Synthesis and ^{13}C NMR Equilibria Studies of (1-^{13}C)Leupeptin and Analogs

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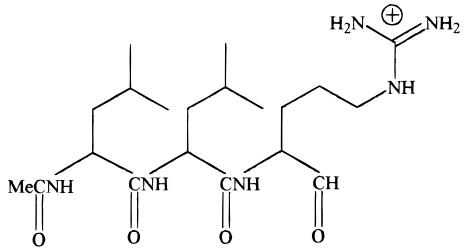
Introduction

Leupeptin, *N*-acetyl-L-leucyl-L-leucyl-L-argininal, **1**, is a well-recognized inhibitor of serine and thiol proteases[1−4] similar to trypsin in specificity. Many of the proteolytic enzymes inhibited by leupeptin are involved in a wide variety of biological processes. These processes include: blood pressure regulation,[5] coagulation,[6] degradation of proteins,[7] virus assembly,[8,9] cell proliferation,[10] and tumor generation.[11,12] Leupeptin has been frequently used as a biochemical tool in the study of proteolytic enzymes and the role they play in biological functions.[5−13] The effectiveness of leupeptin as a protease inhibitor is often attributed to the presence of its C-terminal aldehyde.[14,15] The C-terminal aldehyde of leupeptin is believed to form a stabilized hemiacetal with the serine side chain present in the active site of the enzyme.[16] In thiol enzymes a similar stabilized thiohemiacetal is proposed.[17] The formation of a stabilized hemiacetal as the enzyme−aldehyde complex is extremely pH dependent.[18] The catalytic activity of serine-type proteases depends on a histidine in the active site with a pK_a between 6.7 and 7.0. The enzyme's activity at pH 4.0 is therefore expected to be less than 0.2% of that at pH 8.0. This translates to a 500-fold weaker binding by the enzyme at lower pH. However, aldehydes have been shown to be bound only 2−40-fold more weakly at pH 4.0 than 8.0.[18] Arguments have been proposed as to whether the pH dependence of the stability of the serine protease−aldehyde adducts or the pH dependence of their rates of formation and breakdown are of greater significance for the mechanism of action of these enzymes.[14,19] In either case, the pH dependence of the equilibrium forms of the serine protease inhibitor leupeptin and the rates of interconversion of the inactive forms to the aldehyde form greatly complicate the theories of pH dependence of simple aldehyde−protease complexes.



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Leupeptin and similar argininal compounds have been shown to exist in three covalent forms in aqueous solution.[19−21] These three covalent forms are the hydrate, the aldehyde, and the cyclized carbinol amine (Figure 1).

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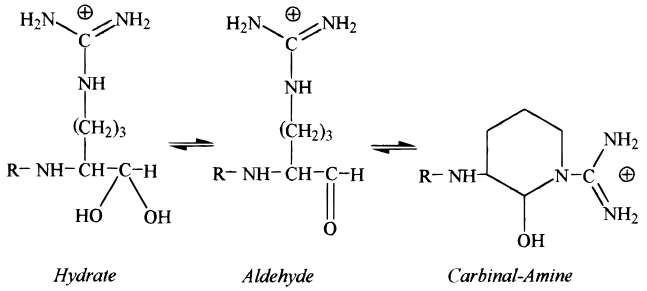

Figure 1. Equilibria forms of leupeptin.

This slow equilibrium has been documented by two-dimensional thin-layer chromatography, ^{1}H NMR, and slightly broadened ^{13}C NMR signals corresponding of each of the three equilibrium forms.[20,21] While frequently described as a "slow equilibrium",[19−21] the rate of interconversion among the three forms has not been established in the literature. Schultz and coworkers[14] have suggested that this equilibrium is pH dependent and may be responsible for the slow observed binding of leupeptin to cathepsin B and papain under acidic pH conditions. However, most trypsin-like serine proteases are typically assayed under slightly basic pH conditions (pH 7.5−9.0). And, no previous investigations have been made of the leupeptin equilibrium under basic pH conditions. Therefore, a ^{13}C NMR study of this equilibrium of leupeptin among the three covalent forms under a variety of pH conditions was undertaken. Due to the poor solubility of leupeptin in aqueous solutions, a synthesis of leupeptin with a ^{13}C label on the aldehyde carbon became necessary. The synthesis of (1-^{13}C)-L-arginine, (1-^{13}C)leupeptin, and nine analogs is described below. Also provided is the resulting ^{13}C NMR data of the labeled protease inhibitors.

Results and Discussion

Synthesis of (1-^{13}C)-L-Arginine. By special request from ICN, (1-^{13}C)arginine can be synthesized and purchased at a quoted cost of $4800 per 25 mg. Due to budget restraints, we chose to seek an economical preparation of (1-^{13}C)-L-arginine. Traditionally arginine has been synthesized by reaction of cyanamide on ornithine.[22,23] However, we found the cost of commerically available (1-^{13}C)-DL-ornithine (nearly $1500 per 100 mg) to also be prohibitive. Therefore, we set out to synthesize (1-^{13}C)-L-arginine from a less expensive source of ^{13}C-labeled carbon. The preparation of (1-^{13}C)arginine is shown in Scheme 1. In this synthetic approach 4-guanidinobutanoic acid (Sigma) was esterified by treatment with thionyl chloride and methanol, and then the guanidino group was protected with a carbobenzoxy (Cbz) group to give methyl N^G-Cbz-4-guanidinobutanoate, **2**. Due to the high basicity and weak nucleophilicity of the guanidino group, a strong base, sodium methoxide, and a 20-fold excess of benzyl chloroformate was necessary. No evidence of the di-Cbz product was observed. The presence of the carbobenzoxy group enhanced the solubility of **2** somewhat. However, compound **2** was still insoluble in traditional solvents used with reducing agents. Methyl N^G-Cbz-4-guanidinobutanoate was dissolved in diglyme (2-methoxyethyl ether), chilled in a dry ice/alcohol bath, and treated with diisobutylaluminum hydride (DIBAL) to form the aldehyde N^G-Cbz-4-guanidinobutanal, **3**. The aldehyde was stirred with [^{13}C]-potassium cyanide and ammonia for 2 days. Acidification and purification then resulted in (1-^{13}C)-N^G-Cbz-DL-arginine hydrochloride, **4**. The carbobenzoxy protecting group was removed by mild catalytic hydrogenolysis (1 atm, 20 °C, 3 h) to give 1-^{13}C-DL-arginine, **5**. The (^{13}C)-DL-arginine was resolved by a method described by Greenstein and Winitiz,[22] to give (1-^{13}C)-L-arginine, **5a**.

Synthesis of 1-^{13}C Leupeptin and Analogs. The preparation of (1-^{13}C)leupeptin and analogs from (1-^{13}C)-L-arginine is shown in Scheme 2. First, the α-amine was protected with a tert-butoxycarbonyl (BOC) group, and then the guanidino group was blocked by reaction with excess benzyl chloroformate. (1-^{13}C)-N^{α}-BOC-N^G-Cbz-L-arginine, **6**, was cyclized to the δ-lactam by reaction with 1,1′-carbonyldiimidazole and immediately reduced to the carbinol amine form of the aldehyde. The aldehyde was converted into the semicarbazone derivative, **7**, for protection and to facilitate purification. Once purified, the BOC protecting group was removed by treatment with 30% trifluoroacetic acid in chloroform. The trifluoroacetate salt of (1-^{13}C)-N^G-Cbz-L-argininal semicarbazone was coupled to the appropriate carbobenzoxy-protected dipeptide acid (Sigma) to form the fully protected (1-^{13}C)leupeptin analog, **8a**−**j**. Both the N-terminal Cbz group and the guanidino protecting group were removed simultaneously by very mild catalytic hydrogenolysis to give the dihydrochloride salt. The N-terminal amino group was then selectively reprotected by careful treatment with acetic anhydride and pyridine. The guanidino group remained protonated in the presence of the pyridine and was therefore unreactive. (1-^{13}C)-N^{α}-Acetyldipeptide-L-argininal semicarbazone hydrochloride, **9a**−**j**, was then reacted with formaldehyde and aqueous HCl. The transimination reaction resulted in (1-^{13}C)leupeptin and analogs, **10a**−**j**. The products were purified by Sephasorb HP chromatography and gave both a positive Sakaguchi[22] test (for guanidino) and 2,4-DNP[24] test (for aldehydes). The products were then characterized by ^{1}H NMR, TLC, and combustion analysis.

