory. $^2H_2$ gas was purchased from Merck and Co.; D depleted water and ^{2}HCl were purchased from Aldrich Chemical Co.; NaO^{2}H (40% in ^2H_2O) was purchased from Wilmad Glass Co. [^{14}C]Arginines were purchased from CEA, France. *Bacillus subtilis* ATCC 6633 spore suspensions were purchased from Baltimore Biological Laboratories. *Streptomyces* L-1689-23 and quantities of streptothricin F were generous gifts from Dr. Donald Borders of Lederle Laboratories. All chemicals were of reagent grade and all solvents were distilled prior to use.

Culture conditions. An improved strain of *Streptomyces* L-1689-23 (produced 200–400 mg/L) was maintained at 5° on agar slants composed of 1% malt extract, 0.4% yeast extract, 4% dextrose, and 2% agar adjusted to pH 7.3. Seed cultures were prepared by innoculating 50–250 mL of medium, containing 0.3% beef extract, 0.5% yeast extract, 0.5% tryptone, 0.1% dextrose, and 2.4% cornstarch with spores from an agar slant. The cultures, contained in Erlenmeyer flasks of 4–5 times the culture volume, were incubated at 29° on a New Brunswick G-25 gyrotory shaker at 200 rpm for 2.5 days. Production media, 250 mL in 1-L Erlenmeyer flasks, consisting of 3.0% cornstarch, 2% molasses, 1.5% soy flour, and 1% $CaCO_3$, were innoculated with 20 mL of seed culture and incubated for 2–2.5 days. Arginines labeled at C-2 were added in a sterile manner through disposable Millipore filters at 12, 20 and 30 hr after innoculation of the production broth. All other labeled arginines were added sterilely at 12 hr after innoculation.

Isolation. The cultures were centrifuged at *ca* 5000 × g for 15 min in an IEC Model K centrifuge. The supernatant was loaded onto an IRC-50 (K^+ form) column (1.6 × 18 cm) and then washed with 300 mL water. The antibiotic was eluted off with 0.3 N HCl, and the ninhydrin-positive fractions were neutralized to pH 6-7 with AG3X4 (OH^- form) resin. After freeze-drying, the residue was extracted with MeOH to remove salts, and the MeOH soln was reduced to dryness, dissolved in 3 mL water and loaded onto a Sephadex LH-20 column (2.5 cm × 225 cm).

After eluting with water, those ninhydrin-positive fractions not containing low R_f impurities (circular TLC with n-PrOH–pyridine–HOAc–water = 5:15:3:17) were combined, bioassayed and lyophilized. The resulting powder was dissolved in *ca* 5 mL MeOH and to this was added a 4-fold molar excess (based on antibiotic bioassay) of methyl orange in a minimal volume of hot water. The soln was stirred 1 hr, taken to dryness by rotary evaporation and lyophilization, taken up in hot MeOH and filtered to remove excess methyl orange. The helianthate salt of streptothricin F was then recrystallized to constant specific radioactivity.

The helianthate was converted to the hydrochloride for NMR studies. The salt was suspended in water, acidified with 1 N HCl to pH 1–2, and centrifuged to remove methyl orange. Residual traces of methyl orange were removed by passing the supernatant through a column of AG3X4 (OH^- form) eluted with acidic water (pH 1); the ninhydrin-positive fractions were adjusted to pH 6–7 with HCl and then lyophilized.

Bioassay. A 0.2 mL aliquot of *B. subtilis* ATCC 6633 spore suspension was pipetted into 10.0 mL sterile saline from a stock suspension. A 0.2 mL aliquot of the spore suspension was evenly spread over Brain Heart Infusion agar (Difco) petri plates. Paper filter disks (Schleicher and Schuell #704-E, 0.5 in dia) were placed on the surface (up to 5 disks per plate) and each moistened with 50 μl of the test soln. The plates were incubated at 37° for 18 hr and antibiotic concentrations determined by comparison of the zone of inhibition with a standard curve.

