A facile synthesis of δ-aminolevulinic acid (ALA) regioselectively labeled with ^{13}C and direct observation of enzymatic transformation from ALA to porphobilinogen (PBG)

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Summary

δ-Aminolevulinic acid (ALA), labeled with ^{13}C at position 1, 2, 3, 4, or 5, was synthesized from ^{13}C-labeled glycine, Meldrum's acid, or bromoacetate. The latter compounds were prepared from ^{13}C-sodium acetate or ^{13}C-acetic acid.

Enzymatic transformation from ALA to porphobilinogen (PBG) was directly observed by ^{13}C-NMR.

Key words; ^{13}C-labeled ALA, ^{13}C-glycine, ^{13}C-Merdrum's acid, ^{13}C-bromoacetate, enzymatic trasformation, ^{13}C-NMR observation

Introduction

Many investigations have been carried out using labeled compounds in biosynthesis and metabolism. With the recent development of high-field FT-NMR spectroscopy, ^{13}C-labeled compounds

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have been widely applied to problems in organic chemistry and biochemistry. ^{13}C-labeled positions of products in biosynthesis can be detected without chemical degradation by ^{13}C FT-NMR spectra[1]. The ^{13}C-labeled position of the using precursor is so important for the study of biosynthetic progress, and it is often necessary to prepare precursors with various labeling position. ^{13}C-Labeled compounds are now commercially available. However, they are still very expensive and are restricted in number.

We have been interested in the regioselective synthesis of ^{13}C-aminolevulinic acid (ALA,**1**), which is known as an intermediate leading to heme (**4**) and vitamin B_{12} (**5**). Recently ALA (**1**), which is also an intermediate in the biosynthesis of chlorophyll (**6**), has been reported as a herbicide[2]. (Figure 1)

Results and Discussion

[5-^{13}C]ALA (**1e**), which is now commercially available, has been synthesized from [2-^{13}C]ethyl malonate[3a] or ethyl 4-oxobutyrate[3b]. However, these procedures are laborious, and commercial (**1e**) is highly expensive. We have developed a convenient synthesis of [5-^{13}C]ALA (**1e**), using [^{13}C]potassium cyanide. Synthesis of (**1e**) is illustrated in Figure 2.

[^{13}C]Copper cyanide (**8**)[4], which was obtained in 90% yield from [^{13}C]potassium cyanide (**7**), was treated with 0.5 equivalent of ethyl succinyl chloride (**9**) in acetonitrile to give cyanoketone (**10**).

In the presence of zinc, (**10**)[5] was reduced at 40°C for 10 min in acetic anhydride and acetic acid with ultrasonic waves (28kHz) to give [5-^{13}C]N-acetyl-ALA ethyl ester (**11**) in high yield. Excess (**8**) could be recovered easily by filtration and this procedure was repeated several times. The overall conversion of (**11**) was 90 %. After hydrolysis of (**11**), white crystalline needles of [5-^{13}C]-ALA (**1e**) were obtained by recrystallization from ethanol-ether. When 6N-hydrochloric acid was replaced by 6N-deuterium

