Synthesis of [1,2-^{13}C]- and [2,3-^{13}C]-Labeled δ-Aminolevulinic Acid

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Summary

[1,2-^{13}C]- and [2,3-^{13}C]-labeled δ-aminolevulinic acids (δ-ALAs) have been prepared by a four-step sequence. [1,2-^{13}C]-ethyl bromoacetate was used to introduce the labels in the 1,2-labeled δ-ALA while [2-^{13}C]-ethyl bromoacetate and [5-^{13}C]-Meldrum's acid were used to introduce the labels in the 2,3-labeled derivative. These amino acid building blocks can be used to prepare heme-containing proteins with labeled hemes according to a previously reported biosynthetic method.

Key Words: doubly ^{13}C-labeled δ-aminolevulinic acid (δ-ALA), ^{13}C-labeled hemes, ^{13}C NMR, heme active site

Introduction

We recently reported a method for the efficient incorporation of isotopes into the active sites of heme-containing proteins.[1] This method utilized a bacterial host and a plasmid into which a synthetic gene encoding for rat liver outer mitochondrial membrane cytochrome b_5, a heme-containing protein, had been inserted.[2] Heme biosynthesis from δ-aminolevulinic acid (δ-ALA) resulted in ≥ 85% labeling of the heme moiety when ^{13}C-labeled δ-ALA was added to a growing culture of *E. coli* cells harboring the recombinant cytochrome b_5 gene.[1] The isotopically enriched heme can be used to facilitate spectroscopic (NMR, IR, Raman, EPR, and Mössbauer) studies of heme protein structure and function, heme transport, heme metabolism, and the influence of heme metabolism in diseases such as jaundice and porphyria.

In a previous study, we used ^{13}C-labeled heme to elucidate the role played by ferricytochrome b_5 heme propionates in binding to ferricytochrome c.[3] The assignment of the ^{13}C NMR signals arising from the heme propionate groups of paramagnetic ferricytochrome b_5 was carried out with protein samples whose heme had been labeled at different positions of the side chains. The presence of the ^{13}C-labeled heme facilitates NMR experiments such as HMQC (heteronuclear multiple quantum coherence) which relies on ^{1}H-^{13}C through-bond correlations and relatively large $^1J_{CH}$ coupling

CCC 0362–4803/97/080669–07$17.50

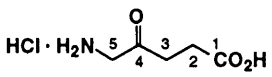
Received 29 January 1997
Revised 15 April 1997

constants. Even with the labeled heme, however, assignment of the carbonyl carbons using HMBC (heteronuclear multiple bond correlation) is difficult because the relaxation rate T_2^{-1} in the paramagnetic protein is generally larger than the coupling constant $^2J_{CH}$. This problem has recently been overcome by applying a new NMR experiment[4] designed for selective ^1H detection of ^1H_n-^{13}C-^{13}C fragments from among a large number of overlapping resonances. In ^{13}C NMR studies of ferricytochrome b_5, this experiment permits assignment of the heme propionate carbonyls.[3]

For the HMQC experiments, ferricytochrome b_5 with heme propionate groups labeled at C-1 or C-2 is sufficient. In the case of the ^1H_n-^{13}C-^{13}C experiments, however, labeling at C-1,2 or C-2,3 of the heme propionates is required. The C-1- and C-2-labeled δ-ALAs are both known[5] but the doubly labeled derivatives have not been previously described. Thus, we report the preparation of [1,2-^{13}C]- and [2,3-^{13}C]-labeled δ-ALAs. These compounds have been used in the biosynthesis of isotopically enriched hemes for ^{13}C-NMR studies of proteins that incorporate this moiety in the active site.