NMR Equilibrium Studies. The NMR samples were prepared by utilizing phosphoric acid-d_3, sodium formate-d, or sodium acetate-d_3, in deuterium oxide (D_2O) with the pH adjusted with either DCl (37%) in D_2O or sodium deuteroxide (40%) in D_2O. The pH of each buffer was measured using an Orion combination electrode with pH values in D_2O corrected for the different response of a pH electrode toward deuterium and protium[25] (meter reading +0.41 for D_2O solutions). Due to limited solubility, each NMR sample contained 5 mg (0.043−0.048 mM) of the appropriate ^{13}C-labeled compound in 0.25 mL of buffered D_2O in a 5 mm NMR tube. NMR spectroscopy was performed at 500 and 100.62 MHz for ^{1}H and ^{13}C on a Bruker AMX-500 NMR with an ASPECT STATION

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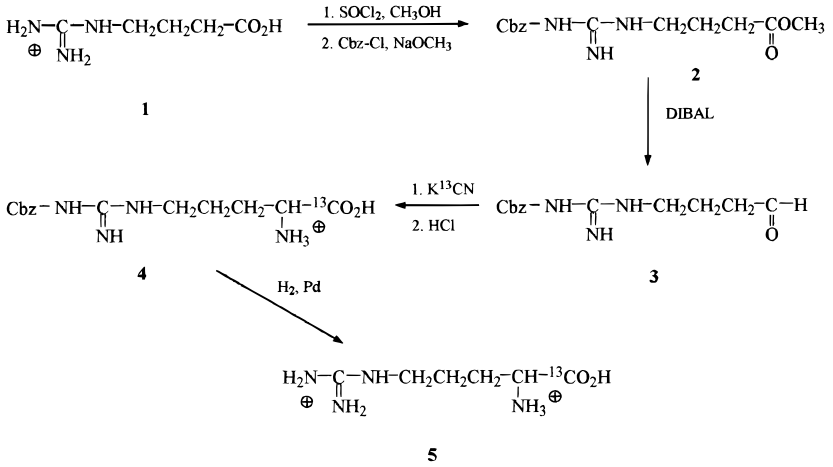
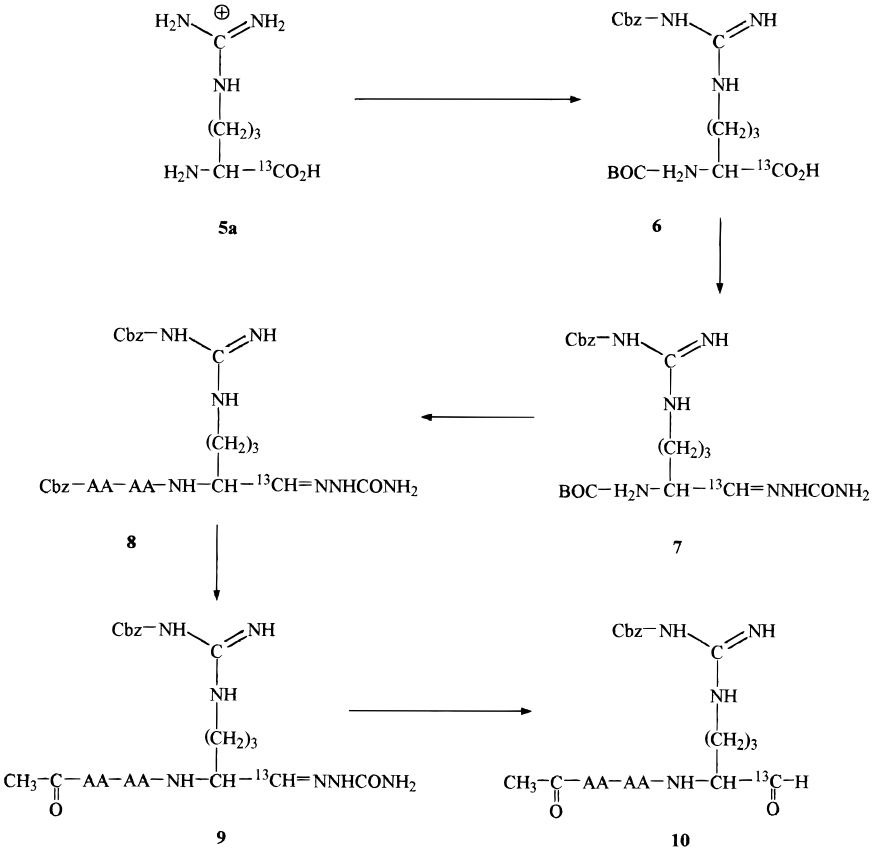
Scheme 1



1 **2** **3** **4** **5**

Scheme 2



5a **6** **7** **8** **9** **10**

data system. Spectra were recorded at 25 °C. Chemical shifts were referenced to the internal sodium 3-(trimethylsilyl)propane sulfonate set at δ 0.0. Spectral widths and the number of data points were adjusted to obtain digital resolution in the frequency domain better than 0.8 or 0.6 Hz/data point for ^{1}H and ^{13}C NMR, respectively. Broad band decoupled ^{13}C NMR spectra, utilizing 12 s pulse delays, were obtained for each compound at eight different pH values. Off-resonance decoupled spectra were also obtained to corroborate peak assignments.

The ^{13}C-labeled carbonyl carbon of argininal in compound **10a** in its three equilibrium forms was observed in acidic solutions (pH 3.0−6.5) at δ 90.3 for the hydrate, δ 77.1 and 76.6 for the *R* and *S* forms of the carbinol amine, and 202.1 for the free aldehyde. In neutral and slightly basic solutions (pH 7.0−8.5) the three equilibrium forms of compound **10a** were observed at δ 92.5 for the hydrate, δ 78.4 and 78.0 for the two forms of the cyclized carbinol amine, and 202.3 for the free aldehyde. At pH 9.5 the signal for the hydrate, the carbinol amine, and the aldehyde equilibria forms were shifted further downfield (the hydrate and carbinol amine by 2−3 ppm, and the aldehyde by 0.3 ppm). This assignment of the slightly broadened ^{13}C NMR signals is in agreement with those made by Scott and coworkers,[16] as well as our earlier work with leupeptin.[20,21] The ^{13}C NMR spectra of the other ^{13}C-labeled analogs, **10b**−**j**, showed similar chemical shifts. Peaks area ratios of the hydrate, free aldehyde, and cyclized carbinol amine for each ^{13}C-labeled leupeptin analog, at pH values ranging from 3.0 to 9.5, are shown in Table 1. Both ^{1}H and ^{13}C NMR data of compounds **10a**−**j** indicate that the hydrated aldehyde

Table 1. Ratio of NMR Signal Intensities (Hydrate/Free Aldehyde/Cyclized Carbinol Amine) at Various pH Values[a]

compd no.	3.0	5.0	6.0	6.5	7.0	7.5	8.5	9.5
10a	9/0/1	9/0/1	7/1/2	6/2/1	2/5/3	3/4/3	1/1/8	1/0/9
10b	10/0/0	9/0/1	8/1/1	7/1/2	3/4/3	3/3/4	1/0/9	1/0/9
10c	9/0/1	9/0/1	8/1/1	6/2/1	2/5/3	2/4/4	1/0/9	1/0/9
10d	9/0/1	9/0/1	6/3/1	5/4/1	2/6/2	2/5/3	2/1/7	1/0/9
10e	9/0/1	9/0/1	7/2/1	6/2/1	3/5/2	2/4/4	1/1/8	1/0/9
10f	9/0/1	9/0/1	8/1/1	6/2/1	3/4/3	3/1/6	1/0/9	1/0/9
10g	9/0/1	9/0/1	9/0/1	8/1/1	5/3/2	4/4/2	1/0/9	1/0/9
10h	9/0/1	9/0/1	5/4/2	4/5/1	1/7/2	1/6/3	2/4/4	1/0/9
10i	9/0/1	9/0/1	6/3/1	5/4/1	1/8/1	1/7/2	1/6/3	1/3/6
10j	9/0/1	9/0/1	8/1/1	7/2/1	1/6/3	2/4/4	1/0/9	1/0/9

[a] ^{13}C NMR broad band decoupled spectra with 12 s pulse delays.