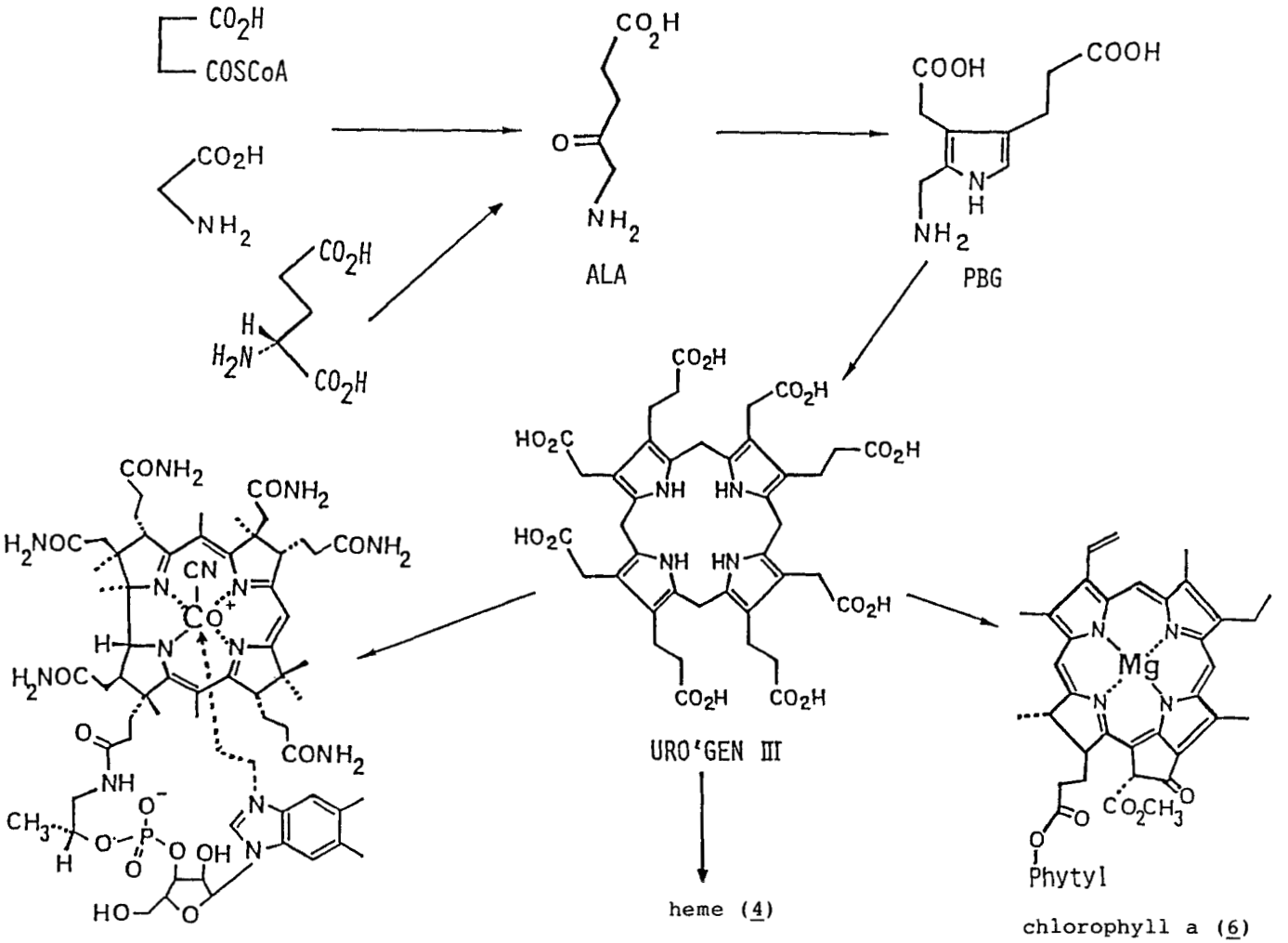
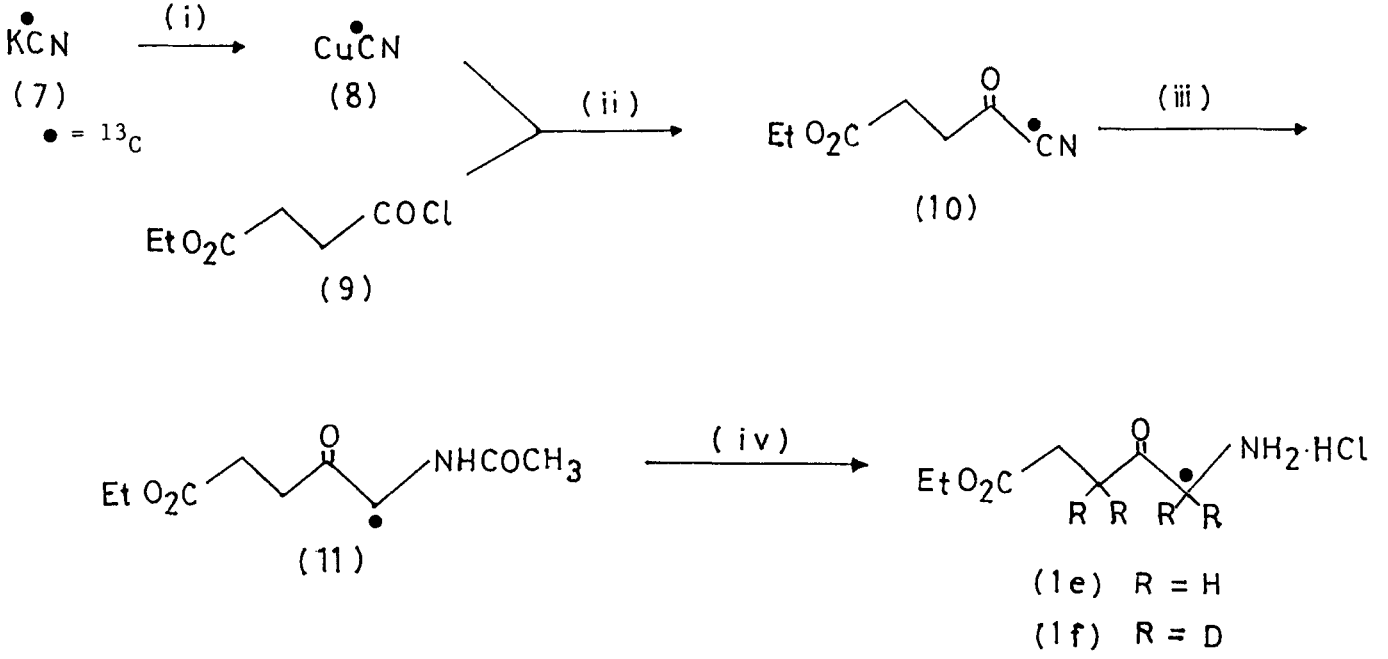


Figure 1. Biosynthesis of heme, V.B_{12}, and chlorophyll



(i) $CuSO_4$ (ii) CH_3CN (iii) ((((, Zn/$AcOH^+$, Ac_2O (28kHz) (iv) 6N-HCl or 6N-DCl

Figure 2. Synthesis of [5-^{13}C]ALA

chloride in the process of hydrolysis, multiple labeled ALA (1f) was obtained in good yield. Synthesis of ALA labeled with ^{13}C at various positions were then undertaken, starting from common raw materials.

Retrosynthetic analysis of ^{13}C-ALA is illustrated in Figure 3. We assume that ALA can be synthesized from bromoacetic acid, Meldrum's acid, and glycine derivatives. So ^{13}C-labeled ALA (1a-e) can be prepared from 1)^{13}C-bromoacetic acid (to [1-^{13}C]-ALA and [2-^{13}C]ALA), 2)^{13}C-Meldrum's acid (to [3-^{13}C]ALA), or 3)^{13}C-glycine derivatives (to [4-^{13}C]ALA and [5-^{13}C]ALA). Those three compouds can be prepared from common precursors; ^{13}C-sodium acetate or ^{13}C-acetic acid. For example,acetic acid leads to bromoacetic acid[6], which is transferred to malonic acid[7], then to Meldrum's acid[8]. Our synthetic route for ^{13}C-ALA is illustrated in Figure 4.