Diethyl bromomalonate. A mixture of diethylmalonate (4 g, 24.9 mmol) and CCl_4 (6 mL) was placed in a 2-neck, 250 mL round bottom flask equipped with a magnetic stirring bar, reflux condenser, and an addition funnel containing Br_2 (4.04 g, 25.6 mmol) in CCl_4 (16 mL). Several milliliters of the Br-soln were added to the flask and the flask then irradiated with a Tensor lamp. As the red color disappeared, more Br_2 was added to maintain a gentle reflux. After addition was complete, the soln was heated at reflux for 1 hr, then cooled and diluted with additional CCl_4. The soln was washed with 5% Na_2CO_3 (2 × 10 mL), dried over $MgSO_4$, filtered, conc *in vacuo*, and distilled to yield

5.95 g (100%) of diethyl bromomalonate: b.p. 228–229° (lit.[44] 233–235°); IR (neat) 2989, 1760, 1741 cm^{-1}; ^1H NMR ($CDCl_3$) δ1.28 (6H, t, J = 7.2 Hz), 4.23 (4H, q, J = 7.2 Hz), 4.87 (1H, s); ^{13}C NMR ($CDCl_3$) δ164.4, 63.2, 42.5, 13.9.

Using diethyl [1,3-$^{13}C_2$]malonate (4 g, 24.7 mmol) two reactions yielded 11.65 g (98%) of diethyl [1,3-$^{13}C_2$]-bromomalonate: ^1H NMR δ1.27 (6H, t, J = 7.3 Hz), 4.2 (4H, q, J = 7.3 Hz, $^3J_{CH}$ = 3.5 Hz), 4.75 (1H, t, $^2J_{CH}$ = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 1:20 dilution with unlabeled compound) δ164.6.

N-(3-*Bromo*-1-*propyl*)-*phthalimide* (**8**). Using a dry box, potassium phthalimide (6.2 g, 33.2 mmol), 1,3-dibromopropane (6.3 g, 31 mmol), hexadecyltri-n-butyl phosphonium bromide (1.75 g, 3.45 mmol) and dry toluene (75 mL) were combined in a flame-dried 250 mL round bottom flask equipped with a stirring bar, reflux condenser, and drying tube. The mixture was then heated at 100° for 3 hr. After cooling, the ppt was filtered and washed with ether (3 × 20 mL), and the combined filtrates were conc *in vacuo* to dryness. The residue was taken up in toluene (50 mL), filtered, and passed through a column of silica gel (3 cm × 9 cm) eluted with toluene. Product-containing fractions were combined, and conc to dryness. The residue was dissolved in ether (50 mL), washed with 10% NaOH, H_2O, sat. brine, and then dried over $MgSO_4$, conc *in vacuo*, and triturated with petroleum ether to yield 2.98 g (51%) of **8**: m.p. 72–73° (lit.[45,46] 73–74°); IR (KBr) 1770, 1700 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.24 (2H, dt, J = 7, 7 Hz), 3.41 (2H, t, J = 7 Hz), 3.82 (2H, t, J = 7 Hz), 7.8 (4H, m); ^{13}C NMR ($CDCl_3$) δ168.1, 133.9, 132.0, 123.2, 36.7, 31.7, 29.8.

Under the same conditions potassium [^{15}N]phthalimide (6.2 g, 33.2 mmol) was converted to **8c** (6.35 g, 71%): m.p. 66–67°; IR (KBr) 1769, 1708 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.28 (2H, m, J_{HH} = 6.6 Hz, $^4J_{NH}$ = 2.1 Hz), 3.4 (2H, t, J = 6.6 Hz), 3.85 (2H, dt, J_{HH} = 6.6 Hz, $^2J_{NH}$ = 1.3 Hz), 7.76 (4H, m); ^{13}C NMR ($CDCl_3$) δ168.2 (d, J_{CN} = 12.7 Hz), 134.7, 132.0 (d, $^2J_{CN}$ = 6.9 Hz), 123.3, 36.7 (d, J_{CN} = 9.8 Hz), 31.7, 29.9.

N-(3-*Iodo*-1-*propyl*)-*phthalimide* (**10**). Compound **8** (3.5 g, 12 mmol) and NaI (7.8 g, 52 mmol) in dry acetone (60 mL) were heated at reflux 12 hr. The mixture was cooled and conc *in vacuo*, and the residue was suspended in water (100 mL) and stirred for 30 min. The solid was filtered off, washed with water, and dried under vacuum 12 hr. Recrystallization from 95% EtOH/H_2O afforded 3.41 g (84%) of **10**: m.p. 87–88° (lit.[47] 88°); IR (KBr) 1769, 1710 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.21 (2H, tt, J = 6.6 Hz), 3.15 (2H, t, J = 6.6 Hz), 3.75 (2H, t, J = 6.6 Hz), 7.73 (4H, m); ^{13}C NMR ($CDCl_3$) δ168.1, 134.0, 131.9, 123.2, 38.6, 32.5, 1.4; MS, *m/e* 315 (6.8), 188 (97.1), 160 (100).

