Biosynthesis of Cylindrospermopsin

David L. Burgoyne,† Thomas K. Hemscheidt,† Richard E. Moore,*,† and Maria T. C. Runnegar‡

Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822, and Department of Medicine, University of Southern California Medical Center, Los Angeles, California 90033

Received August 9, 1999

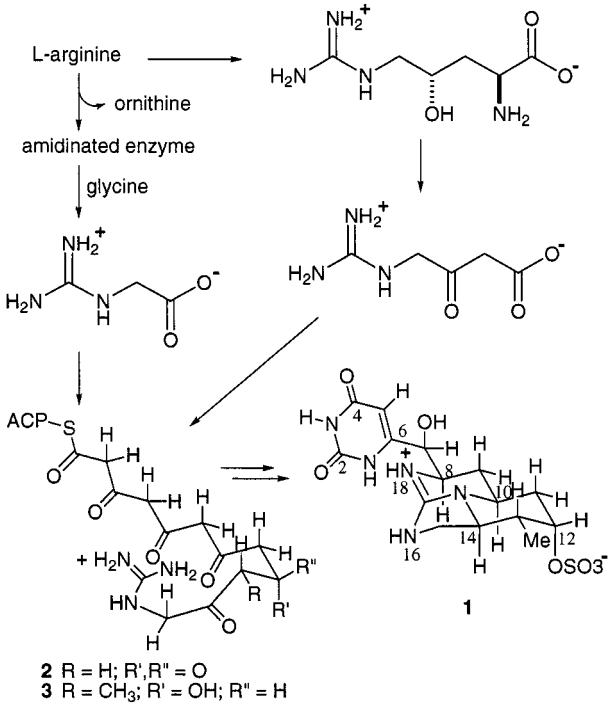
Studies on the biosynthesis of cylindrospermopsin (**1**), a potent hepatotoxin associated with the cyanobacterium *Cylindrospermopsis raciborskii*, indicate that **1** is an acetogenin with guanidinoacetic acid serving as the starter unit of the polyketide chain. Feeding experiments show that C14 and C15 of **1** are derived from C1 and C2 of glycine, respectively, and C4 through C13 arise from five contiguous acetate units attached head to tail. The methyl carbon on C13 originates from the C_1 pool. The starter unit, established by the incorporation of [*guanidino*-^{13}C,α-^{15}N]-guanidinoacetic acid into N16 and C17 of **1**, does not appear to be formed from glycine by known amidination pathways. The origin of the NH−CO−NH segment in the uracil ring is also unknown.

Introduction

Liver poisoning[1] caused by exposure to hepatotoxins released from cyanobacterial blooms growing in domestic water supplies is a serious and growing problem. *Microcystis aeruginosa* has been the most frequently implicated cyanobacterium (blue-green alga) in these poisonings, and the hepatotoxins associated with this cyanophyte are cyclic heptapeptides known as microcystins.[2] In early 1996, for example, 101 of 124 patients who were treated at a hemodialysis center in Caruaru, Brazil with untreated, microcystin-contaminated water from a nearby reservoir acquired acute liver failure, and over 50 persons died.[3]

In 1979 an outbreak of human hepatoenteritis on Palm Island in Northern Queensland, Australia was traced to a different cyanobacterium, *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju.[4] In 1992 it was shown that the hepatotoxin was an alkaloidal polyketide, cylindrospermopsin (**1**), having the molecular composition $C_{15}H_{21}N_5O_7S$. Detailed spectral analysis of **1** led to the identification of its gross structure and relative stereochemistry (7*S**,8*R**,10*S**,12*S**,13*R**,14*S**),[5,6] but its absolute configuration is still unknown. Since 1992 there have been more reports of toxic blooms of *C. raciborskii* and other cylindrospermopsin-producing cyanobacteria.[7−9]

Scheme 1. Proposed Biogeneses of Cylindrospermopsin (1) from L-Arginine[6,a]



[a] Results from feeding experiments described in this paper indicate that the starter unit for the polyketide chain in **1** is guanidinoacetic acid, formed by amidination of glycine. The source of the amidino group in the starter unit is unknown; however, it does not appear to be derived from L-arginine.

Inspection of the structure of cylindrospermopsin (**1**) immediately suggested a polyketide origin for the alkaloid. Two biogenetic pathways were proposed (Scheme 1),[6] and these served as working hypotheses for the design of the feeding experiments described below.

