# SYNTHESIS OF OXYGEN-18 ISOTOPE LABELED AMINO ACIDS AND DIPEPTIDES AND ITS EFFECT ON CARBON-13 NMR

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## SUMMARY

Oxygen-18 isotope labeled at the carboxyl group of glycine, L-alanine and L-proline were synthesized by acid catalyzed exchange or acid hydrolysis of respective methyl ester derivatives of amino acids in $HCl/H_2{}^{18}O$. Quantitative enrichment of glycine was achieved by the acid hydrolysis of amino acetonitrile in $H_2{}^{18}O$. In order to conserve the isotopic enrichment [$^{18}O$] dipeptides were synthesized by solid phase method. [$^{18}O$] isotope effect on [$^{13}C$] NMR of carboxylic carbon of amino acids and carbonyl carbon of peptides was observed. The [$^{18}O$] isotopic shifts observed for the carboxylic carbon of singly and doubly [$^{18}O$] labeled amino acids showed that the [$^{18}O$] isotope effect is additive.

Key Words; [$^{18}O$] isotope labeling; amino acids; dipeptides; [$^{18}O$] isotope effect; [$^{13}C$] NMR

## INTRODUCTION

Oxygen plays a major role in determining the molecular structure and conformation of peptides (1). As it has no radioactive isotopes, oxygen-18 has been the isotope of choice for tracer studies (2). Generally the levels of oxygen-18 have been determined by mass spectrometry. Recent advances in NMR spectroscopy have made it possible to use [$^{13}C$] or [$^{31}P$] NMR for detection of [$^{18}O$]. At high fields, the [$^{18}O$] isotope causes a small change in the chemical shift of a carbon or phosphorus nucleus compared to the corresponding unlabeled materials (3-6). We are interested in developing a method (i.e., [$^{18}O$] isotope effect on the [$^{13}C$] chemical shift) which would allow us to determine the conformation of amino acid and peptide molecules

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in solution. In the present study, we report the synthesis of [$^{18}$O] isotope labeled amino acids and dipeptides, and its effect on natural abundance [$^{13}$C] NMR chemical shifts.

## RESULTS AND DISCUSSION

[$^{18}$O] labeled glycine, L-alanine and L-proline were synthesized by the following methods: a. acid catalyzed exchange of [$^{18}$O] from $H_2{}^{18}O$ (7), b. acid hydrolysis of respective amino acid methyl ester derivatives in $HCl/H_2{}^{18}O$ (8), and c. acid hydrolysis of amino acetonitrile in $HCl/H_2{}^{18}O$ (for glycine only). [$^{18}$O] enrichment by methods 'a' and 'b' are comparable whereas the enrichment by method 'c' is quantitative. The amino group of the [$^{18}$O] amino acids were protected by t-butoxycarbonyl (Boc) group for peptide synthesis.

The [$^{18}$O] isotopes in amino acids are readily exchangeable in solutions. Hence, to avoid isotopic dilution, we employed solid phase

TABLE 1

[$^{18}$O] Enrichments, and Amino Acid Analysis

| Compound | % [$^{18}$O] Enrichment Per Atom | Amino Acid Analysis |
|---|---|---|
| Glycine | 83 (94)[a] | -- |
| L-Alanine | 65 | -- |
| L-Proline | 54 | -- |
| Gly-Gly[b] | 81 | Gly 2.08 |
| Gly-Ala[b] | 81 | Gly 1.01; Ala 1.00 |
| Ala-Ala[b] | 64 | Ala 1.98 |
| Ala-Gly[b] | 63 | Ala 0.99; Gly 1.08 |
| Pro-Pro[b] | 53 | Pro 1.96 |
| Pro-Gly[b] | 50 | Pro 0.98; Gly 1.02 |
| Gly-Pro[b] | 50 | Gly 1.01; Pro 0.96 |

[a] - synthesized by method 'c'; [b] - [$^{18}$O] labeled at the peptide carbonyl oxygen

method (9) to synthesize [$^{18}O$] labeled dipeptides. The labeled dipeptides were homogeneous on tlc (see experimental). The [$^{18}O$] enrichments as determined by mass spectrometry are listed in Table 1.

The [$^{18}O$] isotopic shift on natural abundance [$^{13}C$] NMR of amino acids and peptides is given in Table 2. The additive effect of [$^{18}O$] on [$^{13}C$] NMR of carboxylic carbon resonances of glycine and L-alanine shifted upfield 0.033 ppm upon substitution with one [$^{18}O$] atom and 0.066 ppm upon substitution with two [$^{18}O$] atoms. The additivity observed for the effect of [$^{18}O$] on [$^{13}C$] NMR signals of amino acids is similar to effects previously observed for carboxylic acids (10).

In the case of alanylglycine, the deuterium isotope effect on [$^{13}C$] NMR is also observed (0.080 ppm). The deuterium isotope effect on [$^{16}O$] and [$^{18}O$] carbonyl carbon is seen to be equal (6). For prolylproline, due to the tertiary nature of the peptide nitrogen atom, the deuterium isotope effect is not observed.