Under the same conditions **8c** (6.26 g, 23 mmol) was converted to the iodide **10c** (7.15 g, 99%): m.p. 87–88°; IR (KBr) 1765, 1704 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.23 (2H, dtt, J_{HH} = 6.6 Hz, $^4J_{NH}$ = 2.2 Hz), 3.17 (2H, t, J = 6.6 Hz), 3.76 (2H, dt, J = 6.6 Hz, $^2J_{NH}$ = 1.5 Hz), 7.75 (4H, m); ^{13}C NMR ($CDCl_3$) δ168.1 (d, J_{CN} = 12.7 Hz), 134.0, 131.9 (d, $^2J_{CN}$ = 7.8 Hz), 123.2, 38.6 (d, J_{CN} = 9.8 Hz), 32.5, 1.4; MS, *m/e* 316 (0.5), 189 (90.5), 161 (100).

Diethyl phthalimidomalonate (**9**). Diethyl bromomalonate (9 g, 37.6 mmol) was added to potassium phthalimide (6.1 g, 33 mmol) and hexadecylbutylphosphonium bromide (1.75 g, 3.45 mmol) in anhyd toluene (100 mL). The mixture was stirred 20 hr, then filtered and the filtrate conc *in vacuo*. The residue was taken up in $CHCl_3$ (20 mL) and filtered through a column of silica gel eluted with $CHCl_3$ to remove the catalyst. Product-containing fractions were pooled and concentrated to dryness, and the product recrystallized from ethyl ether–petroleum ether to yield 8.05 g (80%) of **9**: m.p. 75–76° (lit.[46] 73–74°); ^1H NMR ($CDCl_3$) δ1.38 (6H, t, J = 7 Hz), 4.3 (4H, q, J = 7 Hz), 5.45 (1H, s), 7.75 (4H, m); ^{13}C NMR ($CDCl_3$) δ166.4, 164.2, 134.3, 131.6, 123.7, 62.7, 54.5, 14.0; MS, *m/e* 305 (0.4), 232 (52), 187 (100).

Using potassium [^{15}N]phthalimide (1.5 g, 8.1 mmol), 2.3 g (100%) of **9b** was prepared: m.p. 75–76°; IR (KBr) 1758, 1738, 1712 cm^{-1}; ^1H NMR ($CDCl_3$) δ1.3 (6H, t, J = 7 Hz), 4.2 (4H, q, J = 7 Hz), 5.21 (1H, d, $^2J_{NH}$ = 1.8 Hz), 7.65 (4H, m); ^{13}C NMR ($CDCl_3$) δ166.4 (d, J_{CN} = 13.3 Hz), 164.2, 134.4 (d, $^2J_{CN}$ = 8.8 Hz), 131.6, 123.8, 62.7, 54.5 (d, J_{CN} = 12.7 Hz), 14.0; MS, *m/e* 306 (0.3), 233 (76), 188 (100).

Using diethyl [1,3-$^{13}C_2$]bromomalonate (11.68, 48 mmol), the corresponding [$^{13}C_2$]-**9c** was obtained (8.41 g, 56%): m.p. 75–76°; IR (KBr) 1779, 1772, 1706 cm^{-1}; ^1H NMR ($CDCl_3$) δ1.3 (6H, dt, J_{HH} = 7 Hz, $^4J_{CH}$ = 7.3 Hz), 4.23 (4H, dq, J_{HH} = 7 Hz, $^3J_{CH}$ = 3.5 Hz), 5.26 (1H, t, $^2J_{CH}$ = 8.3 Hz), 7.72 (4H, m); ^{13}C NMR ($CDCl_3$, 1:20 dilution with unlabeled compound) δ164.3; MS, *m/e* 307 (0.03), 233 (46.3), 188 (100).