* Phone: (808) 956-7232. FAX: (808) 956-5908. E-mail: moore@gold.chem.hawaii.edu.

† University of Hawaii.

‡ University of Southern California Medical Center.

(1) Falconer, I. R.; Beresford, A. M.; Runnegar, M. T. C. *Med. J. Aust.* **1983**, *1*, 511−514.

(2) Carmichael, W. W.; Beasley, V.; Bunner, D. L.; Eloff, J. N.; Falconer, I.; Gorham, P.; Harada, K.; Krishnamurthy, T.; Min-Juan, Y.; Moore, R. E.; Rinehart, K.; Runnegar, M.; Skulberg, O. M.; Watanabe, M. *Toxicon* **1988**, *26*, 971−973.

(3) Jochimsen, E. M.; Carmichael, W. W.; An, J.-S.; Cardo, D. M.; Cookson, S. T.; Holmes, C. E. M.; Antunes, M. B. de C.; Filho, D. A de M.; Lyra, T. M.; Barreto, V. S. T.; Azevedo, S. M. F. O.; Jarvis, W. R. *N. Engl. J. Med.* **1998**, *338*, 873−878.

(4) Hawkins, P. R.; Runnegar, M. T. C.; Jackson, A. R. B.; Falconer, I. R. *Appl. Environ. Microbiol.* **1985**, *50*, 1292−1295.

(5) Ohtani, I.; Moore, R. E.; Runnegar, M. T. C. *J. Am. Chem Soc.* **1992**, *114*, 1020−1022.

(6) Moore, R. E.; Ohtani, I.; Moore, B. S., De Koning, C. B.; Yoshida W. Y.; Runnegar, M. T. C.; Carmichael, W. W. *Gaz. Chim. Itl.* **1993**, *123*, 329−336.

(7) Harada, K.; Ohtani, I.; Iwamoto, K.; Suzuki, M.; Watanabe, M. F.; Watanabe, M.; Terao, K. *Toxicon* **1994**, *32*, 73−84.

(8) Banker, R.; Carmeli, S.; Hadas, O.; Teltsch, B.; Porat, R.; Sukenik, A. *J. Phycol.* **1997**, *33*, 613−616.

(9) Hawkins, P. R.; Chandrasena, N. R.; Jones, G. J.; Humpage, A. R.; Falconer, I. R. *Toxicon* **1997**, *35*, 341−346.

10.1021/jo991257m CCC: $19.00
Published on Web 12/16/1999

Table 1. Summary of Sodium Acetate Experiments.

		[1,2-^{13}C]acetate		[2-^{13}C, ^{2}H$_3$]acetate		[1-^{13}C,^{18}O$_2$]acetate	
C no.	chemical shift (ppm)	*J* (Hz)	incorpn (%)	isotope shift (ppm)	incorpn (%)	isotope shift (ppm)	incorpn (%)
4	167.8	*a*				0.03	4
5	100.0	67.1	0.8		1		
6	155.7	*a*					4
7	69.7	48.3	0.8		1		
8	53.1	34.9	0.8				4
9	27.7	34.9	0.8	0.3	1		
10	44.5	36.2	0.8				4
11	35.8	36.2	0.8	0.35, 0.7	1		
12	77.7	37.6	0.8			0.04	4
13	39.2	37.6	0.8		2		

[a] Doublet components unobservable due to poor signal-to-noise.

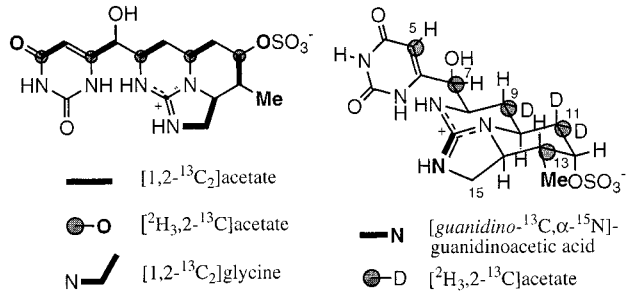


Figure 1. Incorporation of labeled precursors into **1**. The labeled glycines are also incorporated into the methyl carbon as denoted by the boldface **Me**.

