A Simple and Economical Method for the Production of ^{13}C ^{18}O Labeled Fmoc-Aminoacids with High Levels of Enrichment: Applications to Isotope Edited IR Studies of Proteins.

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Supporting Information

Experimental procedures and compound characterization.

General. A 0.25M solution of HCl in the ^{18}O-enriched water was prepared by reacting acetyl chloride (196 mg, 2.5 mmol, 180 uL) with 10.0 ml of the water. ^{18}O-Enriched water (97%) was obtained from Cambridge Isotope Laboratories. The carboxy ^{13}C enriched amino acids were also obtained from this source and were ^{13}C 99% enriched. Dioxane was distilled from sodium and was stored in the dark under nitrogen. Acetyl chloride was freshly distilled before use. FMOC-Cl was from Bachem and was used as received. Other solvents were HPLC grade from Fisher. Hexanes is a commercial mixture of n-hexane and methyl cyclopentane. TLC's were run on EM Sciences Silica Gel 60 plates with UV and ninhydrin (for detection of free amino acid) visualization. Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured in a 1 dcm cell on a Perkin Elmer 241 polarimeter. Mass spectra were determined on a Micromass Platform LCZ electrospray ionization mass spectrometer in the positive ion mode.

General procedure for the preparation of N-(9-fluorenylmethoxycarbonyl-L-amino acids. The amino acid was dissolved or suspended in water (4 - 5 mL / mmol) and sodium carbonate decahydrate (2.5 mol equiv) added. The mixture was stirred until everything dissolved, the resulting solution cooled in an ice bath and a volume of dioxane equal to 60% of the amount of water used was added slowly. Partial precipitation of the amino acid salt occured. A solution of 9-fluorenylmethylchloroformate (1.05 to 1.1 mol equiv) dissolved in a volume of dioxane equal to the dioxane volume used above was added dropwise with vigorous stirring over 10 to 15 min. The resulting mixture was stirred at ice bath temperature for 2 hr and then at room temperature for about 6 hr. The solvent was evaporated in *vacuo* (2mm at 20°), and the solid remaining dissolved in water. The volume depends on the amino acid used and could be as high as 100 mL/mmol. The pH is normally around 9. The aqueous phase was extracted with four portions of ether. The pH was adjusted to 2 to 3 with 3M hydrochloric acid. The FMOC derivative usually precipitates as a solid. The mixture is extracted 4 times with ethyl acetate. The combined ethyl acetate extracts were washed with saturated brine and then dried over magnesium sulfate, filtered and the solvent evaporated leaving a white solid foam. The foam was shaken for a few minutes with hexanes and the hexanes decanted. The solid was crystallized from a suitable solvent, typically from ethyl acetate/hexanes or , in the case of FMOC-L-valine, from methylene chloride/hexanes.

(N-9-Fluorenylmethoxycarbonyl)-L-isoleucine-1-^{13}C: L-Isoleucine-1-^{13}C (1.00g, 7.6 mmol) was dissolved in 30 mL of water containing sodium carbonate decahydrate (5.43g, 19.0 mmol). The solution was cooled in an ice bath and 19 mL of dioxane added slowly over 1 min with stirring. Towards the end of the addition a fine white powder began to precipitate. A solution of FMOC-Cl (2.17g, 8.4 mmol) in 19 mL of dioxane was added dropwise with rapid stirring over 10 min.