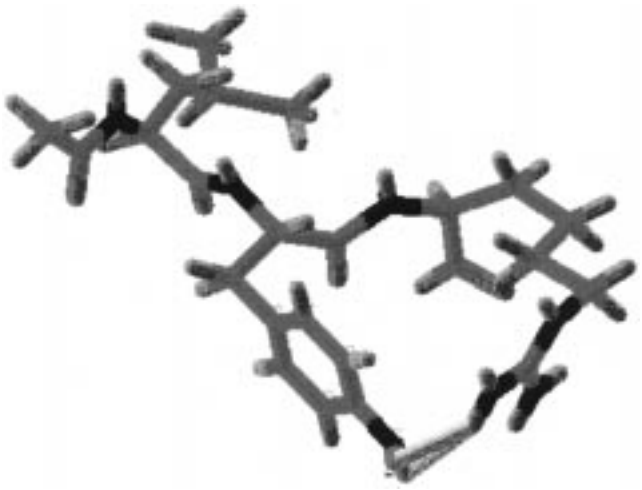


Figure 2. Molecular modeling of **10i** showing hydrogen bonding of guanidinium NH of argininal P_1 side chain to the nitro oxygens of the P_2 *p*-nitrophenylalanyl side chain (light blue = C, dark blue = N, red = O, gray = H, and hydrogen bonds are shown in yellow).

(see Figure 1) is the major form at lower pH (3.0−6.0). At basic pH (8.5 and 9.5), most of the ^{13}C-labeled compounds showed the cyclized carbinol amine as the dominant form, with the hydrated aldehyde as a secondary form. Very little free aldehyde was observed under extremely basic conditions. For (1-^{13}C)leupeptin, **10a**, and similar analogs, a narrow pH range of 7.0−7.5 showed the greatest proportion of the biologically active free aldehyde form. Acetyl-leucyl-cyclohexylalanyl-(1-^{13}C)argininal, **10j**, as well as compounds **10b,c,f,g** that have only stearic alterations show little variation from **10a**. The pH range of dominant free aldehyde was significantly wider in the NMR studies of acetyl-leucyl-phenylalanyl-(1-^{13}C)argininal, **10e**, and wider still for acetyl-leucyl-*p*-chlorophenylalanyl-(1-^{3}C)argininal, **10h**, and acetyl-leucyl-*p*-nitrophenylalanyl-(1-^{13}C)argininal, **10i**. This widening of the pH range of available free aldehyde is more likely to be due to an electronic rather than a stearic affect. However, hydrogen bonding of the guanidinium arginine side chain to the π system of the *p*-nitrophenylalanine side chain in compound **10i**, as shown in Figure 2, also appears to prevent the cyclization of the guanidino nitrogen with the C-terminal aldehyde.

Biaci and Gyger-Marazzi[26] reported that the inhibition of cathepsin B by leupeptin occurs after a significant lag phase with a half-time of several minutes. These authors suggest that the lag phase for inhibition is evidence for a transient intermediate that proceeds tight binding by leupeptin. However, Schultz coworkers[17] propose that the slow approach to steady-state inhibition of cathepsin B and papain at nanomolar concentrations of leupeptin is due to the stability of the thiohemiacetal adduct, as well as to a low concentration of the active form of the inhibitor. Since thiol proteases are normally assayed under acidic pH conditions, our data confirming the lack of a significant amount of the free aldehyde form of leupeptin at these pH conditions may help explain the observed slow binding of leupeptin by cathepsin B and papain.

Biochemical assay of our synthetic (1-^{13}C)leupeptin and analogs as protease inhibitors will be conducted as soon as an available standard enzyme assay technique can be modified for use with the labeled compounds. Due to apparent kinetic isotope effects, we have not been able to establish accurate K_i values for (1-^{13}C)leupeptin and analogs because the rates of inhibition for these compounds are outside the parameters for standard spectrophotometric enzyme assay techniques. Once valid K_i values are established, an investigation and comparison of the rates of inhibition with the relative amounts of free aldehyde at specific pHs will be reported.

Experimental Section

Materials. All solvents used were of ACS purity and were purchased either from Fisher Scientific or Aldrich. The starting material, 4-guanidinobutanoic acid, and the carbobenzoxy-protected dipeptides were purchased from Sigma Chemical Co. (St. Louis, MO). The [^{13}C]potassium cyanide was purchased from Cambridge Isotope Laboratories. All other reagents used were purchased from Aldrich. The purity of each major intermediate or product was checked by TLC (silica gel 60-F254 plates, E. Merck) and in some cases HPLC (Perkin Elmer LCI-100 TriDet, reversed phase C_{18} analytical column, 5 μm). Unless otherwise indicated, the intermediates and product were homogeneous by TLC and HPLC. The structures of the final product and each intermediate were verified by ^1H NMR (Hitachi R-1200, 60 MHz). Aqueous solutions were evaporated under the vacuum of an oil pump using a rotary evaporator equipped with a low-temperature circulating bath (−40 °C). Organic solvents were removed on a rotary evaporator under the vacuum of a water aspirator with bath temperatures of 40 °C or lower.

Methyl 4-Guanidinobutanoate Hydrochloride. A solution of 4-guanidinobutanoic acid (5 g, 34.4 mmol) in 50 mL of thionyl chloride (Aldrich) was stirred at room temperature for 18 h. The solution was then chilled in a dry ice/acetone bath to −78 °C. Methanol (200 mL, precooled in a dry ice/acetone bath) was slowly (over 5 min) and carefully added to the cold thionyl chloride solution. (*This step should always be performed in a fume hood with an open system to avoid the pressure build-up of escaping gases!*) The mixture was allowed to slowly (over 3 h) warm to room temperature while being stirred. The solution was then concentrated under reduced pressure to about one-fourth of its original volume. The concentrated solution was triturated with diethyl ether to produce a crude solid. The solid material was then recrystallized four times from methanol/diethyl ether to give methyl 4-guanidinobutanoate·HCl (5.5 g, 93% yield): HPLC R_v = 3.75 mL (acetonitrile/water, 7/3, v/v, C_{18}, 5 μm); ^1H NMR (D_2O) δ 3.7 (s, 3H), 2.9−3.2 (t, 2H, J = 7 Hz), 1.7−2.0 (m, 2H). Anal. Calcd for $C_6H_{15}N_3O_2Cl$: C, 36.65; H, 7.69; N, 21.37. Found: C, 36.63; H, 7.68; N, 21.38.

Methyl N^G-Carbobenzoxy-4-guanidinobutanoate (2). A solution of methyl 4-guanidinobutanoate hydrochloride (5.0 g, 25.6 mmol) in 100 mL of methanol was mixed with a solution of

(26) Biaci, A.; Gyger-Marazzi, M. *Eur. J. Biochem.* **1982**, *129*, 33−41.

13.8 g (256 mmol) of sodium methoxide in 100 mL of methanol. The combined mixture was chilled in an ice/water bath. Benzyl chloroformate (72 mL total, 512 mmol) was added, in 10 mL increments every 20 min, to the cold, rapidly stirring solution. After each 10 mL addition of benzyl chloroformate, 15 mL of a precooled solution of sodium methoxide (20 g, 370 mmol) in 100 mL of methanol was also added. The reaction mixture was stirred in an ice/water bath for an additional 2 h and then at room temperature for 18 h. The reaction mixture was then neutralized with 100 mL of 1.0 M aqueous HCl. A 1 mL portion of the mixture was removed and diluted with 5 mL of distilled water. The pH of this portion was tested (pH = 6.5). The reaction mixture was then concentrated to about 100 mL under reduced pressure. The concentrated mixture was diluted with ethyl acetate/isopropyl alcohol (300 mL of 6/4, v/v) and stirred for 2 h at room temperature. The undissolved sodium chloride byproduct was removed by filtration. The solvent was removed by evaporation under reduced pressure and the crude product was redissolved in 200 mL of the ethyl acetate/isopropyl alcohol (6/4, v/v) solution. The mixture was filtered again and the solvent was evaporated under reduced pressure. The product was redissolved in 20 mL of isopropyl alcohol and then precipitated with the addition of 200 mL of ethyl ether. The crude solid was recrystallized four times from isopropyl alcohol/ether to give **2** (4.9 g, 65% yield): TLC R_f 0.13 (12% ethanol/ethyl acetate, silica gel F254); HPLC R_v = 6.25 mL (acetonitrile/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 7.4 (s, 5H), 5.1 (s, 2H), 3.9−4.3 (m, 2H), 3.6 (s, 3H), 2.9−3.3 (t, 3H, J = 7 Hz), 2.2−2.7 (m, 2H), 1.6−2.1 (m, 2H). Anal. Calcd for $C_{14}H_{19}N_3O_4$: C, 57.33; H, 6.53; N, 14.33. Found: C, 57.29; H, 6.55; N, 14.36.