[1-^{13}C]ALA (1a) was synthesized as follows. [1-^{13}C]Sodium acetate (12a') was treated with bromine and benzoyl bromide, and was then added anhydrous ethanol to give [1-^{13}C]ethyl bromoacetate (13a) in 59% yield. (13a) was also obtained in 80 % yield

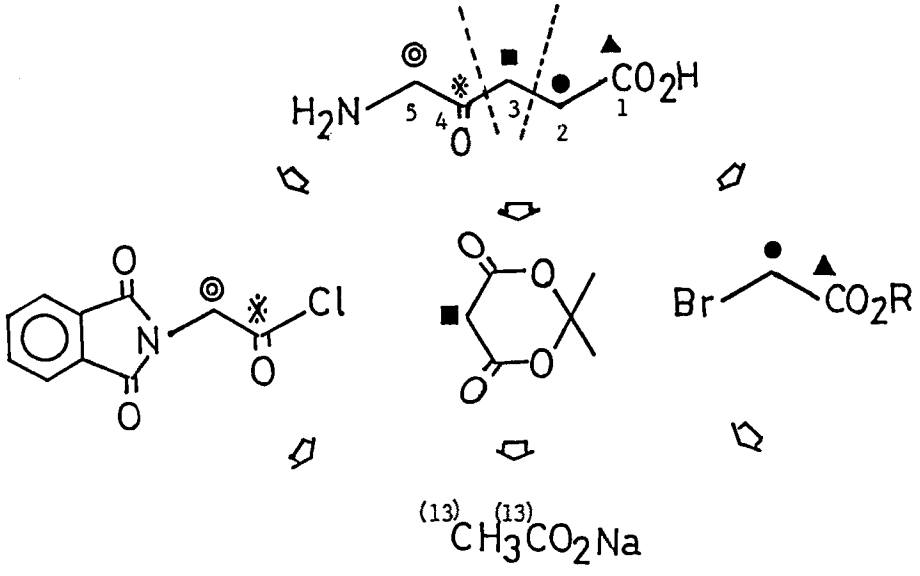


Figure 3. Retrosynthetic analysis of ^{13}C-ALA

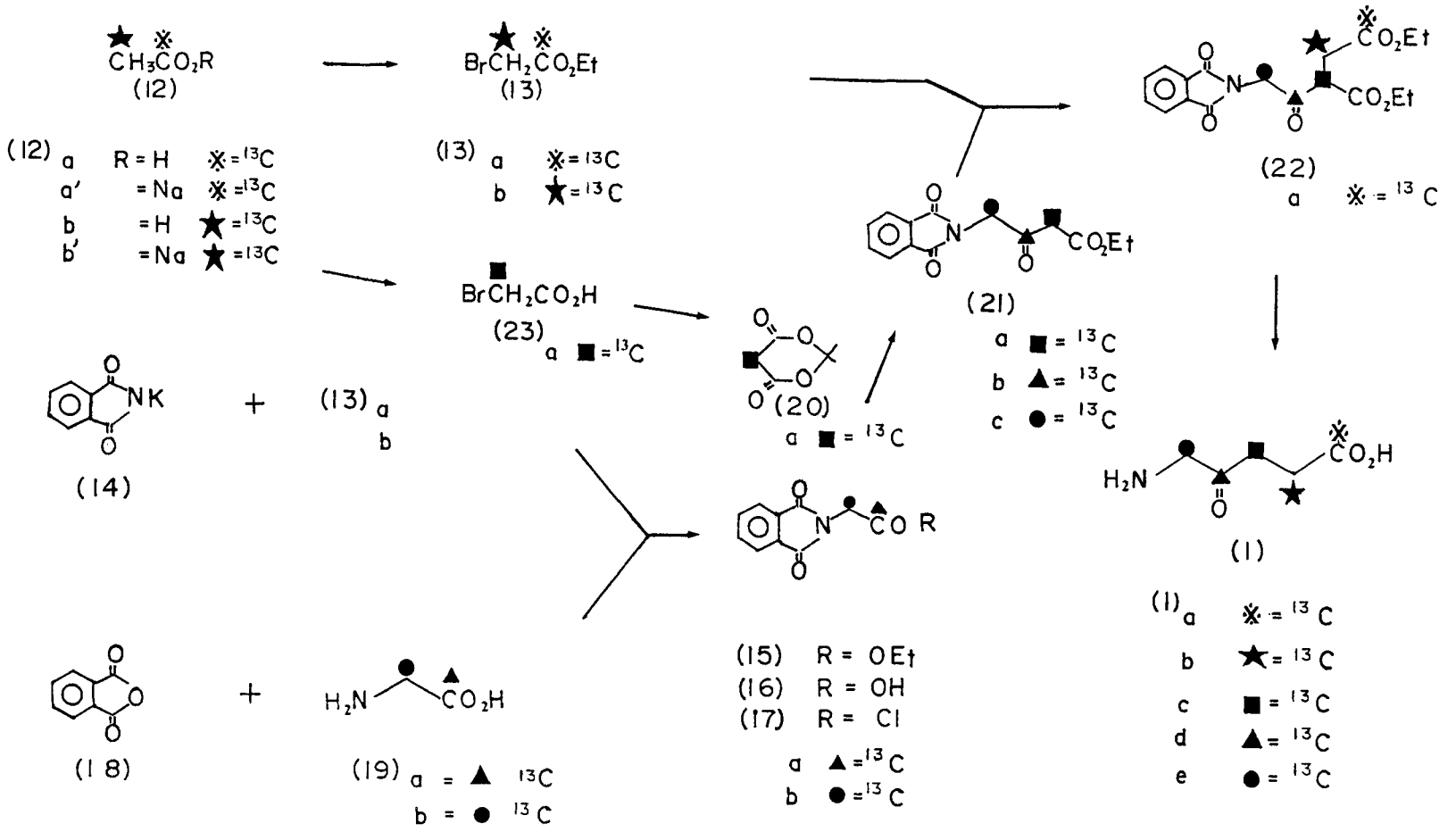


Figure 4. Scheme of ^{13}C-ALA synthesis

from [1-^{13}C]acetic acid (12a). (13a) was treated with ethyl phthalimidoacetoacetate (21) and sodium hydride in 1,2-dimethoxyethane at room temperature for 2 days to give [1-^{13}C]-ethyl-3-ethoxycarbonyl-5-phthalimidolevulinate (22a) in 74% yield. The resulting diester (22a) was hydrolyzed with a mixture of glacial acetic acid and conc. hydrochloric acid (1:1) overnight to give [1-^{13}C]ALA (1a) in high yield.

Starting from [2-^{13}C]sodium acetate (12b'), [2-^{13}C]ALA (1e) was synthesized in the same manner.

For the synthesis of [4-^{13}C]ALA (1d) or [5-^{13}C]ALA (1e), the following method was applied.