DL-*Ornithine hydrochloride* (**12**). NaH (160 mg, 50% oil dispersion) in a flame-dried 100 mL 3-neck flask equipped with a stirring bar and drying tube and under N_2 was treated with abs EtOH (3 mL). The mixture was heated to 60° with an oil bath, and **9** (1 g, 3.3 mmol) was added, yielding a yellow-colored soln. After 5 min, the solvent was removed under high vacuum and heated to 120° for 30 min. The vacuum was replaced with N_2, and **10** (1.3 g, 3.3 mmol) in anhyd toluene (10 mL) was added. The solid lumps were quickly broken up with a spatula and the mixture was stirred while the solvent was slowly removed at aspirator pressure. The system was then heated to 60° and evacuated at 0.1 mm for 30 min. After again replacing the vacuum with N_2, the residue was heated to 160° for 3 hr, and then cooled and slurried with $CHCl_3$. This was filtered, the solid further washed with $CHCl_3$, and the combined filtrates conc to dryness to yield the crude **11** (1.9 g). This **11** was directly hydrolyzed by dissolving in 10 mL each of H_2O, conc HCl, and glacial HOAc, and heating at 100° overnight. After cooling, phthalic acid was removed by filtration and washed with water, and the combined filtrates were conc to dryness. The residue was taken up in water (5 mL), adjusted to pH 8 with NaOH aq, and EtOH added to the cloud point. Crystallization was effected in a refrigerator overnight, and the product recrystallized to give 340 mg (62%) of **12**: m. p. >200° (lit.[46,48] 230° dec).

Under these conditions, **9b** (2.81 g, 9.2 mmol) and **10** (3.02 g, 9.6 mmol) yielded 4.75 g of crude **11b** (99%), which was hydrolyzed to yield 0.64 g of **12b** (41% overall): m.p. >200° (dec); IR (KBr) 2120, 1630 cm^{-1}; ^1H NMR (D_2O) δ1.78 (2H, m), 1.96 (2H, m), 3.08 (2H, t, J = 7.6 Hz), 3.81 (1H, t, J = 6.1 Hz); ^{13}C NMR (D_2O) δ174.8, 54.9 (d, J_{CN} = 5.2 Hz), 39.8, 38.2, 23.6.

Beginning with **9c** (2.9 g, 9.5 mmol) and **10c** (3.13 g, 10.0 mmol), 0.92 g (58%) of **12c** was obtained: m.p. >200° (dec); IR (KBr) 1635, 1625 cm^{-1}; ^1H NMR (D_2O) δ1.81 (2H, m), 1.96 (2H, m), 3.08 (2H, t, J = 7.6 Hz), 3.81 (1H, dt, J_{HH} = 5.4 Hz, $^2J_{CH}$ = 5.4 Hz); ^{13}C NMR (D_2O) δ171.5, 51.6 (d, J_{CH} = 26.9 Hz), 36.4 (d, J_{CN} = 2.5 Hz), 28.4, 25.0.

From diethyl phthalimidomalonate (1.06 g, 3.5 mmol) and **10d** (1.154 g, 3.7 mmol), **12d** was prepared (0.36 g, 62%): m.p. >200° (dec), IR (KBr) 1618 cm^{-1}; ^1H NMR (D_2O) δ1.84 (2H, m), 2.09 (2H, dm, J_{CH} = 124.2 Hz), 3.09 (2H, dt, J_{HH} = 7.6 Hz, $^3J_{CH}$ = 3.4 Hz), 3.81 (1H, dt, J_{HH} = 8.5 Hz, $^2J_{CH}$ = 4.4 Hz).

From **10f** (1.154 g, 3.7 mmol), **12f** (0.16 g, 25%) was obtained: m.p. >200° (dec); IR (KBr) 1621 cm^{-1}; ^1H NMR (D_2O) δ1.89 (2H, m), 3.16 (2H, t, J = 7.7 Hz), (1H, m).