***N*^{G}-Carbobenzoxy-4-guanidinobutanal (3).** A solution of 4.5 g (15.3 mmol) of methyl *N*^{G}-carbobenzoxy-4-guanidinobutanoate (**2**) in 100 mL of anhydrous diglyme (2-methoxy ethyl ether) was chilled in a dry ice/ethanol−water (2/3, v/v) bath to −30 °C under a N_2 atmosphere, vigorously stirred, and slowly treated with 60 mL of 1.0 M diisobutyl aluminum hydride solution in tetrahydrofuran (Aldrich) over 30 min. The reaction mixture was stirred at −30 °C under N_2 for an additional 2 h and then quenched with 100 mL of 0.1 M aqueous HCl. The reaction mixture was then partitioned between the phases of 400 mL of ethyl acetate, 100 mL of 1-butanol, and 400 mL of 10% sodium potassium tartarate. The organic layer was washed with 100 mL of distilled water and dried over anhydrous magnesium sulfate. The organic layer was then concentrated under reduced pressure and the remainder of the solvent was evaporated in vacuo. The crude product was purified by silica gel chromatography (60 cm × 2 cm, 1 × d) using 95/5 ethyl acetate/ethanol as the mobile phase to give 2.8 g (69% yield) of **3**: TLC R_f 0.18 (12% ethanol/ethyl acetate, silica gel F254); ^{1}H NMR (DMSO-d_6) δ 9.5 (s, 0.8H, CHO), 7.5 (s, 5H), 5.8−6.1 (m, 0.2H, carbinol amine), 5.2 (s, 2H), 3.9−4.4 (m, 2H), 3.0−3.3 (t, J = 7 Hz, 2H), 2.1−2.6 (m, 2H), 1.5−2.0 (m, 2H). Anal. Calcd for $C_{13}H_{17}N_3O_3$: C, 59.30; H, 6.51; N, 15.96. Found: C, 59.27; H, 6.53; N, 15.99.

***N*^{G}-Carbobenzoxy-[1-^{13}C]-DL-arginine Hydrochloride (4).** A solution of 2.5 g (9.5 mmol) of *N*^{G}-carbobenzoxy-4-guanidinobutanal (**3**) in 10 mL of 5% aqueous methanol was saturated with ammonia. (NH_3(g) was vigorously bubbled through the solution for 1 h at room temperature.) The solution was then mixed with a solution of 650 mg [^{13}C]potassium cyanide (ICN Stable Isotopes) and 1.1 g of ammonium chloride in 10 mL of saturated aqueous ammonia. The mixture was stirred at room temperature for 48 h. The solvent was then removed in vacuo and 20 mL of cold 12 M HCl was added. The mixture was refluxed for 5 h and then stirred for 24 h at room temperature. The solvent was evaporated in vacuo and the product was purified on Dowex 50W X-4 (90 cm × 3 cm, 1 × d) using 2 M aqueous HCl. The ninhydrin-positive product was dissolved in 100 mL of methanol, mixed with Darco G-60 decolorizing carbon, filtered, and concentrated to about 25 mL under reduced pressure. Crystallization was induced by the addition of 200 mL of cold diethyl ether, to give 2.1 g (65% yield) of **4**: HPLC R_v = 4.7 mL (acetonitrile/water, 6/4, v/v, C_{18}, 5 μm); ^{1}H NMR (methanol-d_4) δ 11.5 (m, 1H), 7.3 (s, 5H), 5.8−6.0 (m, 2H), 5.2 (s, 2H), 4.9−5.1 (m, 2H), 4.0−4.2 (m, 1H), 3.0−3.3 (t, J = 7 Hz, 2H), 2.2−2.6 (m, 2H), 1.5−2.1 (m, 2H). Anal. Calcd for $^{13}CC_{13}H_{21}N_4O_4Cl$: C, 48.92; H, 6.12; N, 16.20. Found: C, 49.15; H, 6.09; N, 16.25.

(1-^{13}C)-DL-Arginine Dihydrochloride (5). A solution of 2.0 g (5.8 mmol) of *N*^{G}-carbobenzoxy-1-^{13}C-DL-arginine hydrochloride (**4**) in 200 mL of methanol and 0.5 mL of 1 M aqueous HCl, containing 0.7 g of prewetted 10% palladium on carbon, was stirred under hydrogen (1 atm) at 20 °C for 3 h. The catalyst was removed by filtration and the solvent evaporated under reduced pressure. The Sakaguchi[22]-positive product was triturated with acetonitrile to give 1.3 g (93% yield) of **5**: mp = 224−227 °C; HPLC R_v = 3.55 mL (0.1% TCA in acetonitrile/water, 6/4, v/v, C_{18}, 5 μm); ^{1}H NMR (deuterium oxide) δ 4.1−4.4 (m, 1 H), 3.2−3.6 (m, 2H), 1.8−2.2 (m, 4H). Anal. Calcd for $^{13}CC_5H_{16}N_4O_2Cl_2$: C, 29.45; H, 6.50; N, 22.58. Found: C, 29.59; H, 6.48; N, 22.62.

(1-^{13}C)-L-Arginine Dihydrochloride (5a). (1-^{13}C)-DL-arginine hydrochloride (2.5 g) was resolved as its acetyl derivative by the method described by Greenstein and Winitz[22] to give 0.88 g (70% yield) of the title compound: mp = 227−230 °C; $[\alpha]^{25}_D$ = +24° (c = 2, 1 M HCl); HPLC R_v = 3.54 mL (0.1% TCA in acetonitrile/water, 6/4, v/v, C_{18}, 5 μm); ^{1}H NMR (10% DCl in D_2O) δ 4.3−4.6 (m, 1H), 3.3−3.7 (m, 2H), 1.7−2.1 (m, 4H). Anal. Calcd for $^{13}CC_5H_{16}N_4O_2Cl_2$: C, 29.45; H, 6.50; N, 22.58. Found: C, 29.57; H, 6.51; N, 22.56.

***N*$^\alpha$-BOC-*N*^{G}-Cbz-(1-^{13}C)-L-arginine (6).** A solution of **5a** (1.0 g, 4.1 mmol) in 20 mL of distilled water was treated with 3.0 mL of triethyl amine and stirred at room temperature for 20 min. A solution of 4.0 g (16.2 mmol) of BOC-ON (2-*tert*-butoxycarbonyloxyimino-2-phenylacetonitrile, Aldrich) in 20 mL of 1,4-dioxane was added to the aqueous solution of **5a**. The mixture was warmed to 40 °C to complete dissolution and then stirred overnight at room temperature. The solvent was then evaporated under reduced pressure and the product triturated with ether. The *N*$^\alpha$-BOC-(1-^{13}C)-L-arginine hydrochloride was purified by reverse phase prep plate chromatography (Whatman PLKC18F Linear-K) using 95% ethanol as a mobile phase. ^{1}H NMR verified the presence of only one BOC group and a positive Sakaguchi test verified the presence of the free guanidinium group. *N*$^\alpha$-BOC-(1-^{13}C)-L-arginine hydrochloride (0.95 g, 3.1 mmol) was dissolved in 50 mL methanol and treated with 15 mL of 1 M sodium methoxide in methanol and chilled in an ice−water bath. Benzyl chloroformate (8.0 mL, 57 mmol) was added in 1 mL increments every 20 min to the cold, rapidly stirring methanolic solution. After each 1 mL addition of benzyl chloroformate, 1 mL of a precooled 1 M sodium methoxide in methanol was also added. The reaction mixture was stirred in an ice bath for an additional 2 h and then at room temperature for 18 h. The reaction mixture was then neutralized by the addition of 80 mL of 0.5 M aqueous HCl. A 5-drop aliquot of the mixture was removed and diluted with 2 mL of distilled water, and the pH was tested (pH = 6.5). Aqueous NaOH (5 mL, 1.0 M) was then added to the reaction mixture with stirring. Another 5-drop aliquot was removed and diluted with 2 mL of distilled water, and the pH was tested (pH = 7.6). The reaction mixture was then concentrated under reduced pressure. The resulting concentrate was diluted with 150 mL of 10% acetonitrile/ethyl acetate and stirred for 1 h. The undissolved sodium chloride was removed by filtration. The solvent was then removed under reduced pressure and the crude *N*$^\alpha$-BOC-*N*^{G}-Cbz-(1-^{13}C)-L-arginine was purified by silica gel chromatography using 5% methanol/ethyl acetate as a mobile phase to give **6** (1.5 g, 64%): TLC R_f = 0.27 (ethyl acetate/methanol, 9/1, v.v); HPLC R_v = 9.95 mL (acetonitrlle/water, 8/2, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.5 (m, 2H), 7.2 (s, 5H), 5.2 (s, 2H), 4.4−4.5 (m, 1H), 3.4−3.6 (t, J = 7 Hz, 2H), 1.8−2.2 (m, 4H), 1.1 (s, 9H). Anal. Calcd for $^{13}CC_{18}H_{28}N_4O_6$: C, 55.98; H, 6.89; N, 13.68. Found: C, 56.19; H, 6.91; N, 13.65.