Phthalic anhydride (18) and [1-^{13}C]glycine (19a) were melted at 165°C for 15 min to give [1-^{13}C]N-phthaloyl glycine (16a) in 92 % yield. (19a) was prepared from [1-^{13}C]ethyl bromoacetate (13a). (16a) was treated with thionyl chloride to give [1-^{13}C]-phthalimido acetyl chloride (17a) in 95% yield. It was treated with 2,2 -dimethyl-4,6-dioxo-1,3-dioxane (Meldrum's acid,20) in dichloromethane and pyridine, and then with dry ethanol to give β-keto ester (21b) in 80% yield. (21b) was reacted with ethyl bromoacetate (13) and sodium hydride, and then hydrolyzed with acid to give [4-^{13}C]ALA (1d). Following this synthetic method, [5-^{13}C]ALA (1e) was obtained, using (17b).

[3-^{13}C]ALA was prepared in the same manner, using [2-^{13}C]-Meldrum's acid (20a), which was acquired from [2-^{13}C]bromoacetate (23) following the method of literature[7].

With these synthesized ^{13}C-labeled ALA in hands, we have investigated the enzymatic transformation of ALA (1) to porphobilinogen (PBG,2). PBG(2) is also known as an intermediate in the biosynthesis of heme (4) and V.B_{12} (5). Formation of PBG (2) *in vivo* from two molecules of ALA is catalyzed by ALA dehydratase (EC 4.2.1.24). This enzyme was purified from human erythrocytes or rat liver by the modified procedure of Anderson and Desnick[9],

except that Zn^{2+} (10uM) and dithiothreitol (0.1 mM) were added during the purification. The specific activity of the preparation was 4.85 units/mg of protein, defining one unit of enzymatic activity as the amount forming 1 umol of PBG/h at 37°C. The determination of those enzymatic reaction was usually taken up by the absorbance of UV spectra at 555 nm, which was based on Ehrlich's reagents. ^{13}C-Labeled ALA (1a-e) was enzymaticaly converted into ^{13}C-labeled PBG (2), which was observed by ^{13}C-NMR. ^{13}C-NMR shows directly the structure of ^{13}C-labeled PBG without chemical degradation. For example, the signal of [3-^{13}C]-ALA at 36.8 ppm disappeared rapidly. On the other hand, the signals at 22.4 ppm and 118.4 ppm, which belong respectively to [4-^{13}C,8-^{13}C]enriched PBG, became larger. (Figure 5)

Recently, the inhibitor of ALA dehydratase was isolated by one member of our group from the marrow of rabbits.[10] Using this inhibitor, we would like to study on the minute mechanism of ALA dehydratase by ^{13}C-NMR. It wil provide a new field for the study of enzyme mechanism.

Dioxane=67.40ppm, each spectrum after 500-3000 times accumulation)

Experimental

General. All melting points were taken on a Yazawa hotstage microscope apparatus type BY-1 and were uncorrected. IR spectra were measured with a Hitachi 215 spectrometer. Absorption maxima are reported in reciprocal centimeters. ^{1}H-NMR spectra were taken in the designated solvents on a Hitachi R-24B or a JEOL-GX-400 spectrometer, using tetramethylsilane (TMS) or sodium 3-trimethyl propionate-d_4 (TSP) as an internal standard. All chemical shifts and coupling constants are shown as values in ppm and Hz, respectively. ^{13}C NMR spectra were recorded on a JEOL-FX-60 or JEOL-GX 400 spectrometer. Signals are reported in ppm downfield from TMS. UV spectra were measured with a JASCO UVIDEC-610C spectrometer.