N-(3-*Cyanoethyl*)-*phthalimide* (**14**). A soln of NaCN (0.96 g, 19.6 mmol) and **13** (5 g, 19.6 mmol) in DMSO (15 mL) in a 50 mL round bottom flask was stirred and heated at 65° for 6 hr. The mixture was reduced to half-volume under high vacuum, and then poured onto ice. After stirring 1 hr, the ppt was filtered off, washed with cold water (2 × 20 mL) and dried under vacuum to given 3.9 g (99%) of **14**: m.p. 150–151° (lit.[49] 153–154°); IR (KBr) 2241, 1762, 1705 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.78 (2H, t, J = 6.6 Hz), 3.96 (2H, t, J = 6.6 Hz), 7.7 (4H, m); ^{13}C NMR ($CDCl_3$) δ167.4, 134.4, 132.6, 123.2, 117.0, 33.6, 17.2; MS *m/e* 200 (6.5), 173 (13.5), 160 (100).

Using sodium [^{13}C]cyanide (3.37 g, 67.2 mmol, 90.3 at.% enriched), **14d** was prepared in 95% yield (17.83 g): m.p.

150–151°; IR (KBr) 2942, 1761, 1705 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.8 (2H, dt, J_{HH} = 6.8 Hz, $^2J_{CH}$ = 6.7 Hz), 3.96 (2H, dt, J_{HH} = 6.8 Hz, $^2J_{CH}$ = 6.7 Hz), 3.96 (2H, dt, J_{HH} = 6.8 Hz, $^3J_{CH}$ = 3.3 Hz), 7.75 (4H, m); ^{13}C NMR ($CDCl_3$) δ167.4, 134.4, 132.6, 123.2, 117.0, 33.6 (d, $^2J_{CC}$ = 2.6 Hz), 17.2 (d, J_{CC} = 58.3 Hz); MS, *m/e* 201 (6.6), 173 (13.3), 160 (100).

N-(3-(p-*Toluenesulfonamido*)-1-*propyl*)-*phthalimide* (**16**). The nitrile **14** (2 g, 10.0 mmol) in abs EtOH (75 mL) and conc HCl (1 mL) was reduced in the presence of Pt catalyst (0.05 g PtO_2) under H_2. When one quiv of H_2 had been taken up (450 mL), the mixture was filtered, the catalyst washed with 95% EtOH (20 mL), and the combined filtrates conc *in cavuo*. The residue was placed on high vacuum overnight, then dissolved in CH_2Cl_2 (100 mL) and treated with *p*-toluenesulfonyl chloride (1.9 g, 10.0 mmol) and Et_3N (3 mL). After stirring 10 hr, solvent was removed *in vacuo*, the residue dried overnight at reduced pressure, and then recrystallized from 95% EtOH–water to give 2.2 g (60%) of **16**: m.p. 154–155°; IR (KBr) 1765, 1705 cm^{-1}; ^1H NMR ($CDCl_3$) δ1.82 (2H, tt, J = 8.0 Hz), 2.38 (3H, s), 3.0 (2H, t, J = 8.0 Hz), 3.69 (2H, t, J = 8.0 Hz), 5.34 (1H, m), 7.20 (2H, d, J = 7 Hz), 7.70 (6H, m); ^{13}C NMR ($CDCl_3$) δ 167.0, 144.8, 134.1, 133.8, 129.6, 127.1, 123.4, 120.1, 40.1, 34.6, 28.9, 21.5.

Compound **14d** (10.5 g, 52.4 mmol) was reduced in three portions, and each immediately treated with Et_3N (3 equiv), and tosyl chloride (1 equiv) in CH_2Cl_2 (100 mL). Work-up afforded a combined yield of **16d** (5.15 g, 27%): m.p. 154–155°; IR (KBr) 1761, 1701 cm^{-1}; ^1H NMR ($CDCl_3$) δ1.82 (2H, m), 2.40 (3H, s), 3.0 (2H, dm, J_{HH} = 7.5 Hz, J_{CH} = 90 Hz), 3.70 (2H, m), 5.53 (1H, m), 7.24 (2H, m), 7.7 (6H, m); ^{13}C NMR ($CDCl_3$) δ167.0, 144.8, 134.1, 133.8, 129.6, 127.1, 123.4, 120.1, 40.1, 34.7 (d, J_{CC} = 40.0 Hz), 28.9, 21.5.