Results and Discussion

C. raciborskii was cultured in the laboratory as previously described.[4] When it appeared that the cyanobacterium was healthy and actively growing, the labeled precursor was added to the culture medium. After 2 days the culture was harvested, and an aqueous extract of the ultrasonicated, freeze-dried cyanobacterium was prepared. The crude extract was fractionated by successive gel filtration on Toyopearl HW40F (1:1 MeOH/H_2O), reversed-phase column chromatography (C18, 10% MeOH/H_2O), and reversed-phase HPLC (C8, 2% MeOH in H_2O) to give the labeled cylindrospermopsin (**1**).

To determine whether **1** was a polyketide, and if so, how many intact acetate units were involved in its construction, uniformly $^{13}C_2$-labeled sodium acetate (mixed with unlabeled sodium acetate to minimize incorporation of intact $^{13}C_2$-acetate units into the same molecule) was fed to the cyanobacterium. The ^{13}C NMR spectrum of the labeled **1** exhibited 10 spin-coupled doublets that indicated that the polyketide-derived portion contained five acetate units (Table 1). Analysis of the coupling constants determined that intact acetate units were incorporated into the carbon pairs C13−C12 (J_{cc}= 37.6 Hz), C11−C10 (J_{cc}= 36.2 Hz), C9−C8 (J_{cc}= 34.9 Hz), C7−C6 (J_{cc}= 48.3 Hz), and C5−C4 (J_{cc}= 67.1 Hz) (Figure 1). The measured level of ^{13}C enrichment was 0.8%. The incorporation of an intact acetate unit into C13−C12 precluded the origin of these carbons from arginine[6] as shown in Scheme 1.

To determine that C4, C6, C8, C10, and C12 were derived from C1 of acetate and moreover that the oxygens attached to C4 and C12 of **1** were derived from the oxygen of acetate, sodium [1-^{13}C,$^{18}O_2$]acetate was administered to the cyanobacterium. The ^{13}C NMR spectrum of the labeled **1** showed enhanced ^{13}C signals for C6, C8, and C10 that were coincidental with the natural abundance lines. In addition, resonances were observed for C4 and C12 that were isotopically shifted upfield from the natural abundance lines by 0.03 and 0.04 ppm, respectively. These results confirmed the incorporation of five acetate units into **1** and indicated that the oxygens attached to C4 and C12 were derived directly from acetate.

To probe the fate of the acetate protons during the biosynthesis, sodium [$^2H_3$,2-^{13}C]acetate was fed to the cyanobacterium. The ^{2}H-decoupled ^{13}C NMR spectrum of the labeled **1** exhibited enhanced signals for C5, C7, and C13 and isotopically shifted ^{13}C signals for C9 and C11 (Table 1). A HETCOR experiment revealed that the C9 methylene carbon possessed one proton and one deuterium, whereas the isotopically shifted C11 methylene carbon had two deuterium atoms on it. The chemical shift of the cross-peak clearly showed that the proton on C9 was axial and therefore the deuterium was equatorial. The lack of isotopically shifted signals for C5, C7, and C13 indicated that the deuterium atoms from these acetate derived carbons were lost during biosynthesis.

Because C12 and C13 are acetate-derived, the alternative glycine pathway shown in Scheme 1 was evaluated. First [1,2-$^{13}C_2$]glycine was fed to the cyanobacterium. The ^{13}C NMR spectrum of the labeled **1** revealed spin-coupled doublets for the C14 and C15 signals ($^1J_{CC}$= 35 Hz) with an enrichment of 3.5%. The central singlet for C15 was also enhanced, by approximately 1.5-fold. These results indicate that the biosynthetic pathway involves incorporation of C1 and C2 of glycine into the C14 and C15 of cylindrospermopsin (**1**), respectively. However, there also appears to be a pathway operating in the cyanobacterium that allows the exchange of C1 in glycine, and this leads to **1** that has ^{13}C label at C15 only.[10] This feeding experiment also showed that the methyl carbon on C13 originates from the C_1 pool, as the signal at 13.7 ppm was enhanced by 5%.

To further probe the role of glycine in the biosynthesis and specifically to determine if the ^{15}N of glycine is retained, [1,2-$^{13}C_2$,^{15}N]glycine was fed to the cyanobacterium. The ^{13}C NMR of the labeled **1** showed the C14 signal as a spin-coupled doublet (35 Hz) and the C15 signal as a doublet of doublets (J_{CN} = 8 Hz, J_{CC} = 35 Hz). The data clearly indicated that glycine was being incorporated as an intact unit into C14, C15, and N16 of **1**. However, a doublet signal was also seen for C15 (J_{CN} = 8 Hz), where the natural abundance line and the

(10) Exchange of C1 of glycine with CO_2-derived carbon atoms has been observed in mitochondria. Sato, T.; Kochi, H.; Sato, N.; Kikuchi, G. *J. Biochem.* **1969**, *65*, 77−83.

downfield component of the doublet were coincident. This result indicated that the pathway responsible for the exchange of C1 in glycine does not involve an exchange of the glycine nitrogen also.