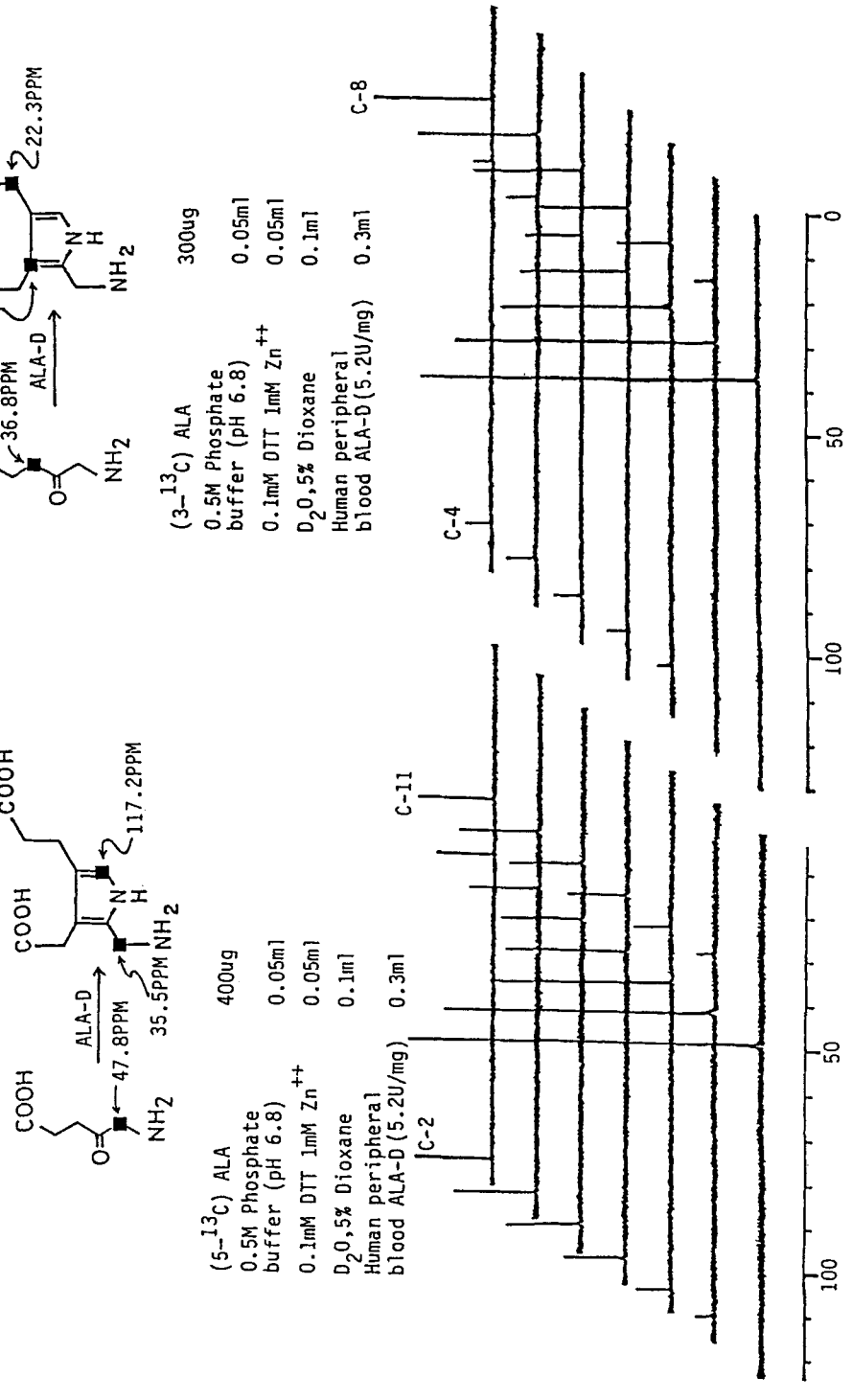


Figure 5. ^{13}C-NMR spectra of enzyme reaction (ALA--PBG) (100MHz,

Mass spectra were obtained from a JEOL JMS-6ISG2 double focusing spectrometer using the direct insertion method at 70eV ionizing energy. One Fab.Mass spectrum was measured on JEOL DX-302 spectrometer by treatment with JMA- DA-5000 data systemes. Fragment ion peaks and their intensities in parentheses are given in m/z and relative intensities to the base peak,respectively.

[5-^{13}C]aminolevulinic acid hydrochloride (1e)[3a,3b,5]

[^{13}C]Copper cyanide (8)[4]

A solution of [^{13}C]potassium cyanide (90% atom ^{13}C,2.0g,30 mmol) and sodium sulfite (4.0g 31.8mmol) in 0.5N NaOH (120ml) was added dropwise to an aqueous solution of 1M copper sulfate (80ml) over 40 min. It was stirred at room temperature for 10 min and was centrifuged. The precipitate was collected, washed with water, and was dried in vacuo to give (8) (2.63g, 96.0%).

[3-^{13}C]Ethyl cyanoformyl propionate (10)[5]

To a suspension of the above [^{13}C]cyanide (8) in dry acetonitrile (6 ml), a solution of 2.47g (15mmol) of carboethoxy propionylchloride (9) in dry acetonitrile (3 ml) was added slowly. It was refluxed for 30 min and the solvent was evaporated off. The dark brown residue was extracted with dichloromethane to give a crude product (10) (2.59g).

[5-^{13}C]ethyl (N-acetyl)-5-amino-4-oxo-pentanoate (11)[5]

A zinc powder (7.2g) was suspended in acetic acid-acetic anhydride (1:1,40ml), using ultrasonic wave at 40°C (Nihonseiki FF-120,28kHz). To this suspension, a solution of the above nitrile (10) in acetic acid - acetic anhydride (6 ml) was added dropwise over 15 min. It was stirred for 90 min with ultrasonic wave. The precipitate was filtered off, and the filtrate was evaporated in vacuo. The yellow residue was dissolved in 20 ml of ether and was extracted with water (20mlx2). The water layer was freeze-dried to give yellow solid, which was purified with column chromatography (SiO_2) to give colorless crystals (11) (1.45g). Excess copper

cyanide in 2) were recovered and were utilized in 3) to give another (11) (1.27g). Total conversion of (11) was 89.8%, m.p. 42°C; ^{1}H-NMR ($CDCl_3$) 1.25 (t,3H,J=7.0Hz,CH_3), 2.05 (s,3H,$COCH_3$), 2.65 (t,3H,J=5.5Hz,CH_2), 2.75 (t,3H,J=5.8Hz,CH_2), 4.12 (q, 2H,J=7.0Hz,O-CH_2), 4.20 (d,0.2H,J=4.6Hz,NH-CH_2), 4.20 (dd,1.8H,J= 4.6Hz,$^{1}J_{^{13}C-H}$=137.9Hz,NH-$^{13}CH_2$), 6.5 (br,1H,NH);^{13}C-NMR ($CDCl_3$) 49.2 (5-^{13}C); MS m/z 202 (M^+).

[5-^{13}C]Aminolevulinic acid hydrochloride (1e)

6N-HCl (6ml) was added to aminoketone (11) (227mg,1.12mmol) and refluxed for 2hr. It was evaporated in vacuo to give crystal (1e) (187mg,98.8%), m.p.145° C;^{1}H-NMR (D_2O) 2.71(t,2H,J=6.1 Hz,CH_2), 2.90 (t,2H,J=5.8Hz,CH_2), 4.13 (s,0.2H,CO-CH_2-N), 4.13 (d,1.8H,$^{1}J_{^{13}C-H}$=143.4Hz,CO-$^{13}CH_2$N); ^{13}C-NMR (D_2O) 47.8 (5-^{13}C).