***N*$^\alpha$-BOC-*N*^{G}-Cbz-(1-^{13}C)-L-Argininal Semicarbazone (7).** *N*$^\alpha$-BOC-*N*^{G}-Cbz-(1-^{13}C)-L-arginine (5.8 g, 14.2 mmol) was dissolved in 100 mL of freshly distilled tetrahydrofuran (THF) and stirred under N_2 for 1 h. The mixture was chilled in ice/H_2O bath and treated with 2.8 g of 1,1′-carbonyldiimidazole. The mixture was stirred at 5 °C for 1 h and then chilled in a dry ice−alcohol/H_2O, 3/1 by vol, (−40 °C) bath under N_2. $LiAlH_4$ (35 mL, 1 M) in THF (Aldrich) was added dropwise over 20 min. The reaction mixture was allowed to stir at −40 °C for an additional 20 min under N_2. The reaction was then quenched

by the addition of 100 mL of 0.1 M aqueous HCl. The mixture was then partitioned between the phases of dichloromethane (400 mL) and 5% sodium potassium tartarate (400 mL). The organic phase was washed with distilled water (100 mL), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The crude product was dissolved in 100 mL of 95% ethanol and treated with 100 mL of a solution containing 2.7 g semicarbazide hydrochloride in 3% aqueous sodium bicarbonate. The mixture was stirred for 18 h at 25 °C and then partitioned between the phases of ethyl acetate (200 mL) and 5% sodium potassium tartarate (200 mL). The organic phase was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude semicarbazone was purified by silica gel column chromatography (75 × 2.5 cm) using 3% methanol/ethyl acetate as the mobile phase to produce 2.2 g (34%) of **7**: TLC $R_f = 0.36$ (5% methanol in ethyl acetate); HPLC $R_v = 4.05$ mL (acetonitrile/water, 8/2, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.4−9.6 (m, 1H), 8.2−8.5 (m, 2H), 7.2 (s, 5H), 6.9 (d, $J = 7$ Hz, 1H), 5.8−6.1 (m, 3H), 5.2 (s, 2H), 4.0−4.2 (m, 1H), 3.1−3.3 (t, $J = 7$ Hz, 2H), 1.8−2.2 (m, 4H), 1.1 (s, 9H). Anal. Calcd for $^{13}CC_{19}H_{30}N_7O_5$: C, 53.66; H, 6.73; N, 21.81. Found: C, 53.87; H, 6.71; N, 21.86.

N^G-Cbz-(1-^{13}C)-L-Argininal Semicarbazone Trifluoroacetate. A solution of 2.0 g of **7** in 40 mL of trifluoroacetic acid/chloroform (50/50 by volume) was chilled in an ice/H_2O bath and stirred at 5 °C for 3 h. The solution was concentrated under reduced pressure. The crude product was triturated with anhydrous ether and recrystallized from methanol/ether to give 2.1 g of N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone trifluoroacetate: TLC $R_f = 0.09$, ninhydrin positive, Sakaguchi negative (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (D_2O) δ 7.1 (s 5H), 6.9 (d, $J = 7$ Hz, 1H), 5.2 (s, 2H), 4.0−4.2 (m, 1H), 3.0−3.2 (t, $J = 7$ Hz, 2H), 1.7−2.1 (m, 4H).

Coupling Procedure. A solution of 1 mmol of the appropriate carbobenzoxy-protected dipeptide (Sigma) in 10 mL of anhydrous *N*,*N*-dimethylformamide (DMF) was chilled to −15 °C with vigorous stirring. The solution was treated with 0.140 mL (1 mmol) of triethylamine. After 30 min, 0.097 mL (1 mmol) of ethyl chloroformate was added to the cold reaction mixture. The reaction mixture was stirred at −15 °C for an additional 45 min. A precooled solution containing 0.464 g (1 mmol) of N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone trifluoroacetate (**7**) and 0.280 g (2 mmol) of triethylamine in 10 mL of anhydrous DMF was then added to the reaction mixture. The resulting solution was stirred at 0 °C for 4 h and then overnight at room temperature. The reaction mixture was then partitioned between the phases of ethyl acetate (200 mL) and 0.1 M aqueous NaOH (200 mL). The organic layer was extracted with 10% aqueous $NaHCO_3$ (200 mL), distilled water (200 mL), 0.1 M aqueous HCl (200 mL), and distilled water again (200 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The tripeptide was solidified by the addition of anhydrous ether and then recrystallized from methanol/ether.

Cbz-L-leucyl-L-leucyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8a): TLC $R_f = 0.55$ (ethyl acetate/methanol, 9/1, v/v); HPLC $R_v = 6.33$ mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.4−9.6 (m, 1H), 8.2−84 (m, 2H), 7.4 (s, 5H), 7.0 (s, 5H), 6.8 (d, $J = 7$ Hz, 1H), 5.8−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.6−4.8 (m, 3H), 4.1−4.4 (m, 3H), 3.4−3.6 (t, $J = 7$ Hz, 2H), 1.5−1.8 (m, 10H), 0.9−1.0 (d, $J = 7$ Hz, 12H). Anal. Calcd for $^{13}CC_{34}H_{51}N_9O_7$: C, 59.28; H, 7.22; N, 17.74. Found: C, 59.54; H, 7.19; N, 17.75.

Cbz-L-leucyl-L-valyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8b): TLC $R_f = 0.42$ (ethyl acetate/methanol, 9/1, v/v); HPLC $R_v = 6.21$ mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.5−9.6 (m, 1H), 8.0−8.2 (m, 2H), 7.4 (s, 5H), 7.1 (s, 5H), 6.9 (d, $J = 7$ Hz, 1H), 5.8−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.7−4.9 (m, 3H), 4.2−4.4 (m, 3H), 3.4−3.6 (t, $J = 7$ Hz, 2H), 1.4−1.7 (m, 8H), 1.0−1.2 (d, $J = 7$ Hz, 12H). Anal. Calcd for $^{13}CC_{33}H_{49}N_9O_7$: C, 58.75; H, 7.09; N, 18.09. Found: C, 58.96; H, 7.11; N, 18.07.

Cbz-L-leucyl-L-isoleucyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8c): TLC $R_f = 0.58$ (ethyl acetate/methanol, 9/1, v/v); HPLC $R_v = 6.45$ mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.3−9.5 (m, 1H), 8.1−8.3 (m, 2H), 7.4 (s, 5H), 7.2 (s, 5H), 6.8 (d, $J = 7$ Hz, 1H), 5.7−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.7−4.9 (m, 3H), 4.0−4.3 (m, 3H), 3.3−3.5 (t, $J = 7$ Hz, 2H), 1.3−1.7 (m, 14H), 0.9−1.1 (m, 9H). Anal. Calcd for $^{13}CC_{34}H_{51}N_9O_7$: C, 59.28; H, 7.22; N, 17.74. Found: C, 59.52; H, 7.20; N, 17.73.