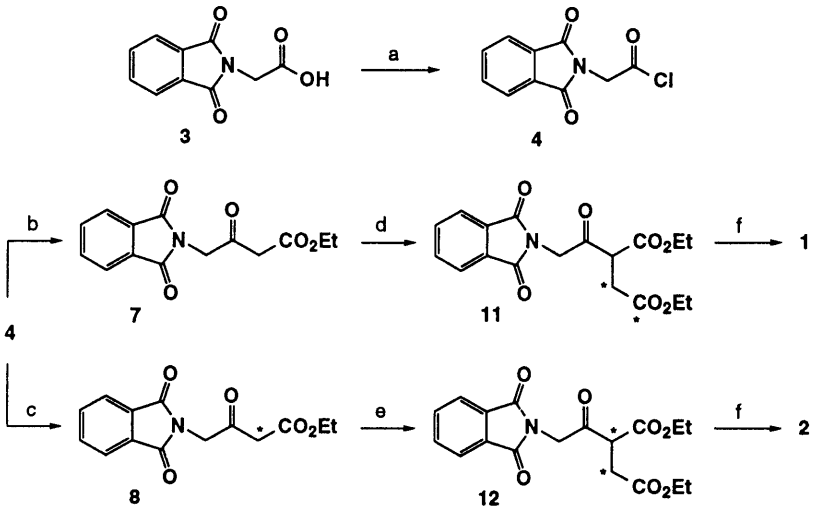
Results and Discussion

The synthesis was adapted from an earlier report by Kajiwara and co-workers[5] who prepared a series of monolabeled δ-ALAs for studies on the enzyme-mediated biosynthesis of porphobilinogen. δ-ALA derivatives, singly labeled at C-1 and C-2, were prepared as described by these authors; the physical and spectral properties matched those reported. For the 1,2- and 2,3-labeled derivatives, **1** and **2** respectively, a similar strategy was employed and this is described below.



1 (1,2-^{13}C)
2 (2,3-^{13}C)

The common starting point was *N*-phthaloylglycine (**3**). This acid was converted to its acid chloride (**4**) by reaction with thionyl chloride in benzene. The acid chloride was sequentially reacted with the anion derived from Meldrum's acid[6] (**5**) and heated under reflux with ethanol to give ethyl γ-phthalimidoacetoacetate (**7**) in an overall yield of 83-88% from **3**. The 2,3-labeled δ-ALA required the use of [5-^{13}C]-Meldrum's acid (**6**) to prepare [2-^{13}C]-ethyl γ-phthalimidoacetoacetate (**8**) since C-2 of **8** becomes C-3 in the final product. Labels at C-1 and C-2 were introduced via alkylation with labeled ethyl bromoacetates **9** and **10**. Deprotonation of **7** using sodium hydride in dry dimethoxy-ethane and reaction with [1,2-^{13}C]-ethyl bromoacetate (**9**) afforded the 1,2-labeled phthalimido diester **11**. Similar treatment of **8** with [2-^{13}C]-ethyl bromoacetate (**10**) afforded the 2,3-labeled phthalimido



Key: (a) $SOCl_2$, PhH, reflux; (b) Meldrum's acid (**5**), pyridine, CH_2Cl_2, 0 °C, then EtOH, reflux; (c) [5-^{13}C] -Meldrum's acid (**6**), pyridine, CH_2Cl_2, 0 °C, then EtOH, reflux; (d) NaH, DME, 0 °C, then $Br^{13}CH_2{}^{13}CO_2Et$ (**9**), 0 → 50 °C; (e) NaH, DME, 0 °C, then $Br^{13}CH_2CO_2Et$ (**10**), 0 → 50 °C; (f) 1:1 HOAc:HCl, reflux, 24 h; Dowex® 50X8-200 ion exchange chromatography

Scheme. Synthesis of [1,2-^{13}C]- and [2,3-^{13}C]-Labeled δ-ALA

diester **12**. Yields for the alkylation ranged from 65-70%. Finally, hydrolysis-decarboxylation of **11** and **12** using 1:1 acetic acid : concentrated hydrochloric acid gave **1** and **2**, respectively. Purification was carried out by ion exchange chromatography on Dowex® 50X8-200 to give the purified amino acid hydrochlorides in 75-77% yield. The synthesis is outlined in the Scheme.

Experimental

General. Solvents were purified in the following manner: Tetrahydrofuran (THF) was distilled from $LiAlH_4$ immediately prior to use; dimethoxyethane (DME) was stored under N_2 over 4-Å molecular sieves, and distilled from $LiAlH_4$ immediately prior to use. Labeled ethyl bromoacetate and malonic acid were obtained from Cambridge Isotope Laboratories, Andover, MA, USA. Other reagents were used as received. All reactions were run under an atmosphere of dry N_2. The saturated NH_4Cl, saturated NaCl, and 6 M HCl used in workup procedures refer to aqueous solutions. Reactions were monitored by one of the following methods: (1) TLC on hard layer silica gel GF plates (Analtech) visualized using UV light, phosphomolybdic acid, or I_2 vapor or (2) capillary GC with FI detection (SE-30 column, 6 m x 0.25 mm i.d., 0.25 μm film thickness) programmed between 50-300 °C. Preparative separations were performed using one of the following methods: (1) flash