[1-^{13}C]Aminolevulinic acid hydrochloride (1a)[11]

[1-^{13}C]-Ethyl-3-ethoxycarbonyl4-oxo-5-phthalimidolevulinate (22a)

A solution of ethyl phthalimidoacetate (21) (847.7mg,3.31mmol) in 7ml of 1,2-dimetoxyethane was added to a stirring suspension of sodium hydride (154.0mg,3.85mmol,60% in paraffin) in 7 ml of 1,2-dimethoxyethane and stirred for 1hr at room temperature under argon atmosphere. A solution of [1-^{13}C]-ethyl bromoacetate (13a) (647mg,3.85 mmol) in 5ml of dry 1,2-dimethoxyethane was added to the above suspension. After stirring for 1 day at room temperature, the reaction mixture was neutralized with 1N hydrochloric acid and extracted with ether. The organic layer was washed with sat.brine, dried over anhydrous sodium sulfate, evaporated, and was recrystallized from ether-hexane to give (22a) as colorless needles (1.25g, 89.7%), m.p. 71-74° C; ^{1}H-NMR ($CDCl_3$) 1.25,1.33 (2t,6H,J=7.0Hz,ester methyl), 2.95 (dd,2H,$^{2}J_{^{13}C-H}$=8.0Hz,methylene at C-2), 4.18,4.29 (2q,4H,J=7.0Hz, estermethylene), 4.19 (t,J=6.8 Hz,1H,11methylene), 4.83 (m,2H,methylene at C-5), 7.83 (AA'BB' system,4H,phenyl); ^{13}C-NMR ($CDCl_3$) 170.8 (1-^{13}C); IR (KBr) 1723, 1745 cm^{-1}(s,C=O); MS m/z 362 (M^+,1.24%).

[1-^{13}C]Aminolevulinic acid hydrochloride (1a)

The above diester (22a) was dissolved in 15ml of glacial acetic acid-conc. hydrochloric acid (1:1), and was refluxed for 1 day under argon. After evaporation, the residual brown powder was dissolved in 20ml of water and was then evaporated to remove the excess hydrogen chloride. The residue was dissolved in 40ml of water and washed with 150 ml of ethyl acetate three times to remove phthalic acid. The water layer was concentrated and the residue was purified by Dowex 50W-X8 column (2.5x12cm), freeze-dried, and was then recrystallized from ethanol-ether to give (1a) as colorless needles (1.25g,71.0%), m.p.146-149°C; ^{1}H-NMR (D_2O) 2.71 (dt,2H,$^{3}J_{^{13}C-H}$=5.8Hz,J=6.3Hz,methylene at C-2), 2.90 (dt,2H,$^{3}J_{^{13}C-H}$=6.1Hz,J=6.3Hz,methylene at C-3), 4.13 (s,2H,methylene at C-5); ^{13}C-NMR(D_2O) 177.4 (1-^{13}C); IR (KBr) 3430 cm^{-1} (m,N-H).

[4-^{13}C]Aminolevulinic acid hydrochloride (1d)

[1-^{13}C]Phthalimidoacetic acid (16a)

[1-^{13}C]Glycine (99 % atom^{13}C,1.0g,13.1mmol) (19a) was mixed with phthalic anhydride (1.95g,13.1mmol) and the mixture was heated at 150-170°C for 10 min while it melted and resolidified. The product was recrystallized from water to give (16a) as colorless needles (2.64g,97.5%), m.p.191-194°C; ^{1}H-NMR (acetone-d_6) 4.40 (d,2H,$^{2}J_{^{13}C-H}$=5.4Hz,$CH_2{}^{13}CO$), 7.82 (s,4H,phenyl); ^{13}C-NMR (acetone-d_6,CD_3=29.76) 168.93 (1-^{13}C); IR (KBr),1690,1720, 1775 cm^{-1} (s,C=O); MS m/z 206 (M^+,0.61%).

[1-^{13}C]Phthalimidoacetyl chloride (17a)[12]

Dried [1-^{13}C]phthalimidoacetic acid (16a) (2.64g,12.8 mmol) was refluxed with thionyl chloride (12ml,61.8mmol) at 60°C for 4hr. The excess thionyl chloride was removed. The resulting pale yellow solid was dissolved in benzene and evaporated. This procedure was repeated in three times to remove thionyl chloride completely. It gave (17a) as pale yellow crystals (2.91g, 99.2%),

^{1}H-NMR ($CDCl_3$), 4.80 (d,2H,$^2J_{^{13}C-H}$=5.8Hz,$CH_2{}^{13}CO$), 7.78 (AA'BB' system, 4H,phenyl).

[3-^{13}C]Ethyl phthalimidoacetoacetate(**21b**)

Meldrum's acid (**20**) (1.95g,13.5mmol) was dissolved in 1ml of dichloromethane and 2ml of pyridine. A solution of [1-^{13}C]-phthalimidoacetyl chloride (**17a**) (2.91g,13.0 mmol) in 2ml of dichloromethane was added dropwise to the above solution of Meldrum's acid at 0°C under argon over a period of 1.5hr. Then the reaction mixture was stirred additionally for 1.5hr at room temperature. The product was washed with 6N hydrochloric acid, sat. brine, dried over anhydrous magnesium sulfate, and evaporated. The residual viscous oil was refluxed with 100ml of abs. ethanol for 3hr, then excess ethanol was removed. The residue was diluted with 100 ml of dichloromethane, washed with sat.sodium bicarbonate, sat. brine, dried over anhydrous sodium sulfate and evaporated. The resulting crude product was recrystallized from ethanol to give (**21b**) as colorless needles (2.33g, 65.4%), m.p.109-111°C (lit;[2] 111°C); ^{1}H-NMR ($CDCl_3$), 1.29 (t,3H,J=7.0Hz, ester methyl), 3.5 (d,2H,$^2J_{^{13}C-H}$=6.2Hz, $^{13}COCH_2CO$), 4.22 (q,2H, J=7.0Hz,ester methylene), 4.62 (d,2H,$^2J_{^{13}C-H}$=5.0Hz,$NCH_2{}^{13}CO$), 7.77 (AA'BB'system,4H,phenyl); ^{13}C-NMR ($CDCl_3$), 194.79 (3-^{13}C); IR (KBr), 1685,1722,1745cm^{-1} (s,C=O); MS m/z 276 (M^+,28.1%).

[4-^{13}C]Ethyl-3-ethoxycarbonyl-4-oxo-5-phthalimidolevulinate (**22d**)

A solution of [3-^{13}C]-ethyl phthalimidoacetoacetate (**21b**) (2.33g, 8.43mmol) in 20ml of 1,2-dimethoxyethane was added to a stirring suspension of sodium hydride (420mg, 10.5 mmol, 60% in paraffine) in 5ml of dry 1,2-dimethoxyethane at 0°C. After addition it was stirred for 1 hr at room temperature under argon. A solution of ethyl bromoacetate (1.76g,10.5mmol) in 5ml of 1,2-dimethoxyethane was added to the above suspension. After stirring for 2 days at room temperature, the reaction mixture was neutra-

lized with 1N hydrochloric acid and extracted with ether. The organic layer was washed with sat. brine, dried over anhydrous sodium sulfate and evaporated to give a crude product, which was used in the following reaction without further purification.