Compound **14** (6 g, 30 mmol) in 1 g batches was reduced with $^2H_2$ in [O-^{2}H] EtOH and ^{2}HCl. Treatment with EtN and tosyl chloride in CH_2Cl_2 afforded 6.41 g (59% overall) of **16f**: m.p. 153–155°; IR (KBr) 1767, 1706 cm^{-1}; ^1H NMR ($CDCl_3$) δ1.81 (2H, t, J = 7.5 Hz), 2.36 (3H, s), 3.69 (2H, t, J = 8.0 Hz), 5.5 (1H, s), 7.24 (2H, d, J = 8 Hz), 7.7 (6H, m).

N-(3-(N'-*Nitroso* - p - *toluenesulfonamido*) - 1 - *propyl*) - *phthalimide* (**17**). Sulfonamide **16** (1.0 g, 2.8 mmol), in Ac_2O (13.95 mL) and glacial AcOH (2.8 mL) was cooled to 0°, and $NaNO_2$ (4.18 g, 61.3 mmol) was added over a 8 hr period to the stirred soln. The mixture was maintained at 0° for an additional 8 hr, and then poured into ice water (50 mL) and stirred 30 min. This was extracted with ether and the combined extracts washed with water, 5% $NaHCO_3$, again with water, and finally with sat NaCl aq. The organic layer was dried ($MgSO_4$), filtered, and conc *in vacuo*, and the residue recrystallized from CH_2Cl_2–petroleum ether to yield 0.74 g (69%) of **17**: m.p. 118–119°; IR (KBr) 1765, 1708 cm^{-1}; ^1H NMR ($CDCl_3$) δ1.81 (2H, tt, J = 6.8 Hz), 2.41 (3H, s), 2.74 (2H, t, J = 6.8 Hz), 3.6 (2H, t, J = 7 Hz), 3.75 (2H, t, J = 7 Hz), 7.28 (2H, d, J = 8 Hz), 7.7 (8H, m).

N-(3-*Hydroxy*-1-*propyl*)-*phthalimide tosylate* (**18**). Compound **17** (0.73 g, 1.87 mmol) and anhyd Na_2CO_3 (0.20 g, 1.87 mmol) in CCl_4 (150 mL) were stirred under N_2 and heated under reflux for 26 hr. After cooling, the solvent was removed *in vacuo* and the residue, in $CHCl_3$, was chromatographed on silica gel, eluting with $CHCl_3$. Product-containing fractions were combined and concentrated to give 0.50 g of **18** (74%): m.p. 147–148°; IR (KBr) 1760, 1699 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.0 (2H, t, J = 6 Hz), 2.4 (3H, s), 3.7 (2H, t, J = 6 Hz), 4.6 (2H, t, J = 6 Hz), 7.2 (2H, d, J = 8 Hz), 7.7 (6H, m).

N-(3-*Iodo*-1-*propyl*)-*phthalimide* (**10**). NaI (0.4 g, 6.9 mmol) was added to tosylate **18** (0.50 g, 1.38 mmol) in acetone (50 mL), and the mixture heated under reflux for 2 hr. The soln was cooled, solvent was removed *in vacuo*, and the residue stirred in water (20 mL) for 1 hr. The solid was filtered off, washed with cold water, and dried *in vacuo*. Recrystallization from 95% EtOH–water yielded 385 mg (88%) of **10**: m.p. 87–88°.

N-(3-*Iodo*-1-*propyl*-[3-^{13}C])-*phthalimide* (**10d**). Using the conditions described, **16d** (5.3 g, 15.25 mmol) in 5 portions were each treated with $NaNO_2$ (4.58 g, 67.1 mmol) slowly added over 8 hr. After an additional 8 hr, the reactions were worked up as above to yield a total of 5.13 g (87%) of **17d**: IR (KBr) 1765, 1702 cm^{-1}. Each product was directly converted to the tosylate to give a total of 3.46 g (75%) of **18d**. This material (3.46 g, 9.91 mmol), NaI (10 g, 66.7 mmol) and acetone (200 mL) in a 500 mL round bottom flask were heated at reflux for 4 hr. After cooling, the solvent was removed *in vacuo*, water (70 mL) was added, and the suspension stirred 1 hr. The solid was removed by filtration, dried under high vacuum, taken up in $CHCl_3$, and chromatographed on silica gel (2.5 cm × 20 cm) eluted with $CHCl_3$ to yield 1.15 g (37%) of **10d**: m.p. 86–88°; IR (KBr) 1757, 1696 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.31 (2H, m), 3.78 (2H, m), 4.0 (2H, t, J = 7.2 Hz), 7.29 (4H, m); ^{13}C NMR ($CDCl_3$, 1:20 dilution with **10**) δ168.1, 134.3, 132.0, 123.4, 38.6, 32.5, 1.8; MS, *m/e* 316 (0.1), 189 (78.5), 160 (100).