Almost all the solid dissolved toward the end of the addition, but a new solid then began to separate. The mixture was stirred at ice bath temperature for 2 hr then at room temperature for 5.5 hr. The solvent was evaporated in *vacuo* (2 mm at 20°) and the solid remaining dissolved in 150 mL of water. The pH of the solution was about 8.5. It was extracted with 4 x 40 mL of ether. The aqueous phase was adjusted to pH 2 with 3M hydrochloric acid. A white solid precipitated. The mixture was extracted with 4 x 50 mL of ethyl acetate and the combined extracts were washed with 40 mL of saturated brine then dried over magnesium sulfate, filtered and the solvent evaporated leaving a white solid weighing 2.65g. The solid was stirred with 40 mL of hexanes for 60 min, the hexanes decanted and the solid dried in *vacuo* a short time. Isolated 2.64g of material. The solid was dissolved in 25 mL of boiling ethyl acetate and 250 mL of hexanes added slowly while warming gently. Crystallization began during this time. The mixture was allowed to cool slowly to room temperature then left at 5° overnight. The solid was isolated by filtration, washed with 2 x 20 mL of hexanes and vacuum dried at room temperature. Isolated 2.35g (87%) of a white flaky solid, m.p. 146-147°, lit 146-147°[1]. TLC: R_f = 0.46 ($CHCl_3$-CH_3OH-CH_3COOH 100:10:1 v/v).

(N-Fluorenylmethoxycarbonyl)-L-alanine-1-^{13}C: Isolated 5.80g (84%) of a white feathery solid, m.p. 153-154° (lit 147-150°[1]) starting from 2.00g (22.2 mmol) of the ^{13}C-labeled L-alanine. TLC: R_f = 0.59 ($CHCl_3$-CH_3OH-CH_3COOH, 50:10:1 v/v).

(N-Fluorenylmethoxycarbonyl)-L-valine-1-^{13}C: Isolated 5.11g (89%) of a white microcrystalline solid, m.p. 143-144° (lit 144-145°[2]) starting from 2.00g (16.9 mmol) of ^{13}C-labeled L-valine. TLC: R_f = 0.55 ($CHCl_3$-CH_3OH-CH_3COOH, 50:10:1 v/v).

(N-Fluorenylmethoxycarbonyl)-L-leucine-1-^{13}C: Isolated 3.47g (88%) of a white crystalline solid, m.p. 152-154° (lit. 152-154°[1]) starting from 1.48g (11.2 mmol) of ^{13}C-labeled L-leucine. TLC: R_f = 0.56 ($CHCl_3$-CH_3OH-CH_3COOH, 100:10:1 v/v).

(N-Fluorenylmethoxycarbonyl)-L-phenylalanine-1-^{13}C: Isolated 2.93g (81%) of a white micro-crystalline solid, m.p. 183-184° with a phase change at 156-158° (lit. 181-182°[1]) from 1.54g (9.3 mmol) of ^{13}C-labeled L-phenylalanine. TLC: R_f = 0.45 ($CHCl_3$-CH_3OH-CH_3COOH, 100:10:1 v/v).

Preparation of ^{18}O enriched FMOC-aminoacids.

^{18}O Enriched N-FMOC-L-Phenylalanine-1-^{13}C: FMOC-L-Phenylalanine-^{13}C (3.53 g, 9.0 mmol) was added to 25 mL of recovered 3:2 dioxane-water which was 0.1M in HCl and had an ^{18}O content of about 90%. The mixture was heated with stirring under reflux under nitrogen for 10 hr. Analysis by mass spectrometry showed an ^{18}O content of about 83%. The solvent was evaporated *in vacuo* and the solid dried a further 6 hr. The crude material was dissolved in 15 mL of hot dioxane (bath temp. 100-102°) and 96% ^{18}O enriched water (10 mL) which was 0.25M in HCl slowly added. Once the reaction mixture had equilibrated to the bath temperature it was a homogeneous solution. The solution was stirred under reflux under nitrogen for 10 hr. A drop was removed and evaporated to dryness and used for analysis by mass spec. The enrichment medium was recovered for reuse by a vacuum distillation at room temperature through a short column into a liquid nitrogen cooled trap. The solid residue was vacuum dried for several hours then crystallized from ethyl acetate - hexanes. Recovered 3.15 g (90%) of the enriched FMOC amino acid with an ^{18}O enrichment level of 95-96%., m.p. 183-185° with a phase change at 157-158°. TLC showed a single spot. $[\alpha]^{22}_D$ = - 38.6° (c,1 DMF) lit. $[\alpha]^{25}_D$ = -38.7 (c,1 DMF)[3].