Cbz-L-leucyl-L-phenylalanyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8d): TLC $R_f = 0.32$ (ethyl acetate/methanol, 9/1, v/v); HPLC $R_v = 7.05$ mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.4−9.6 (m, 1H), 7.8−8.1 (m, 2H), 7.4 (s, 5H), 7.2 (s, 5H), 7.0 (s, 5H), 6.8 (d, $J = 7$ Hz, 1H), 5.9−6.1 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.6−4.8 (m, 3H), 3.9−4.2 (m, 3H), 3.0−3.4 (m, 4H), 1.4−1.7 (m, 7H), 0.9−1.1 (d, $J = 7$ Hz, 6H). Anal. Calcd for $^{13}CC_{37}H_{49}N_9O_7$: C, 61.41; H, 6.63; N, 16.92. Found: C, 61.71; H, 6.66; N, 16.94.

Cbz-L-phenylalanyl-L-leucyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8e): TLC $R_f = 0.25$ (ethyl acetate/methanol, 9/1, v/v); HPLC $R_v = 7.15$ mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.4−9.5 (m, 1H), 7.9−8.1 (m, 2H), 7.4 (s, 5H), 7.2 (s, 5H), 6.9 (s, 5H), 6.8 (d, $J = 7$ Hz, 1H), 5.8−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.5−4.9 (m, 3H), 3.8−4.1 (m, 3H), 2.9−3.3 (m, 4H), 1.3−1.7 (m, 7H), 0.8−1.0 (d, $J = 7$ Hz, 6H). Anal. Calcd for $^{13}CC_{37}H_{49}N_9O_7$: C, 61.41; H, 6.63; N, 16.92. Found: C, 61.69; H, 6.65; N, 16.90.

Cbz-L-leucyl-L-alanyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8f): TLC $R_f = 0.16$ (ethyl acetate/methanol, 9/1, v/v); HPLC $R_v = 3.25$ mL (methanol/water, 6/4, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.4−9.6 (m, 1H), 7.9−8.1 (m, 2H), 7.4 (s, 5H), 7.2 (s, 5H), 6.8 (d, $J = 7$ Hz, 1H), 5.8−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.6−4.9 (m, 3H), 3.7−4.1 (m, 3H), 3.0−3.3 (t, $J = 7$ Hz, 2H), 2.0−2.2 (d, $J = 7$ Hz, 3H), 1.2−1.7 (m, 7H), 0.9−1.1 (d, $J = 7$ Hz, 6H). Anal. Calcd for $^{13}CC_{31}H_{45}N_9O_7$: C, 57.62; H, 6.78; N, 18.85. Found: C, 57.84; H, 6.76; N, 18.81.

Cbz-L-leucyl-β-alanyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8g): TLC $R_f = 0.14$ (ethyl acetate/methanol, 9/1, v/v); HPLC $R_v = 3.45$ mL (methanol/water, 6/4, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.4−9.6 (m, 1H), 7.9-8.2 (m, 2H), 7.4 (s, 5H), 7.2 (s, 5H), 6.8 (d, $J = 7$ Hz, 1H), 5.7−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.6−4.9 (m, 3H), 3.6−4.1 (m, 2H), 2.9−3.4 (m, 6H), 1.2−1.8 (m, 7H), 0.9−1.1 (d, $J = 7$ Hz, 6H). Anal. Calcd for $^{13}CC_{31}H_{45}N_9O_7$: C, 57.62; H, 6.78; N, 18.85. Found: C, 57.87; H, 6.75; N, 18.80.

Cbz-L-leucyl-L-*p*-chlorophenylalanyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8h): TLC $R_f = 0.22$ (acetonitrile/ethyl acetate, 1/2, v/v); HPLC $R_v = 5.88$ mL (methanol/water, 8/2, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.4−9.6 (m, 1H), 8.0−8.3 (m, 2H), 7.1−7.7 (m, 14H), 6.8 (d, $J = 7$ Hz, 1H), 5.7−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.6−4.9 (m, 3H), 3.9−4.2 (m, 3H), 3.0−3.5 (m, 4H), 1.4−1.7 (m, 7H), 0.9−1.1 (d, $J = 7$ Hz, 6H). Anal. Calcd for $^{13}CC_{37}H_{48}N_9O_7Cl$: C, 58.70; H, 6.21; N, 16.18. Found: C, 58.96; H, 6.19; N, 16.15.

Cbz-L-leucyl-L-*p*-nitrophenylalanyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8i): TLC $R_f = 0.14$ (acetonitrile/ethyl acetate, 1/2, v/v); HPLC $R_v = 4.22$ mL (methanol/water, 8/2, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 9.4−9.7 (m, 1H), 8.0−8.3 (m, 2H), 7.0−7.9 (m, 14H), 6.8 (d, $J = 7$ Hz, 1H), 5.7−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.5−4.9 (m, 3H), 3.8−4.2 (m, 3H), 2.8−3.5 (m, 4H), 1.4−1.7 (m, 7H), 0.9−1.1 (d, $J = 7$ Hz, 6H). Anal. Calcd for $^{13}CC_{37}H_{48}N_{10}O_9$: C, 57.91; H, 6.13; N, 17.73. Found: C, 58.19; H, 6.11; N, 17.70.

Cbz-L-leucyl-L-cyclohexylalanyl-N^G-Cbz-(1-^{13}C)-L-argininal semicarbazone (8j): TLC $R_f = 0.18$ (acetonitrile/ethyl acetate, 1/2, v/v); HPLC $R_v = 6.77$ mL (acetonitrile/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO−d_6) δ 9.4−9.7 (m, 1H), 8.0−8.4 (m, 2H), 7.4 (s, 5H), 7.1 (s, 5H), 6.8 (d, $J = 7$ Hz, 1H), 5.6−6.0 (m, 3H), 5.3 (s, 2H), 5.1 (s, 2H), 4.6−4.9 (m, 3H), 3.7−4.1 (m, 3H), 2.9−3.1 (t, $J = 7$ Hz, 2H), 2.0−2.3 (m, 2H), 1.4−1.7 (m, 12H), 0.8−1.2 (m, 12H). Anal. Calcd for $^{13}CC_{37}H_{55}N_9O_7$: C, 60.92; H, 7.38; N, 16.79. Found: C, 61.21; H, 7.36; N, 16.75.

General N-Terminal and Guanidino Deblocking Procedure. A dilute solution containing 0.50 mmol of the fully protected tripeptide (**8a**−**j**) in 200 mL of methanol was treated with a slurry of 10 mg of 10% palladium on carbon in 2.0 mL of 0.10 M HCl. The mixture was stirred under a hydrogen atmosphere (1 atm), with hydrogen bubbling through the mixture, at room temperature for 4 h. The catalyst was removed by filtration and the solution was concentrated under reduced pressure. Excess water was removed by the addition and subsequent evaporation of acetonitrile. The crude soil was recrystallized from methanol/ether. The Sakaguchi- and nin-

hydrin-positive dihydrochloride salt of the tripeptide semicarbazone was dissolved in 10 mL of anhydrous *N*,*N*-dimethylformamide and chilled to 0 °C. The rapidly stirring solution was treated with 40 μL (0.50 mmol) of anhydrous pyridine. The solution was stirred at 0 °C for 30 min and then treated with 47 μL (0.50 mmol) of acetic anhydride. The mixture was stirred at 0 °C for 3 h and then overnight at room temperature. Distilled water (100 μL) was added to quench the reaction. Cold THF (100 mL at 0 °C) was added to precipitate the product. The crude solid was purified by Sephasorb H-P chromatography (120 × 5 cm column) using methanol as the mobile phase. The fractions showing both a positive Sakaguchi[22] and a negative ninhydrin test were pooled and concentrated under reduced pressure.

Acetyl-L-leucyl-L-leucyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9a): TLC R_f = 0.67 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 6.21 mL (0.05% TCA in methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.4−8.5 (m, 3H), 7.8−8.0 (m, 3H), 6.9−7.0 (d, J = 7 Hz, 1H), 5.8−6.0 (m, 3H), 4.6−4.8 (m, 3H), 3.9−4.3 (m, 3H), 3.4−3.6 (t, J = 7 Hz, 2H), 2.4 (s, 3H), 1.5−1.8 (m, 10H), 0.9−1.1 (d, J = 7 Hz, 12H). Anal. Calcd for $^{13}CC_{20}H_{42}N_9O_4Cl$: C, 48.60; H, 8.12; N, 24.19. Found: C, 48.79; H, 8.10; N, 24.11.