The cyanobacterium was also furnished with [2,2-$^2H_2$,1,2-$^{13}C_2$]glycine. The ^{2}H-decoupled ^{13}C NMR spectrum of the labeled **1** revealed four spin-coupled doublets for C15, which were shown by a HETCOR experiment to correspond to species containing C15(H_2), C15(aH,bD), C15(aD,bH), and C15(D_2). In this feeding experiment, however, no evidence was obtained for the exchange of C1 in glycine. The reason for the complete shutdown of the exchange pathway is unclear. The experiment was not repeated.

Once it was established that glycine was the direct precursor of C14, C15, and N16 in **1**, a feeding experiment with guanidinoacetic acid was carried out to establish if it was the starter unit for the polyketide. A doubly labeled guanidinoacetic acid[11] was synthesized by condensing [1-^{13}C]*O*-methylisourea, prepared by heating [^{13}C]urea in methyl *p*-toluenesulfonate, and [^{15}N]glycine in 2 N NaOH. [4-^{13}C,3-^{15}N]guanidinoacetic acid hydrochloride (75 mg) was added to each of four 2 L flasks on day 4 after inoculation, and the cyanobacterium was harvested on day 6. The ^{13}C NMR spectrum of the labeled **1** showed intact incorporation of the guanidino functionality by the presence of a spin-coupled doublet for C17 (155.9 ppm, J_{CN} = 19 Hz). Guanidinoacetic acid therefore appears to be the starter unit for this polyketide.

The guanidino functionality in the starter unit and therefore **1** does not arise from a transfer of an amidino group from arginine in analogy to amidination of glycine in creatine biosynthesis. Two feeding experiments with [U-^{13}C,^{15}N]arginine showed no incorporation into C17, N18, and N19. The possibility that guanidinoacetic acid could arise from condensation of carbamoyl phosphate with glycine followed by transamination of the intermediate ureidoacetic acid, a route that is analogous to the conversion of ornithine to arginine, was also studied. [4-^{13}C,3-^{15}N]Ureidoacetic acid was synthesized and fed to the cyanobacterium, but the ^{13}C NMR spectrum of the **1** isolated from this experiment showed no incorporation of label into N16 and C17. The experiment was repeated once with sodium [1,2-$^{13}C_2$]acetate as an internal standard. The ^{13}C NMR spectrum of the sample of **1** isolated from this experiment showed the expected spin-coupling pattern in the acetate-derived portion of the toxin, but again incorporation of [4-^{13}C,3-^{15}N]ureidoacetic acid into N16 and C17 was not detectable.

Mechanistic Implications. The feeding experiments described above clearly indicate that cylindrospermopsin has a polyketide origin. A glycine-related precursor, guanidinoacetic acid, appears to be the starter unit for the polyketide chain, as uniformly labeled [^{13}C,^{15}N]glycine and [3-^{15}N,4-^{13}C]guanidinoacetic acid are both incorporated into **1**. The origin of the amidino group in the starter unit, however, is unknown. It does not appear to arise by a transamidination process from arginine, nor does it seem to be produced via a ureidoacetic acid intermediate. The methyl group on the polyketide chain originates from the C_1 pool via *S*-adenosylmethionine, as shown by all of the [1,2-$^{13}C_2$]glycine feeding experiments.

On the basis of the information obtained from the various acetate feeding experiments, in particular the one with [2-^{13}C,$^2H_3$]acetate, several plausible pathways can be proposed for the biogenesis of **1**. The backbone of the polyketide is most probably assembled by either a modular (type I) or nonmodular (type II) polyketide synthase (PKS) from guanidinoacetic acid and five acetate units. Two processing steps (C-methylation and ketoreduction) and three cyclizations involving the guanidino group occur either as the polyketide chain is being elongated or after it has been completed. (9*S**,10*S**)-12-Guanidino-9-hydroxy-10-methyl-3,5,7,11-tetraoxododecanoate ACP thioester (**3**) is particularly attractive as an intermediate. Molecular modeling studies suggest that **3** forms a very stable conformation in which the *Z* proton on N19 and the proton of the hydroxyl group on C9 are both strongly hydrogen-bonded to the ketone carbonyl oxygens on C7 and C11 (Figure 2). The four H-bond distances in the model are between 1.85 and 2.10 Å. The modeling studies suggest that polyketide **3** readily assumes a favorable conformation for the formation of rings A, B, and C in **1**. The cyclizations may therefore be spontaneous events that are not under enzymatic control.