[4-^{13}C]Aminolevurinic acid hydrochloride(1d)

The above crude product (22d) was dissolved in 20ml of glacial acetic acid-conc. hydrochloric acid (1:1), and the mixture was refluxed overnight at 120°C under argon. After evaporation, the brownish residue was dissolved in 50 ml of water and was washed with 150 ml of ethyl acetate three times to remove phthalic acid. The water layer was concentrated and the residue was purified by Dowex 50W-X8 column (2.5x12cm), freeze-dried, and then recrystallized from ethanol-ether to give colorless needles (1d, 391.0mg,27.5%), m.p.146-149°C; ^{1}H-NMR (D_2O) 2.71 (dt,2H, $^{3}J_{^{13}C-H}$=6.7Hz,J=5.7Hz,methylene at C-2), 2.90 (dt,2H,$^{2}J_{^{13}C-H}$=6.1 Hz, J=5.7Hz,methylene at C-3), 4.13 (d,2H,$^{2}J_{^{13}C-H}$=3.9Hz, methylene at C-5); ^{13}C-NMR (D_2O) 204.9 (4-^{13}C); IR (KBr) 3430 cm^{-1} (m,N-H),1725,1685 (s,C=O).

[2-^{13}C]Aminolevulinic acid hydrochloride (1b)[13]

This compound was prepared from [2-^{13}C]ethyl bromoacetate (13b), (1b,579mg,69.6%,from 13b) in the same procedure as [1-^{13}C]aminolevulinic acid (1a). The data of the product are as follows.

[2-^{13}C]ALA HCl (1b), m.p.148°C; ^{1}H-NMR (D_2O) 2.71 (dt,1.8H, $^{1}J_{^{13}C-H}$=131Hz,J=6.5Hz,methylene at^{13}C-2), 2.71 (t,0.2H,J=6.5Hz, methylene at^{12}C-2), 2.88 (t,2H,J=6.5Hz,methylene at C-3), 4.13 (s,2H,methylene at C-5); ^{13}C-NMR (D_2O) 31.6 (s,^{13}C-2); IR (KBr) 3430cm^{-1} (m,N-H).

[3-^{13}C]Amino levulinic acid hydrochloride (1c)[14]

This compound was synthesized from [2-^{13}C]Meldrum's acid (20a), (1c,1.285g,27.3%,from 20a) in the same way as [1-^{13}C]-ALA HCl (1a) or [2-^{13}C]ALA HCl (1b).

[2-^{13}C]Meldrum's acid (20a)[8]

A suspension of [2-^{13}C]malonic acid (99%atom^{13}C, 5.00g) in

acetic anhydride (6.0ml) was treated with 0.2 ml of conc.sulfuric acid at 0 C. To this solution was added dry acetone (4.0ml) dropwise over a period of 10 min. The mixture was kept stirring for 30 min at room temperature and was stood in a freezer (-20°C) for 18 hr. The crystalline precipitates were collected on a suction filter under reduced pressure. The white precipitates were washed with 20 ml of ice-cooled 0.5N sulfuric acid, then with ice-cooled water until the odour of acetic acid disappeared. It was recrystallized from acetone-ether-hexane to give (20a) as colorless needles (5.72g,82.6%), m.p.92-95°C; ^{1}H-NMR ($CDCl_3$) 3.62 (d,2H,$J_{^{13}C-H}$ =134.2Hz,$^{13}CH_2$), 1.79 (s,6H, CH_3); IR 1790,1750 cm^{-1} (C=O); ^{13}C-NMR ($CDCl_3$,$CDCl_3$=77.0) 36.15 (2-^{13}C); MS m/z 130 (M^+-15,5.51%).

[2-^{13}C]Ethyl phthalimidoacetoacetate (21b) ,^{1}H-NMR ($CDCl_3$) 1.31 (t,3H,J=7.2Hz,ester methyl), 3.58(d,2H,$J_{^{13}C-H}$=131.2Hz), 4.24 (q,2H,J=7.2Hz,estermethylene),4.76(s,2H,CO-$^{13}CH_2$-CO), 7.88,7.75(m,4H,phenyl); ^{13}C-NMR ($CDCl_3$,$CDCl_3$=77.0) 46.90 (2-^{13}C); MS m/z 276 (M^+,2.2%).

[3-^{13}C]Ethyl-3-ethoxycarbonyl-4-oxo-5-phthalimidolevulinate (22c), ^{1}H-NMR ($CDCl_3$) 1.26 (t,3H,J=7.2Hz,ester methyl), 1.35 (t, 2H,J=7.2Hz,ester methyl), 2.94 (ddd,1H,$^{2}J_{^{13}C-H}$=4.6Hz,J=17.6Hz,J= 6.1Hz,methylene at C-2), 3.02 (ddd,1H,$^{2}J_{^{13}C-H}$=4.6Hz,J=17.6Hz,J= 7.5Hz,methylene at C-2), 4.15 (ddd,1H,$J_{^{13}C-H}$=139.6Hz,J=7.5Hz,J=6. 1Hz,methyne at C-3), 4.16 (q,2H,J=7.2Hz,ester methylene), 4.28 (q, 2H,J=7.2 Hz,ester methylene), 4.73 (d,2H,J=18.0Hz,methylene at C-5), 4.94 (d,2H,J=18.0Hz,methylene at C-5), 7.88,7.74 (m,4H,phenyl); ^{13}C-NMR ($CDCl_3$,$CDCl_3$=77.0) 52.05 (3-^{13}C); MS m/z 362 (M^+, 0.12%).