TABLE 2

[$^{18}O$] Isotope Effect on [$^{13}C$] NMR Chemical Shift of Amino Acids and Dipeptides

| Compound | Functional Group | [$^{18}O$] Isotope Shift[a] (ppm upfield from the corresponding [$^{16}O$] compound) |
|---|---|---|
| Glycine[b] | $-C^{16}O^{18}OH$ | 0.033 |
| | $-C^{18}O^{18}OH$ | 0.066 |
| L-Alanine[c] | $-C^{16}O^{18}OH$ | 0.033 |
| | $-C^{18}O^{18}OH$ | 0.066 |
| Ala-Gly[d] | $-C^{18}O-NH-$ | 0.028 |
| Pro-Pro[e] | $-C^{18}O-N<$ | 0.026 |

[a] - Solvent $H_2O/D_2O$ (4:1); chemical shifts are measured with reference to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)
[b] - Acquisition time = 4.09 sec; number of scans = 1,543
[c] - Acquisition time = 8.19 sec; number of scans = 1,928
[d] - Acquisition time = 4.09 sec; number of scans = 8,765
[e] - Acquisition time = 4.09 sec; number of scans = 4,422

In conclusion, the [$^{18}O$] isotope effect on [$^{13}C$] NMR chemical shift of amino acids and peptides may serve as a probe for future conformational studies of amino acids and peptides.

## EXPERIMENTAL

Glycine, L-alanine and L-proline were purchased from Sigma Chemical Company, St.Louis, MO and $H_2{}^{18}O$ (95 atom percent [$^{18}O$]) was obtained from Monsanto Research Corporation, Miamisburg, OH. Silica gel G 60 F-254 precoated thin layer chromatography plates were supplied by the Brinkman, Inc., Westbury, NY. We employed a Schwarz/Mann Peptide Synthesizer for the synthesis of [$^{18}O$] labeled dipeptides and a Finnigan MAT 4510 GC/MS spectrometer to measure the [$^{18}O$] enrichment. Amino acid analysis was carried out on a Durrum D-500 amino acid analyzer.

Synthesis of [$^{18}O$] Dipeptides:

a. [$^{18}O$] enrichment of amino acids:

Enriched $H_2{}^{18}O$ was saturated with dry hydrogen chloride gas, and up to 1 g/ml of amino acid or methyl ester derivative of amino acid or amino acetonitrile (for glycine only) was dissolved, degased, sealed under vacuum and kept at 105°C for 16-20 hrs under standard conditions of protein acid hydrolysis with minimal racemization and decomposition (11). Following removal and quantitative recovery of $H_2{}^{18}O$ by high vacuum distillation at room temperature, the [$^{18}O$] amino acid was dissolved in minimum amount of water and lyophilized two times. No side products were detected by amino acid analysis or by tlc when plates were developed with a neutral, basic, or acidic solvent (methanol - water - pyridine (20:5:1), n-propanol - water (7:3), n-propanol - ammonium hydroxide (7:1), and n-butanol - acetic acid - water (4:1:1) by volume).

b. Preparation of [$^{18}O$] Boc-amino acid:

The amino group of [$^{18}O$] amino acids were protected by t-butoxycarbonyl (Boc) group as published elsewhere (12).

c. Synthesis of [$^{18}$O] dipeptides by solid phase method (9):

Solid phase method was chosen in order to avoid isotopic dilution during synthesis even though solution method is preferred for synthesizing smaller peptides. The assembled peptides were cleaved from the resin by treating them with hydrofluoric acid and purified by column chromatography on a 20 x 2.5 cm column packed with 20 g of silica gel. The sample was applied as a solution in methanol - chloroform (1:2) and elution was accomplished with methanol - chloroform (2:1). 5 ml of fractions were collected and were analyzed by tlc (chloroform - methanol - acetic acid (14:2:1) or chloroform - methanol (19:1)). Appropriate fractions were pooled and evaporated to dryness, the residue was dissolved in water, and the solution immediately lyophilized. The purified [$^{18}$O] dipeptides, identical with the unlabeled commercial samples, were ascertained by tlc (methanol - chloroform (6:3), chloroform - methanol - ammonia (12:9:4), 1-butanol - ethyl acetate - acetic acid - water (1:1:1:1), and 1-butanol - acetic acid - water (4:1:1)) and by amino acid analysis (Table 1).

Natural abundance [$^{13}$C] NMR spectra were obtained by using a Nicolet NT-200 MHz spectrometer. Samples were prepared in deionized water/$D_2O$ (4:1) to provide an instrumental lock. A total of 1,500 - 9,000 scans (1,000 Hz sweep width, 4.09 or 8.19 sec acquisition time (Table 2), 90° pulse angle) and a 16K data block were used to record the spectra in the fourier transform mode. The exponential weighing factor for the free induction decay was 0.2 Hz.

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