A proposed pathway that is consistent with results of the [2-^{13}C,$^2H_3$]acetate feeding experiment is shown in Scheme 2. No evidence was obtained on how the uracil ring is constructed.

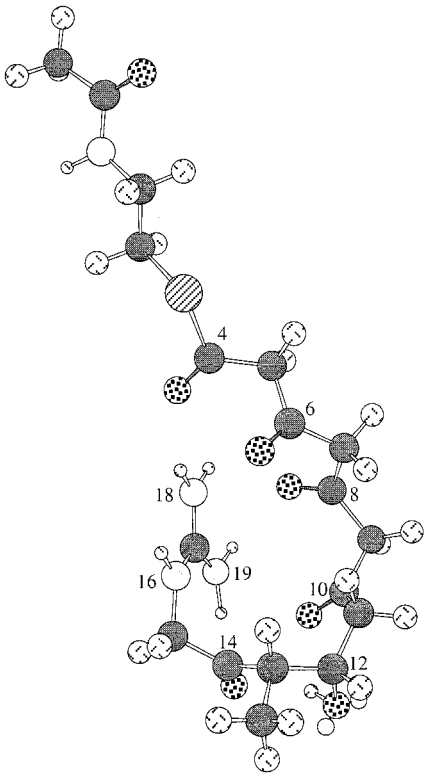


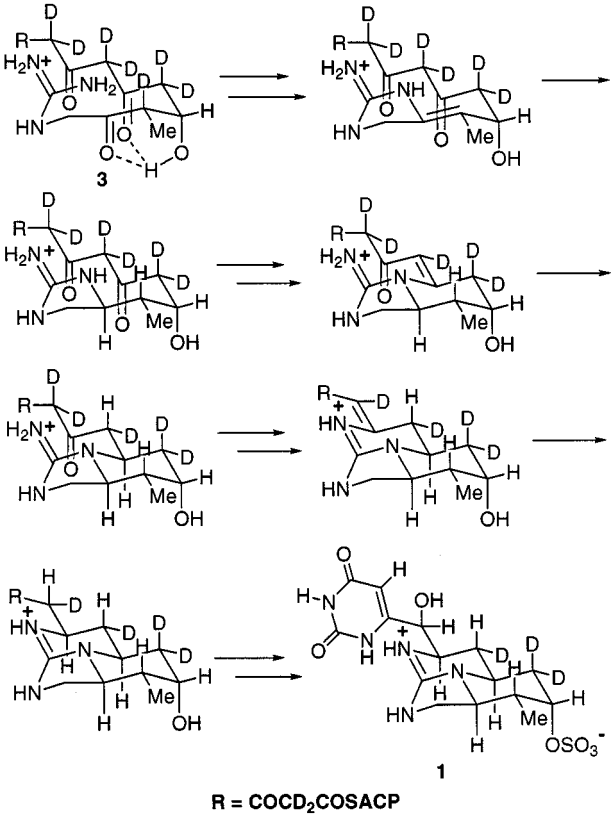
Figure 2. Minimum energy conformation of the putative intermediate (9*S**,10*S**)-12-guanidino-9-hydroxy-10-methyl-3,5,7,11-tetraoxododecanoate thioester. For the MM2 calculation (CS Chem 3D Pro) the polyketide has been attached to *N*-acetylcysteamine as a thioester. Cysteamine is the first unit of the phosphopantetheinyl arm on the acyl carrier protein of the polyketide synthase. The cylindrospermopsin numbering scheme appears on the drawing.

Experimental Section

General Procedures. ^{13}C NMR spectra and HETCOR experiments on cylindrospermopsin (**1**) were obtained at 125

(11) Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H. *Annalen* **1953**, *583*, 129−149.

Scheme 2. Proposed Reductive Cyclization of the Putative (9*S, 10*S**)-12-Guanidino-9-hydroxy-10-methyl-3,5,7,11-tetraoxododecanoate ACP Thioester 3, Labeled with Deuterium at Carbons Derived from C2 of Acetate, to the Tricyclic Guanidine System Found in Cylindrospermopsin (1).**



MHz in D_2O acidified with DCl. All ^{13}C-, ^{15}N-, ^{2}H-, and ^{18}O-labeled compounds used in this study were 99 atom %.