N - (3 - *Iodo* - 1 - *propyl* - [3 - $^2H_2$]) - *phthalimide* (**10f**). The deuterated sulfonamide **16f** (6.41 g, 17.7 mmol) in six portions was converted to the N-nitrososulfonamide (3.84 g, 74%), thence to the tosylate and finally to the iodide **10f** (1.27 g, 30% overall from the sulfonamide): m.p. 86–88°; IR (KBr) 1755, 1694 cm^{-1}; ^1H NMR ($CDCl_3$) δ2.24 (2H, t, J = 6.7 Hz), 3.78 (2H, t, J = 6.7 Hz), 7.79 (4H, m); ^{13}C NMR ($CDCl_3$) δ169.0, 133.7, 132.1, 123.0, 38.5, 2.4; MS, *m/e* 317 (7.3), 190 (98.5), 160 (100).

O-*Methyl isourea tosylate*. Urea (1.0 g, 16.6 mmol) and freshly-distilled methyl tosylate (6.2 g, 33 mmol) in a flame-dried 25 mL round bottom flask, equipped with a stirring bar and drying tube, was heated at 100° for 5 hr. After cooling, acetone (45 mL) was added, the mixture stirred 1 hr at room temp, and then kept at 5° overnight. The crystalline product was collected, washed with cold acetone (15 mL), dried under vacuum, and recrystallized from dioxane to yield 2.34 g (57%) of the isourea salt: m.p. 134–135° (lit.[50] 134–135°); IR (KBr) 3155, 1690, 1656 cm^{-1}.

From [^{13}C]urea (0.32 g, 5.33 mmol, 90.3 at.% enriched), 0.38 g (42%) of [^{13}C]isourea salt was obtained: m.p. 135–136°; IR (KBr) 3160, 1684 cm^{-1}.

From [^{15}N]urea (0.32 g, 5.33 mmol, 98 at.% enriched), 0.63 (48%) of the [^{15}N]isourea salt was obtained: m.p. 134–135°; IR (KBr) 3150, 1681 cm^{-1}.

DL-*Arginine* (**4**). Ornithine hydrochloride (0.22 g, 1.29 mmol) in water (2 mL) was added to a boiling soln of $CuCO_3 \cdot Cu(OH)_2$ (0.43 g, 1.94 mmol) in water (5 mL). After a few min, the ppt was filtered off from the hot soln and washed with hot water until the filtrate was colorless. The combined filtrates were conc to 1 mL and O-methylisourea tosylate (0.32 g, 1.29 mmol) in water (3 mL) was added. The pH was adjusted to 9.8 with 10% NaOH and the mixture stirred 3 days at room temp. The pH was then adjusted to 1 with conc HCl, and H_2S was bubbled into the mixture for 15 min. After filtering and washing the ppt with water, the filtrates were boiled 15 min and then conc to dryness *in vacuo*.

The residue (0.76 g) was taken up in water (5 mL) and treated with flavianic acid (0.98 g) in boiling water (10 mL). The mixture was allowed to cool and was placed in a refrigerator overnight, whereupon the ppt was collected by filtration and dried under high vacuum. Hydrolysis of the flavianate salt was affected by heating in conc HCl (3 mL) for 2 hr. The ppt was filtered and washed with cold conc HCl until the filtrate was a light yellow. After concentrating the filtrate, the residue was taken up in water, decolorized with Norit, and crystallized from 95% EtOH–water to yield arginine (0.26 g, 94%): m.p. >200°; IR (KBr) 1660, 1625 cm^{-1}; ^1H NMR (D_2O) δ1.82 (4H, m), 3.25 (2H, m), 3.77 (1H, m).

Starting with **10b** (0.22 g, 1.29 mmol) and [^{13}C]-isourea tosylate (0.32 g, 1.29 mmol), **4b** (0.08 g, 27%) was obtained: m.p. >200° (dec); IR (KBr) 1660, 1625 cm^{-1}; ^1H NMR (D_2O) δ1.82 (4H, m), 3.25 (2H, m), 3.77 (1H, m).