chromatography[7] on silica gel (Grace, grade 62, 60-200 mesh) containing UV-active phosphor (Sylvania no. 2282) with band elution monitored using a hand-held UV lamp and (2) ion-exchange chromatography[5] using Dowex® 50X8-200 cationic exchange resin with band elution monitored by paper chromatography developed with 0.2% ninhydrin in EtOH. Melting points are uncorrected. IR spectra are referenced to polystyrene. ^{1}H and ^{13}C NMR spectra were measured in the indicated solvents at 400 and 100 MHz, respectively, and where appropriate, are referenced to internal Me_4Si. High resolution mass spectra (HRMS, EI/DP) were obtained at 70 eV; MS-LSIMS spectra were run in a matrix of 3-nitrobenzyl alcohol using Cs^+ at 16 keV.

Phthalimidoacetyl Chloride (4). A solution of 1.91 g (9.32 mmol) of **3** and 5.54 g (3.40 mL, 46.6 mmol) of thionyl chloride in 20 mL of benzene was stirred under reflux for 12 h. The reaction mixture was cooled and concentrated under vacuum. To remove excess thionyl chloride, the resulting yellow oil was redissolved in 25 mL of benzene and concentrated under vacuum (2x). Acid chloride **4** was isolated as light yellow crystals and used without further purification, crude mp 77-78 °C. IR (thin film) 1804, 1771, 1727, 1608, 1403, 779, 718 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.92 (m, 2 H), 7.80 (m, 2 H), 4.83 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 169.1, 166.5, 134.6, 131.4, 123.9, 47.5; HRMS *m/e* Calcd for $C_{10}H_6{}^{35}ClNO_3$: 223.0037. Found: 223.0032.

Meldrum's Acid (5) and [5-^{13}C]-Meldrum's Acid (6). The general procedure of Davidson and Bernhard was used.[6] Purification was accomplished by dissolving the crude product in a minimum of hot acetone, adding an equal volume of 1:2 ether : hexane, and cooling. The product was isolated by filtration and dried under vacuum. Compound **5** was obtained in 62% as white needles, mp 94-95 °C (lit[6] mp 94-95 °C). The spectral data for **5** were: IR (thin film) 1788, 1749, 1396, 1373, 1067, 975, 945, 829 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.64 (s, 2 H), 1.79 (s, 6 H); ^{13}C NMR ($CDCl_3$) δ 162.9, 106.2, 36.1, 27.5; HRMS *m/e* Calcd for $C_6H_8O_4$: 144.0422. Found: 144.0417. For the preparation of the labeled compound, 1.25 g (11.9 mmol) of [2-^{13}C]-malonic acid was used. The product was isolated and purified as above to give a 66% yield of **6**, mp 94-95 °C (lit[6] mp 94-95 °C). The spectral data for **6** were: IR (thin film) 1787, 1748, 1394, 1372, 1066, 973, 943, 828 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.64 (d, 2 H, J = 134.0 Hz), 1.79 (s, 6 H); ^{13}C NMR ($CDCl_3$) δ 36.1 (^{13}C-5); HRMS *m/e* Calcd for $^{12}C_5{}^{13}CH_8O_4$: 145.0456. Found: 145.0449.

Representative Procedure for the Two-Carbon Chain Extension Using Meldrum's Acid: Ethyl γ-Phthalimidoacetoacetate (7). The general procedure of Kajiwara and co-workers[5] was used. To a 0 °C solution of 1.40 g (9.72 mmol) of **5** in 2 mL of pyridine and 1 mL of CH_2Cl_2 was added 2.08 g (9.31 mmol) of **4** in 1 mL of CH_2Cl_2 dropwise over 1 h. The reaction was

stirred as it warmed to rt. The reaction mixture was washed with 6 M HCl followed by saturated NaCl, then dried ($MgSO_4$) and concentrated under vacuum. The resulting orange solid was dissolved in 100 mL of absolute EtOH and heated under reflux for 3 h. The reaction mixture was concentrated under vacuum and the crude product was recrystallized from absolute EtOH to afford 2.01 g (7.31 mmol, 78%) of **7** as light yellow needles, mp 110-111 °C (lit[5] mp 109-111 °C). IR (thin film) 1774, 1745, 1722, 1416, 1392, 725 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.87 (m, 2 H), 7.75 (m, 2 H), 4.68 (s, 2 H), 4.23 (q, 2 H, J = 7.2 Hz), 3.60 (s, 2 H), 1.31 (t, 3 H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 194.8, 167.4, 166.1, 134.2, 131.9, 123.5, 61.8, 46.8, 46.5, 13.9; HRMS *m/e* Calcd for $C_{14}H_{13}NO_5$: 275.0793. Found: 275.0788.