General Culture Conditions. Unialgal cultures were grown in modified Chu-10 medium buffered with 0.02 M HEPES (*N*-2′-hydroxyethylpiperazine-*N*′-2-ethanesufonic acid; pH adjusted to 7.55 before sterilization). Stock inoculum of *C. raciborskii* in Chu−HEPES (pH adjusted to 7.5 with 0.17 g $NaNO_3$/L added) was centrifuged for 15 min at 3000 rpm in a Sorvall centrifuge in sterile bottles. The pellet was resuspended in approximately 200 mL of fresh Chu−HEPES with nitrate. This inoculum was added in equal portions to four 2 L conical flasks containing Chu−HEPES and allowed to grow. All cultures were incubated at 25 ± 1 °C and subjected to continuous illumination from cool white fluorescent tubes at an intensity of about 90 $\mu E \cdot s^{-1} \cdot m^{-2}$ (LI-COR 188B quantum meter) and continuous aeration (CO_2 in air). When it appeared that the cultures were growing actively, the precursor was added and the cyanobacterium was allowed to grow further for 1−4 days in the presence of the precursor. The cyanobacterium was finally harvested by centrifugation at 10 000 rpm in a Sorvall centrifuge for 20 min.

General Isolation Procedure. The freeze-dried cyanobacterium was extracted twice with distilled water (2 × 30 mL) at 4 °C for 1 h. The combined extracts were evaporated to dryness in vacuo, the residue suspended in a minimum amount of 1:1 MeOH/H_2O, and the mixture applied to a Toyopearl HW40F precolumn (2 cm × 6 cm) connected to a Toyopearl HW40F column (4 cm × 30 cm). Fractions were eluted with 1:1 MeOH/H_2O at a flow rate of 0.8 mL/min. The fractions that eluted from 200 to 315 mL were combined and purified further by reversed-phase flash chromatography on a 2 cm × 30 cm column of ODS YMC GEL (120A) using successively H_2O (100 mL), 1:9 MeOH/H_2O (400 mL), 1:1 MeOH/H_2O (150 mL), and MeOH (200 mL) as the eluants. Final purification of the cylindrospermopsin (**1**) from the 10% MeOH/H_2O fraction was accomplished by reversed-phase HPLC on a 1 cm × 25 cm column of Econosil C8 (10 μm) with 98% H_2O/MeOH (flow rate 2.0 mL/min). ^{13}C NMR spectrum of **1**: δ 13.7 (13-Me), 28.3 (C9), 36.2 (C11), 39.7 (C13), 45.0 (C10), 48.3 (C15), 53.6 (C8), 57.8 (C14), 70.2 (C7), 78.1 (C12), 100.0 (C5), 156.1 (C2), 156.5 (C6), 167.8 (C4).

Feeding Experiments. A. Sodium [1,2-$^{13}C_2$]Acetate. This precursor (50 mg) and unlabeled acetate (50 mg) was administered to each of four 2 L cultures on day 29 after inoculation. The cultures were harvested on day 31 by centrifugation, and the cyanobacterium was lyophilized to yield 516 mg of freeze-dried material from which 2.1 mg of labeled **1** was isolated. Its ^{13}C NMR spectrum showed equally enriched doublet signals (0.8%) at δ 28.3 (C9, J_{CC} = 34.9 Hz), 36.2 (C11, J_{CC} = 36.2 Hz), 39.7 (C13, J_{CC} = 37.6 Hz), 45.0 (C10, J_{CC} = 36.2 Hz), 53.6 (C8, J_{CC} = 34.9 Hz), 70.2 (C7, J_{CC} = 48.3 Hz), 78.1 (C12, J_{CC} = 37.6 Hz), and 100.0 (C5, J_{CC} = 67.1 Hz). The signals for C6 and C4, however, could not be seen clearly, and therefore the enrichment and J_{CC} values could not be determined for these carbons.

B. Sodium [1-^{13}C,$^{18}O_2$]Acetate. Labeled acetate (50 mg) and unlabeled acetate (100 mg) were added to each of four 2 L flasks on day 5 after inoculation. The cultures were harvested on day 9 and yielded a total of 825 mg of freeze-dried cyanobacterium from which 6.8 mg of labeled **1** was isolated. The ^{13}C NMR spectrum showed equally enriched signals (4%) at δ 45.0, 53.6, 78.10/78.14 (12-^{16}O/12-^{18}O), 156.5, and 167.80/167.83 (4-^{16}O/4-^{18}O).