Acetyl-L-leucyl-L-valyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9b): TLC R_f = 0.63 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 6.56 mL (0.05% TCA in methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO−d_6) δ 8.4−8.6 (m, 3H), 7.8−8.0 (m, 3H), 6.9−7.0 (d, J = 7 Hz, 1H), 5.7−6.0 (m, 3H), 4.6−4.8 (m, 3H), 4.0−4.3 (m, 3H), 3.4−3.6 (t, J = 7 Hz, 2H), 2.5 (s, 3H), 1.5−1.8 (m, 8H), 0.9−1.1 (d, J = 7 Hz, 12H). Anal. Calcd for $^{13}CC_{19}H_{40}N_9O_4Cl$: C, 47.57; H, 7.95; N, 24.86. Found: C, 47.79; H, 7.93; N, 24.90.

Acetyl-L-leucyl-L-isoleucyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9c): TLC R_f = 0.65 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 6.87 mL (0.05% TCA in methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.3−8.5 (m, 3H), 7.8−8.1 (m, 3H), 6.9−7.0 (d, J = 7 Hz, 1H), 5.8−6.0 (m, 3H), 4.5−4.7 (m, 3H), 3.9−4.3 (m, 3H), 3.4−3.6 (t, J = 7 Hz, 2H), 2.4 (s, 3H), 1.5−1.8 (m, 13H), 0.8−1.0 (m, 9H). Anal. Calcd for $^{13}CC_{20}H_{42}N_9O_4Cl$: C, 48.60; H, 8.12; N, 24.19. Found: C, 48.82; H, 8.14; N, 24.16.

Acetyl-L-leucyl-L-phenylalanyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9d): TLC R_f = 0.72 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 5.27 mL (0.05% TCA in methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.3−8.5 (m, 3H), 7.8−8.1 (m, 3H), 7.1 (s, 5H), 6.9−7.0 (d, J = 7 Hz, 1H), 5.7−6.0 (m, 3H), 4.5−4.9 (m, 3H), 3.8−4.2 (m, 3H), 3.1−3.5 (m, 4H), 2.4 (s, 3H), 1.3−1.7 (m, 7H), 0.8−1.0 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{23}H_{40}N_9O_4Cl$: C, 52.11; H, 7.26; N, 22.71. Found: C, 52.35; H, 7.23; N, 22.67.

Acetyl-L-phenylalanyl-L-leucyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9e): TLC R_f = 0.70 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 6.01 mL (0.05% TCA in methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.3−8.6 (m, 3H), 7.9−8.1 (m, 3H), 7.0 (s, 5H), 6.8−6.9 (d, J = 7 Hz, 1H), 5.8−6.0 (m, 3H), 4.5−4.9 (m, 3H), 3.8−4.2 (m, 3H), 3.1−3.5 (m, 4H), 2.5 (s, 3H), 1.4−1.7 (m, 7H), 0.8−1.0 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{23}H_{40}N_9O_4Cl$: C, 52.11; H, 7.26; N, 22.71. Found: C, 52.33; H, 7.24; N, 22.69.

Acetyl-L-leucyl-L-alanyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9f): TLC R_f = 0.79 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 3.73 mL (0.05% TCA in methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.4−8.6 (m, 3H), 7.9−8.2 (m, 3H), 6.9−7.0 (d, J = 7 Hz, 1H), 5.7−6.0 (m, 3H), 4.6−4.9 (m, 3H), 3.7−4.2 (m, 3H), 3.0−3.3 (t, J = 7 Hz, 2H), 2.5 (s, 3H), 2.0−2.2 (d, J = 7 Hz, 3H), 1.2−1.6 (m, 7H), 0.9−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{17}H_{36}N_9O_4Cl$: C, 45.35; H, 7.58; N, 26.32. Found: C, 45.54; H, 7.56; N, 26.28.

Acetyl-L-leucyl-β-alanyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9g): TLC R_f = 0.81 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 3.93 mL (0.05% TCA in methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.4−8.7 (m, 3H), 7.9−8.2 (m, 3H), 6.8−6.9 (d, J = 7 Hz, 1H), 5.7−6.0 (m, 3H), 4.6−4.9 (m, 3H), 3.7−4.1 (m, 3H), 2.9−3.4 (m, 6H), 2.4 (s, 3H), 2.0−2.2 (d, J = 7 Hz, 3H), 1.2−1.6 (m, 7H), 0.9−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{17}H_{36}N_9O_4Cl$: C, 45.35; H, 7.58; N, 26.32. Found: C, 45.52; H, 7.55; N, 26.26.

Acetyl-L-leucyl-L-*p*-chlorophenylalanyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9h): TLC R_f = 0.71 (ethanol/water, 7/3, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 4.21 mL (0.05% TCA in methanol/water, 8/2, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.4−8.8 (m, 3H), 7.6−8.1 (m, 7H), 6.9−7.0 (d, J = 7 Hz, 1H), 5.7−6.0 (m, 3H), 4.6−4.9 (m, 3H), 3.7−4.1 (m, 3H), 3.0−3.5 (m, 4H), 2.5 (s, 3H), 1.4−1.7 (m, 7H), 0.9−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{23}H_{39}N_9O_4Cl_2$: C, 49.07; H, 6.67; N, 21.38. Found: C, 49.29; H, 6.65; N, 21.34.

Acetyl-L-leucyl-L-*p*-nitrophenylalanyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9i): TLC R_f = 0.77 (ethanol/water, 7/3, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 3.98 mL (0.05% TCA in methanol/water, 8/2, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.5−8.8 (m, 3H), 7.5−8.2 (m, 7H), 6.9−7.0 (d, J = 7 Hz, 1H), 5.7−6.0 (m, 3H), 4.7−4.9 (m, 3H), 3.7−4.0 (m, 3H), 3.1−3.5 (m, 4H), 2.4 (s, 3H), 1.4−1.7 (m, 7H), 0.9−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{23}H_{39}N_{10}O_6Cl$: C, 48.20; H, 6.55; N, 23.24. Found: C, 48.43; H, 6.52; N, 23.40.

Acetyl-L-leucyl-L-cyclohexylalanyl-(1-^{13}C)-L-argininal semicarbazone·HCl (9j): TLC R_f = 0.44 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 6.56 mL (0.05% TCA in methanol/water, 7/3, v/v, C_{18}, 5 μm); ^{1}H NMR (DMSO-d_6) δ 8.3−8.6 (m, 3H), 7.9−8.2 (m, 3H), 6.9−7.0 (d, J = 7 Hz, 1H), 5.7−6.0 (m, 3H), 4.5−4.9 (m, 3H), 3.7−4.2 (m, 3H), 2.9−3.1 (t, J = 7 Hz, 2H), 2.5 (s, 3H), 2.0−2.3 (m, 2H), 1.3−1.7 (m, 12H), 0.8−1.2 (m, 12H). Anal. Calcd for $^{13}CC_{23}H_{46}N_9O_4Cl$: C, 51.55; H, 8.26; N, 22.47. Found: C, 51.79; H, 8.30; N, 22.44.

General Procedure for Deblocking the C-Terminal Aldehyde. The acetyl tripeptide semicarbazone hydrochloride (0.33 mmol) **9a**−**j** was dissolved in 5 mL of methanol and 1 mL of 0.5 M aqueous HCl and chilled to 0 °C. The cold, rapidly stirring solution was treated with 0.75 μL (1.0 mmol) of 37% formaldehyde. The mixture was stirred at 0 °C for 4 h. The product was solidified by the addition of 100 mL of cold THF. The crude solid was purified via Sephasorb H-P (120 × 5 cm column) using methanol as the mobile phase. The fractions demonstrating both positive Sakaguchi and 2,4-DNP tests were pooled and concentrated under reduced pressure. The resulting acetyl-tripeptide aldehydes **10a**−**j** were solidified by the addition of anhydrous ether and were recrystallized from methanol/ether.