[2-^{13}C]-Labeled Ethyl γ-Phthalimidoacetoacetate (8). This compound was prepared as described above from 1.63 g (7.29 mmol) of **4** and 1.10 g (7.64 mmol) of **6**. Recrystallization of the crude product from absolute EtOH gave 1.61 g (5.83 mmol, 80%) of **8** as light yellow needles, mp 110-111 °C (lit[5] mp 109-111 °C). IR (thin film) 1772, 1742, 1720, 1415, 1390, 722 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.87 (m, 2 H), 7.75 (m, 2 H), 4.68 (s, 2 H), 4.23 (q, 2 H, J = 7.2 Hz), 3.59 (d, 2 H, J = 131.1 Hz), 1.30 (t, 3 H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 46.8 (^{13}C-2); HRMS *m/e* Calcd for $^{12}C_{13}{}^{13}CH_{13}NO_5$: 276.0827. Found: 276.0832.

Representative Procedure for the Alkylation of Ethyl γ-Phthalimidoacetoacetate: [1,2-^{13}C]-Ethyl 3-Ethoxycarbonyl-4-oxo-5-phthalimidopentanoate (11). The general procedure of Kajiwara and co-workers[5] was used. To a stirred solution of 0.17 g (7.10 mmol) of oil-free NaH in 10 mL of DME at 0 °C was added 1.62 g (5.90 mmol) of **7** in 2 mL of DME dropwise at rt over 30 min. To this solution was added 1.00 g (5.92 mmol) of [1,2-^{13}C]-ethyl bromoacetate (**9**) in 2 mL of DME dropwise during 30 min. The mixture was slowly warmed to 50 °C and stirred for 12 h. The reaction was cooled, poured into NH_4Cl and extracted with ether (3x). The combined organic extracts were washed with saturated NH_4Cl (2x), H_2O (1x), saturated NaCl (1x), dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified by flash chromatography[7] on a 60 cm x 2.5 cm silica gel column eluted with increasing concentrations of ether in hexanes to give 1.50 g (4.13 mmol, 70%) of **11** as a white solid, mp 71-73 °C. IR (thin film) 1772, 1741, 1718, 1412, 1388, 715 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.87 (m, 2 H), 7.74 (m, 2 H), 4.94 (d, 1 H, J = 18.0 Hz), 4.73 (d, 1 H, J = 18.0 Hz), 4.28 (m, 2 H), 4.17 (m, 3 H), 2.99 (dm, 2 H, J = 132.2 Hz), 1.35 (t, 3 H, J = 7.2 Hz), 1.27 (t, 3 H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 170.8 (d, ^{13}C-1, J = 58.9 Hz), 32.1 (d, ^{13}C-2, J = 58.9 Hz); HRMS *m/e* Calcd for $^{12}C_{16}{}^{13}C_2H_{19}NO_7$: 363.1228. Found: 363.1224.

[2,3-^{13}C]-Ethyl 3-Ethoxycarbonyl-4-oxo-5-phthalimidopentanoate (12). This compound was prepared as described above from 1.40 g (5.07 mmol) of **8** and 0.86 g (5.12 mmol) of [2-^{13}C]-ethyl bromoacetate (**10**) to afford 1.27 g (3.50 mmol, 69%) of **12** as a white solid, mp 72-74 °C; IR (thin film) 1771, 1741, 1718, 1413, 1388, 713 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.87 (m, 2 H), 7.74 (m, 2 H), 4.94 (d, 1 H, J = 18.0 Hz), 4.73 (d, 2 H, J = 18.0 Hz), 4.28 (m, 2 H), 4.16 (q, 2 H, J = 7.2 Hz), 4.17 (dm, 1 H, J = 128.4 Hz), 2.99 (dm, 2 H, J = 132.2 Hz), 1.35 (t, 3 H, J = 7.2 Hz), 1.27 (t, 3 H, J = 1.72 Hz); ^{13}C NMR ($CDCl_3$) δ 51.6 (d, ^{13}C-3, J = 38.8 Hz), 32.1 (d, ^{13}C-2, J = 39.0 Hz); HRMS *m/e* Calcd for $^{12}C_{16}{}^{13}C_2H_{19}NO_7$: 363.1228. Found: 363.1221.