C. Sodium [2-^{13}C,$^{2}H_3$]Acetate. Labeled acetate (50 mg) and unlabeled acetate (100 mg) were added to each of four 2 L flasks 24 h after inoculation. The cultures were allowed to grow for 3 days and then harvested and lyophilized to yield a total of 3 g of freeze-dried cyanobacterium from which 2.7 mg of labeled **1** was isolated. The ^{13}C NMR spectrum showed equally enriched signals (1%) at δ 28.3/28.0 (9-H_2/9-DH), 36.2/35.9/35.6 (11-H_2/11-DH/11-D_2), 39.7, 70.2, and 100.0.

D. [1,2-$^{13}C_2$]Glycine. The labeled amino acid (20 mg) was added to each of four 2 L flasks on day 4 after inoculation. On day 6 the cultures were harvested, and the cyanobacterium was lyophilized to yield 1.57 g of freeze-dried material from which 5 mg of labeled **1** was isolated. The ^{13}C NMR spectrum showed enriched signals at δ 13.7 (s; 5%), 48.3 (s, 1.5% and d, J_{CC} = 35 Hz; 3.5%), and 57.8 (d, J_{CC} = 35 Hz; 3.5%).

E. [1,2-$^{13}C_2$,^{15}N]Glycine. The labeled amino acid (20 mg) was added to each of four 2 L flasks on day 4 after inoculation. The cultures were harvested on day 6, and the cyanobacterium was lyophilized to give 750 mg of freeze-dried material from which 5.4 mg of **1** was isolated. The ^{13}C NMR spectrum showed enriched signals at δ 13.7 (8%), 48.3 (d, J_{CN} = 8 Hz, 2%; dd, J_{CC} = 35 Hz, J_{CN} = 8 Hz, 7%), 57.9 (d, J_{CC} = 35 Hz, 7%).

F. [1,2-$^{13}C_2$,2,2-$^{2}H_2$]Glycine. [1,2-$^{13}C_2$]Glycine (100 mg, 1.27 mmol) was dissolved in 2 N NaOH (2 mL), and the mixture was cooled in an ice bath. Benzoyl chloride (186 μL) was added, and the mixture was stirred at 0 °C for 2 h. The reaction mixture was acidified with 1 N HCl and evaporated to dryness in vacuo. Silica gel chromatography of the residue yielded white crystals of [1,2-$^{13}C_2$]hippuric acid, mp 184−187 °C (lit.[11] 187−188 °C): ^{1}H NMR (CD_3OD, 300 MHz) δ 4.05 (s, 2H), 7.4−7.6 (m, 3H), 7.85 (d, 2H) ppm; ^{13}C NMR (CD_3OD, 75 MHz) δ 42, 128, 130, 133, 135, 170, 173. The [1,2-$^{13}C_2$]hippuric acid was dissolved in acetic acid-*d* (3 mL), acetic anhydride (145 μL) was added, and the mixture was refluxed for 30 min and then cooled to room temperature. Next D_2O (2 mL) was added, and the mixture was stirred for 1 h and finally evaporated to dryness. The above procedure was repeated two more times. The labeled hippuric acid was then hydrolyzed in 6 N DCl at reflux for 12 h ,and the hydrolysate was subjected to reversed-phase flash chromatography (ODS, H_2O eluant) followed by ion exchange chromatography (Dowex 50 × 8) to give pure [1,2-$^{13}C_2$,2,2-$^{2}H_2$]glycine (81 mg, 1.03 mmol, 81% yield): ^{13}C NMR (D_2O, 125 MHz, ^{2}H decoupled) δ 42 (d), 171 (d).

[1,2-$^{13}C_2$,2,2-$^{2}H_2$]Glycine hydrochloride (23 mg) was added to each of four 2 L flasks, 24 h after inoculation. The cultures were allowed to grow for 3 days. Harvesting yielded 505 mg

of freeze-dried cyanobacterium from which 2.5 mg of labeled **1** was isolated. The ^{13}C NMR showed enriched signals at δ 48.44 (s, C15), 48.43 (d, J_{CC} = 35 Hz, C15-H_2), 48.19 (d, J_{CC} = 35 Hz, C15-H_aD_b), 48.10 (d, J_{CC} = 35 Hz, C15-D_aH_b), 47.85 (d, J_{CC}=35 Hz, C15-D_2).