[3-^{13}C]ALA HCl (1c,1.285g,27.3% from 20a), m.p.147 C; ^{1}H-NMR (D_2O) 2.70 (t,2H,J=6.3Hz,methylene at C-2), 2.89 (dt,1.8H,$^{1}J_{^{13}C-H}$ =126.5Hz,J=6.3Hz,methylene at ^{13}C-3), 2.89 (t,0.2H,J=6.3Hz,methylene at ^{12}C-3), 4.12 (s,2H,methylene at C-5); ^{13}C-NMR (D_2O) 36.8 (3-^{13}C); IR (KBr) 3430 cm^{-1} (sh,N-H); FAB-MS m/z 133 (M^++1).

[2-^{13}C]Ethyl bromoacetate (13b)[15]

[2-^{13}C]Sodium acetate (12b') (820mg,10mmol) and benzoic acid (1.1g,9.0mmol) was placed in a round-bottomed flask and was then added benzoyl bromide (7.5ml,64mmol). The mixture was heated at 120 C for 5hr. The produced acetyl bromide was distilled into a receiving flask. Dry bromine (2.6ml,50mmol) was added to the flask at -78°C over 5 min. After addition, the mixture was stirred at room temperature for a few minutes, and was then refluxed for 5hr. After cooling to room temperature, excess bromine was blown out with N_2 gas. To the residue was added dry ethanol (2.4ml,40 mmol) at -78°C over 10min, and was stirred at room temperature for 12 hr. The product was neutralized with sat. sodium bicarbonate (10ml) and was extracted with ether (3x30ml). The combined organic layer was washed with water (2x10ml), dried over anhydrous magnesium sulfate, and evaporated to give (13a) as a slightly yellow oil (991mg,59.3%), ^{1}H-NMR ($CDCl_3$) 1.35 (t,3H,J=7.0Hz,ester methyl), 3.15 (d,2H,$^1J_{^{13}C-H}$=76Hz,methylene) 4.20 (q,2H,J=7.0Hz,ester methylene); IR (neat) 1735 cm^{-1} (C=O); ^{13}C-NMR ($CDCl_3$) 25.9 (2-^{13}C).

[1-^{13}C]Ethyl bromoacetate (13a)[15]

This compound was prepared from [1-^{13}C]sodium acetate (12a') in the same procedure as we just described the above (13b), ^{1}H-NMR ($CDCl_3$) 1.35 (t,3H,J=7.0Hz,ester methyl), 3.77 (d,2H,$^2J_{^{13}C-H}$=6.0Hz,methylene), 4.20 (dq,2H,$^3J_{^{13}C-H}$=6.0Hz,J=7.0Hz,ester methylene); IR (neat) 1735 cm^{-1} (C=O); ^{13}C-NMR ($CDCl_3$) 167.2 (1-^{13}C).

[2-^{13}C]Ethyl phthalimidoacetoacetate (15b)

Ethyl-[2-^{13}C]bromoacetate (939mg,5.59mmol) (13b) and phthalimide potassium salt (14) (1.15g,6.22mmol) in 7.6ml of dimethylformate was refluxed for 3 hr. The reaction mixture was extracted with chloroform (4x15ml). The combined organic layer was neutralized with 0.1N sodium hydroxide (10ml), washed with water (2x15ml), dried over anhydrous sodium sulfate, and evaporated to

give a crude product. It was recrystallized from ethanol to give (**15b**) as colorless needles (975mg,75.0%), ^{1}H-NMR ($CDCl_3$) 1.25 (t,3H,J=7.0Hz,ester methyl), 3.72 (d,$^{1}J_{^{13}C-H}$=68Hz,methylene), 4.29 (q,2H,J=7.0Hz,ester methylene), 7.78 (m,4H,aromatic-H); ^{13}C-NMR ($CDCl_3$), 38.98 (2-^{13}C); IR (KBr) 1776,1732,1720cm^{-1} (C=O); MS m/z 234 (M^+).

[2-^{13}C]Glycine (**19b**)

The above ethyl[2-^{13}C]phthalimidoacetoacetate (**15b**) (250mg, 1.07 mmol) was dissolved in 6.25ml of 6N HCl and was refluxed for 5hr. The reaction mixture was diluted with equal volume of water and was washed with ethyl acetate (3x10ml). The water layer was evaporated and the residue was purified by Dowex 50X4 to give (**19b**) as a white powder (108mg, 90.0%), ^{1}H-NMR (D_2O) 3.70 (dd,2H, $^{2}J_{^{13}C-H}$=72Hz,J=72Hz methylene); ^{13}C-NMR (D_2O) 49.93 (s,^{13}C methylene); IR (KBr) 3100,1600 (C=O) cm^{-1}; MS m/z 76 (M^+,8.9%).

Enzyme reaction

ALA dehydratase	0.3ml
^{13}C labeled ALA	500μg
D_2O	0.1ml
$ZnSO_4$ (1mM) and DTT (100mM) solution	0.05ml
0.5M sodium phosphate buffer,pH 6.4	0.05ml

ALA dehydratase[10] was isolated from human peripheral blood. The purified enzyme has a specific activity of 12.6units/mg when assayed by Granick Mauzerall method.[16] In a 5mm NMR tube was placed 0.1ml of ^{13}C-labeled aminolevulinic acid solution in D_2O containing dioxane as an internal standard. 0.05ml of 0.5M sodium phosphate buffer (pH 6.8) and same volume of zinc sulfate (1 mM)- dithiothreitol (100mM) was added to the aminolevulinic acid solution. After addition of 0.3ml of aminolevulinic acid dehydratase and rapid mixing, the mixture was incubated in a NMR tube.

Proton decoupled ^{13}C NMR spectra were measured in sodium phosphate buffer (pH 6.8) in a 5 mm NMR tube using a JNM-GX-400

spectrometer at 100.4 MHz. These spectra were run at 20°C using a pulse width 5.5 μs (45°puls) and spectral width of 25000 Hz. Internal lock on D_2O was used and the chemical shifts were related to dioxane (67.4 ppm). (A): 1000 scans beginning 10 min after mixing. (B)-(D):2000 scans taken after former spectrum. (E)-(G) were the results of 3000 scans respectively.

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