Representative Procedure for the Hydrolysis-Decarboxylation of the Phthalimido β-Keto Esters: [1,2-^{13}C]-Labeled δ-Aminolevulinic Acid Hydrochloride ([1,2-^{13}C]-5-Amino-4-oxopentanoic Acid Hydrochloride, 1). A solution of 1.50 g (4.13 mmol) of **11** in 10 mL of 1:1 HOAc : HCl was stirred at reflux for 24 h, then cooled and concentrated under vacuum. The resulting tan solid was dissolved in 30 mL of distilled water and concentrated to dryness to remove excess acid. The off-white solid was redissolved in 25 mL of water and washed with 10 mL of ethyl acetate (3x); the aqueous layer was saved and concentrated under vacuum. The crude amino acid was purified by ion exchange chromatography using Dowex® 50X8-200 cationic exchange resin.[5] A typical procedure involved rinsing 20 mL (solid volume) of the resin with 0.001 M HCl to clean and activate the resin. The resin was packed into a medium-pore glass-fritted column using 0.001 M HCl as the solvent. The crude product (ca. 600 mg) was dissolved in 1 mL of 0.001 M HCl and loaded on top of the resin plug. The column was eluted with 10 volumes of 0.001 M HCl (ca. 200 mL) to remove impurities. The eluting solvent was changed to 1 M HCl and the amino acid hydrochloride was eluted in 2-mL fractions. Fractions were assayed using paper chromatography visualized with 0.2% ninhydrin in EtOH. Concentration of the product-containing fractions afforded 525 mg (3.13 mmol, 76%) of **1** as a light yellow solid, mp 150-152 °C (dec). IR (thin film) 3600-2133, 1717 cm^{-1}. A 25-mg sample of labeled δ-ALA·HCl was dissolved in 2 mL of D_2O and concentrated to dryness (3x) before preparing the NMR sample. ^{1}H NMR (D_2O) δ 3.93 (s, 2 H), 2.68 (s, 2 H), 2.53 (dq, 2 H, J = 141.4, 6.5 Hz); ^{13}C (D_2O) δ 177.8 (d, ^{13}C-1, J = 54.5 Hz), 28.3 (d, ^{13}C-2, J = 55.2 Hz); MS-LSIMS *m/e* 134 (M^+ + 1).

[1,2-^{13}C]-Labeled δ-Aminolevulinic Acid Hydrochloride ([2,3-^{13}C]-5-Amino-4-oxopentanoic Acid Hydrochloride, 2). This compound was prepared as described above from 1.24 g (3.42 mmol) of **12** and 8 mL of 1:1 HOAc : HCl to afford 436 mg (2.60 mmol, 76%) of **2** as a light yellow solid, mp 150-151 °C (dec); IR (thin film) 3605-2108, 1718 cm^{-1}. A 25-mg sample of

labeled δ-ALA·HCl was dissolved in 2 mL of D_2O and concentrated to dryness (3x) before preparing the NMR sample. ^1H NMR (D_2O) δ 3.94 (s, 2 H), 2.69 (dq, 2 H, J = 128.9, 6.2 Hz), 2.53 (dq, 2 H, J = 130.7, 6.2 Hz); ^{13}C (D_2O) δ 36.9 (d, ^{13}C-3, J = 38.0 Hz), 29.9 (d, ^{13}C-2, J = 37.8 Hz); MS-LSIMS *m/e* 134 ($M^+ + 1$).

Acknowledgement

The authors are grateful to the American Heart Association (9507904S) and NIH (GM50503) for support of this research. The authors also acknowledge Cambridge Isotope Laboratories for a CIL Research Grant. C.L.S. wishes to thank Oklahoma State University Foundation for a Jonas Distinguished Graduate Fellowship and the Department of Chemistry for a Skinner Fellowship. Partial support for our high resolution NMR instruments (NSF, W. M. Keck Foundation, State of Oklahoma and Oklahoma State University) and mass spectrometers (NSF, Oklahoma Center for the Advancement of Science and Technology) is also appreciated.

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