Similarly, **10c** (0.4 g, 2.37 mmol) and isourea tosylate

(0.875 g, 3.55 mmol) afforded 0.29 g (58%) of **4c**: m.p. >200° (dec); IR (KBr) 3381, 3167, 1654, 1610 cm^{-1}; ^1H NMR (D_2O) δ1.69 (2H, m), 1.93 (2H, m), 3.27 (2H, t, J = 6.9 Hz), 3.79 (1H, dd, J_{HH} = 5.4 Hz, $^2J_{CH}$ = 5.4 Hz); ^{13}C NMR (D_2O) δ177.9, 159.1, 55.1 (d, J_{CC} = 26.6 Hz), 41.4 (d, J_{CN} = 9.4 Hz), 28.4, 24.8.

Compound **10d** (0.2 g, 0.97 mmol) and [^{15}N]isourea tosylate (0.25 g, 1.0 mmol) yielded 0.14 g (89%) of **4d**: m.p. >200° (dec); IR (KBr) 3382, 3181, 1653 cm^{-1}; ^1H NMR (D_2O) δ1.75 (2H, m), 2.07 (2H, dq, J_{HH} = 7.8 Hz, J_{CH} = 131.1 Hz), 3.28 (2H, m), 3.81 (1H, m); ^{13}C NMR (D_2O) δ177.9, 159.1, 55.5 (d, $^3J_{CC}$ = 16.9 Hz), 41.5, 29.6, 24.9.

From **10f** (0.06 g, 0.45 mmol), and isourea tosylate (0.087 g, 0.45 mmol), 0.033 g (42%) of **4f** was obtained: m.p. >200°; IR (KBr) 3378, 3182, 1658 cm^{-1}; ^1H NMR (D_2O) δ1.75 (2H, m), 3.28 (2H, m), 3.81 (1H, s); ^{13}C NMR (D_2O) δ175.1, 157.6, 54.9, 41.3, 27.6, 24.5.

DL-[2-^2H]*Arginine* (**4e**). A soln of arginine (2.0 g, 9.5 mmol) in D_2O (3 mL) was heated at 150° in a sealed tube for 10 hr. After cooling, the tube was opened and the solvent was removed under vacuum, and the residue was purified via the flavianate salt. Reconversion in standard fashion yielded **4e** (0.88 g, 44%): m.p. > 200°; IR (KBr) 3374, 3169, 1667, 1662 cm^{-1}; ^1H NMR (D_2O) δ1.89 (4H, broad t, J = 4 Hz), 3.27 (4H, broad t, J = 4 Hz); ^{13}C NMR (D_2O) δ174.9, 158.0, 55.0, 44.1, 28.1, 24.5.

DL-[2,3,3-$^2H_3$]*Arginine* (**4g**).[34] Arginine (1.05 g, 5 mmol), pyridoxal (0.102 g, 0.5 mmol), and aluminum sulfate (0.083 g, 0.125 mmol) were dissolved in D_2O (10 mL) in a 100 mL round bottom flask wrapped in Al-foil. The mixture was frozen and lyophilized and redissolved in D_2O (10 mL) containing pyridine (0.21 mL, 2.5 mmol). This mixture was frozen, evacuated to 10 mm Hg pressure, sealed, and then placed in a 100° oven for 2 days. The flask was cooled and the contents poured into water (100 mL) containing sodium oxalate (0.134 g, 1 mmol), collidine (0.33 mL, 2.5 mmol) and HCl (2.5 mmol). This mixture was adjusted to pH 5 with 3N HCl and loaded onto a 1.25 cm × 23 cm column of Dowex 100-X8 (H^+) in water. The column was washed with water (500 mL), and arginine was eluted with 0.25N NaOH. Ninhydrin positive fractions were pooled, decolorized with Norit, and lyophilized to yield 0.85 g (80%) of **4g**: m.p. >200°; IR (KBr) 3377, 3243, 1653, 1618 cm^{-1}; ^1H NMR (D_2O) δ1.71 (2H, t, J = 7.0 Hz), 3.28 (2H, t, J = 7.0 Hz); ^{13}C NMR (D_2O) δ175.1, 157.6, 54.9, 41.3, 27.6, 24.5.

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