^{18}O-Enriched N-FMOC-L-Alanine-1-^{13}C: Prepared from N-FMOC-L-alanine-1-^{13}C (5.8g, 18.6 mmol) in 28 mL of ~ 94% ^{18}O-enriched 1:1 dioxane-water. Only one equilibration step of 3 hr. was used. Recovered 5.44 g (94%) of material with an ^{18}O content of 87%, m.p. 152-153°.$[\alpha]^{22}_{D}$ = -18.5° (c,1 DMF) lit. $[\alpha]^{20}_{D}$ = -18.1°(c,1 DMF) lit.[13]

^{18}O-Enriched N-FMOC-L-Leucine-1-^{13}C: Prepared from N-FMOC-L-leucine-1-^{13}C (4.57g, 12.9 mmol) in 20 mlLof 3:2 dioxane-^{18}O water which was 0.1M in HCl. Equilibration was done in a two step process with a time of about 10-12 hr in each case. Recovered 4.24g (93%) of material with an ^{18}O content of 94%, m.p. 153-154°. $[\alpha]^{20}_{D}$ = -25.6° (c,1 DMF) lit. $[\alpha]^{20}_{D}$ = -25.8°[4].

^{18}O-Enriched N-FMOC-L-Isoleucine-1-^{13}C: Prepared from N-FMOC-L-isoleucine-1-^{13}C (2.35g, 6.6 mmol). The initial enrichment medium was 20 mL of 93-94% enriched 3:2 dioxane-water which was 0.1M in HCl. It required 34 hr to bring the ^{18}O content to 68%. Decomposition was becoming noticeable (TLC) so no attempt was made to reach a true equilibrium. The second equilibration was done in 15 mL of > 96% enriched 3:2 dioxane-water which was also 0.1M in HCl. The second equilibration time was 30 hr. Recovered 1.87g (80%) with an ^{18}O content of 90%, m.p. 146-147°. $[\alpha]^{21}_{D}$ = -11.9° (c,1 DMF), lit. $[\alpha]^{20}_{D}$ = -11.5° (c,1 DMF)[5].

^{18}O-Enriched N-FMOC-L-Valine-1-^{13}C: Prepared from N-FMOC-L-valine-1-^{13}C (2.04g, 6.0 mmol). The initial equilibration in 3:2 dioxane-water (0.1M in HCl) was allowed to proceed for about 20 hr and brought the ^{18}O level to about 77%. The second enrichment was done in 96% ^{18}O

enriched 3:2 dioxane-water for 20hr. Recovered 1.86g (91%) with an ^{18}O content of about 93%, m.p. 143-144°. $[\alpha]^{21}_D$ = -16.7° (c, 0.98 DMF), lit. $[\alpha]^{25}_D$ = -17.5° (c, 1 DMF)[1].

Preparation of Ala57 $^{13}C=^{18}O$ HP-36 and spectroscopy studies. label HP-36 with a single $^{13}C=^{18}O$ label at Ala-57 was prepared using standard Fmoc solid phase peptide synthesis protocols and purified using reverse phase HPLC as described in reference 6. HPLC buffers used HCL instead of TFA as the ion pairing agent since residual TFA can interfere with FTIR and is hard to remove. The identity of the purified protein was confirmed by MALDI Mass spectroscopy. FTIR experiments were performed at Los Alamos National Laboratory as described in reference 6. Briefly, experiments were conducted using a Bio-rad FTS-40A FTIR instrument equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The spectra were the rest of 256 scans recorded with a resolution of 2 cm^{-1}. The protein was exchanged in D_2O and the lyophilized sample dissolved in 10mM deuterated sodium phosphate pD 5.8. pD refers to the uncorrected (for D_2O) pH meter reading.

References

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