G. [4-^{13}C,3-^{15}N]Guanidinoacetic Acid. [^{13}C]Urea (323 mg, 5.35 mmol) and methyl *p*-toluenesulfonate (2.00 g, 10.62 mmol) were combined and heated under nitrogen for 5 h and then cooled to room temperature. Acetone (20 mL) was added, and the mixture was stirred at room temperature for 2 h. After the mixture cooled overnight at 4 °C, the liquid portion was decanted, and the solid was washed with ethyl ether (2 × 20 mL). Crystallization from 1,4-dioxane yielded the *O*-methylisourea *p*-toluenesulfonate salt (849 mg, 3.22 mmol, 60%): mp 133−135 °C; ^1H NMR (D_2O, 300 MHz) δ 2.34 (s, 3H), 3.92 (d, J_{HC} = 4 Hz, 3H), 7.31 (d, J = 8 Hz, 2H), 7.68 (d, J = 8 Hz, 2H). The *O*-methylisourea salt (487 mg, 1.84 mmol) in 8 N NaOH (230 μL) and H_2O (575 μL) was added to a stirred solution of [^{15}N]glycine (115 mg, 1.53 mmol) in H_2O (575 μL). After 30 min a white precipitate formed. Stirring was continued for an additional 2 h. The precipitate of guanidinoacetic acid was collected, washed with 1:1 ethyl ether/absolute ethanol (3 × 10 mL), and dissolved in 1 N HCl. Evaporation in vacuo gave the hydrochloride salt as a white solid (163 mg, 1.07 mmol, 70%): ^1H NMR (D_2O, 300 MHz) δ 4.09 (bd, J_{HC} = 4.8 Hz); ^{13}C NMR (D_2O, 75 MHz) δ 44.1 (d, J_{CN} = 12.6 Hz), 158.8 (d, J_{CN} = 22.2 Hz), 173.7.

[4-^{13}C,3-^{15}N]Guanidinoacetic acid (75 mg) was added to each of four 2 L flasks, and the cultures were allowed to grow for 2 days. Harvesting yielded 700 mg of freeze-dried cyanobacterium from which 3 mg of labeled **1** was obtained. The ^{13}C NMR spectrum showed an enriched signal at δ 155.9 (d, J_{CN} = 19 Hz) for C17.

H. [4-^{13}C,3-^{15}N]Ureidoacetic Acid. Potassium [^{13}C]cyanate (0.25 g, 3.1 mmol) was added to 2 mL of H_2O containing [2-^{15}N]-glycine (0.235 g, 3.1 mmol). The mixture was heated at reflux for 4 h, cooled to room temperature, and acidified by the addition of 6 N HCl. The crystalline precipitate was collected by filtration, washed with a small amount of cold water, and dried in vacuo to give colorless crystals of the labeled ureidoacetic acid (0.298 g. 80%), mp 170−172 (dec): ^{13}C NMR (D_2O, 125 MHz) δ 41.8 (d, J_{CN} = 7.5 Hz), 162.7 (d, J_{CN} = 21.1 Hz), 172.0.

[4-^{13}C,3-^{15}N]Ureidoacetic acid (25 mg was added to each of four 2 L flasks, and the cultures were allowed to grow for 4 days. Harvesting yielded 1.95 g of freeze-dried cyanobacterium from which 4 mg of **1** was obtained. The ^{13}C NMR spectrum showed no enrichment in the signal for C17 at 155.9 ppm.

A second feeding experiment was carried out with [4-^{13}C,3-^{15}N]ureidoacetic acid (25 mg) and sodium [1,2-$^{13}C_2$]acetate (50 mg) in admixture with unlabeled acetate (50 mg). The ^{13}C NMR spectrum of the labeled **1** that was isolated again showed no enrichment in the signal for C17 at 155.9 ppm, but enrichment in the acetate-derived carbon atoms (see Experiment A) was evident.

Acknowledgment. This research was supported by NSF grant CHE-9530794.

Supporting Information Available: NMR Spectra of **1** isolated from feeding experiments with [1-^{13}C,$^{18}O_2$]acetate, [1,2-$^{13}C_2$,2-^{15}N]glycine, and [1,2-$^{13}C_2$]glycine (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JO991257M