Acetyl-L-leucyl-L-leucyl-(1-^{13}C)-L-argininal·HCl (10a): TLC R_f = 0.37 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 5.64 mL (0.07% TCA in ethanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −28.4° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 10.0 (s, 0.4H, CHO), 7.6−7.9 (m, 3H), 6.0 (m, 0.6H, carbinol amine), 5.4 (m, 2H), 4.6−4.7 (m, 1H), 4.1−4.3 (m, 3H), 3.0−3.3 (t, J = 7 Hz, 2H), 2.5 (s, 3H), 1.3−1.7 (m, 10H), 0.9−1.1 (d, J = 7 Hz, 12H). Anal. Calcd for $^{13}CC_{19}H_{39}N_6O_4Cl$: C, 51.98; H, 8.47; N, 18.11. Found: C, 52.08; H, 8.44; N, 18.06.

Acetyl-L-leucyl-L-valyl-(1-^{13}C)-L-argininal·HCl (10b): TLC R_f = 0.65 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 5.27 mL (0.07% TCA in ethanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −35.0° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 10.1 (s, 0.4H, CHO), 7.5−7.9 (m, 3H), 5.9 (m, 0.6H, carbinol amine), 5.4 (m, 2H), 4.6−4.7 (m, 1H), 4.1−4.4 (m, 3H), 3.0−3.3 (t, J = 7 Hz, 2H), 2.5 (s, 3H), 1.5−1.8 (m, 8H), 0.9−1.1 (d, J = 7 Hz, 12H). Anal. Calcd for $^{13}CC_{18}H_{37}N_6O_4Cl$: C, 50.94; H, 8.29; N, 18.68. Found: C, 51.02; H, 8.37; N, 18.61.

Acetyl-L-leucyl-L-isoleucyl-(1-^{13}C)-L-argininal·HCl (10c): TLC R_f = 0.27 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 5.39 mL (0.07% TCA in ethanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −29.6° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 10.1 (s, 0.4H, HCO), 7.3−7.7 (m, 3H), 6.1 (m, 0.6H, carbinol amine), 5.6 (m, 2H), 4.6−4.7 (m, 1H), 4.2−4.3 (m, 3H), 3.0−3.2 (t, J = 7 Hz, 2H), 2.6 (s, 3H), 1.1−1.5 (m, 10H), 0.8−1.0 (d, J = 7 Hz, 12H). Anal. Calcd for $^{13}CC_{19}H_{39}N_6O_4Cl$: C, 51.98; H, 8.47; N, 18.11. Found: C, 52.17; H, 8.52; N, 18.04.

Acetyl-L-leucyl-L-phenylalanyl-(1-^{13}C)-L-argininal·HCl (10d): TLC R_f = 0.56 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 6.79 mL (0.07% TCA in ethanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha[^{25}_D$ = −31.3° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 7.6−7.9 (m, 3H), 7.1 (s, 5H), 6.1 (m, 1H, carbinol amine), 5.7 (m, 2H), 4.7−4.9 (m, 1H), 4.1−4.3 (m, 3H), 3.0−3.3 (m, 4H), 2.4 (s, 3H), 1.3−1.7 (m, 7H), 0.9−

1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{22}H_{37}N_6O_4Cl$: C, 55.67; H, 7.49; N, 16.87. Found: C, 55.73; H, 7.64; N, 16.76.

Acetyl-L-phenylalanyl-L-leucyl-(1-^{13}C)-L-argininal·HCl (10e): TLC R_f = 0.63 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 6.94 mL (0.07% TCA in ethanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −41.4° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 9.9 (s, 0.3H, CHO), 7.6−7.8 (m, 3H), 7.1 (s, 5H), 6.0 (m, 0.7H, carbinol amine), 5.6 (m, 2H), 4.9−5.0 (m, 1H), 4.2−4.4 (m, 3H), 3.0−3.2 (m, 4H), 2.3 (s, 3H), 1.3−1.6 (m, 7H), 1.0−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{22}H_{37}N_6O_4Cl$: C, 55.67; H, 7.49; N, 16.87. Found: C, 55.76; H, 7.59; N, 16.75.

Acetyl-L-leucyl-L-alanyl-(1-^{13}C)-L-argininal·HCl (10f): TLC R_f = 0.74 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 4.44 mL (0.07% TCA in ethanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −22.5° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 10.0 (s, 0.2H, CHO), 7.7−7.9 (m, 3H), 6.0 (m, 0.8H, carbinol amine), 5.4 (m, 2H), 4.5−4.7 (m, 1H), 4.1−4.3 (m, 3H), 3.0−3.3 (t, J = 7 Hz, 2H), 2.5 (s, 3H), 2.0−2.2 (d, J = 7 Hz, 3H), 1.3−1.7 (m, 7H), 0.9−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{16}H_{33}N_6O_4Cl$: C, 48.63; H, 7.88; N, 19.92. Found: C, 48.69; H, 7.94; N, 19.83.

Acetyl-L-leucyl-β-alanyl-(1-^{13}C)-L-argininal·HCl (10g): TLC R_f = 0.79 (ethanol/water, 6/4, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 4.54 mL (0.07% TCA in ethanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −17.9° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 10.1 (s, 0.1H, CHO), 7.7−7.9 (m, 3H), 6.0 (m, 0.9H, carbinol amine), 5.3 (m, 2H), 4.6−4.7 (m, 1H), 3.6−4.1 (m, 2H), 2.9−3.5 (m, 6H), 2.5 (s, 3H), 1.2−1.7 (m, 7H), 0.9−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{16}H_{33}N_6O_4Cl$: C, 48.63; H, 7.88; N, 19.92. Found: C, 48.71; H, 7.93; N, 19.81.

Acetyl-L-leucyl-L-*p*-chlorophenylalanyl-(1-^{13}C)-L-argininal·HCl (10h): TLC R_f = 0.46 (methanol/water, 7/3, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 6.89 mL (0.07% TCA in ethanol/water, 5/5, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −33.5° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 10.3 (s, 0.4H, CHO), 7.7−7.8 (m, 3H), 7.0−7.5 (m, 7H), 5.9 (m, 0.6H, carbinol amine), 5.7 (m, 2H), 4.9−5.0 (m, 1H), 4.1−4.4 (m, 3H), 3.0−3.3 (m, 4H), 2.4 (s, 3H), 1.3−1.6 (m, 7H), 1.0−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{22}H_{36}N_6O_4Cl_2$: C, 52.07; H, 6.81; N, 15.78. Found: C, 52.19; H, 6.89; N, 15.65.

Acetyl-L-leucyl-L-*p*-nitrophenylalanyl-(1-^{13}C)-L-argininal·HCl (10i): TLC R_f = 0.66 (methanol/water, 7/3, on Whatman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 5.34 mL (0.07% TCA in ethanol/water, 5/5, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −39.4° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 10.2 (s, 0.3H, CHO), 7.7−7.9 (m, 3H), 7.2−7.6 (m, 7H), 6.0 (m, 0.7H, carbinol amine), 5.7 (m, 2H), 4.9−5.0 (m, 1H), 4.0−4.4 (m, 3H), 3.0−3.4 (m, 4H), 2.5 (s, 3H), 1.3−1.6 (m, 7H), 1.0−1.1 (d, J = 7 Hz, 6H). Anal. Calcd for $^{13}CC_{22}H_{36}N_7O_6Cl$: C, 51.06; H, 6.68; N, 18.06. Found: C, 51.21; H, 6.73; N, 18.19.

Acetyl-L-leucyl-L-cyclohexylalanyl-(1-^{13}C)-L-argininal·HCl (10j): TLC R_f = 0.27 (ethanol/water, 6/4, on Whtaman $MKC_{18}F$ reversed phase TLC); HPLC R_v = 8.14 mL (0.07% TCA in ethanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_D$ = −28.3° (*c* 1.0, methanol); ^{1}H NMR (DMSO-d_6) δ 10.1 (s, 0.8H, CHO), 7.7−7.9 (m, 3H), 6.0 (m, 0.2H, carbinol amine), 5.4 (m, 2H), 4.5−4.6 (m, 1H), 4.0−4.3 (m, 3H), 3.0−3.3 (t, J = 7 Hz, 2H), 2.5 (s, 3H), 2.0−2.3 (m, 2H), 1.3−1.7 (m, 12H), 0.8−1.2 (m, 12H). Anal. Calcd for $^{13}CC_{22}H_{43}N_6O_4Cl$: C, 55.01; H, 8.51; N, 16.67. Found: C, 55.24; H, 8.59; N, 16.54.

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