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Multiple-Turnover Isotopic Labeling of Fmoc- and Boc-Protected Amino Acids with Oxygen Isotopes

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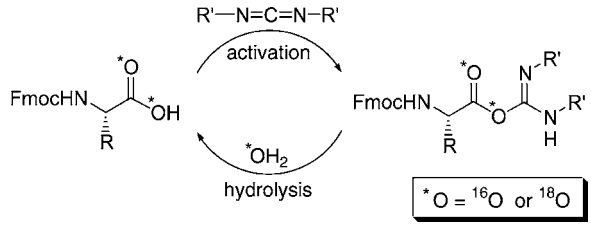
ABSTRACT



An efficient method for the selective isotopic labeling of carboxylic acids is reported. By reacting an amino acid with excess carbodiimide and $^{18}OH_2$, a kinetically enhanced multiple turnover reaction provides the ^{18}O-labeled product in high yield and excellent isotopic enrichment. This reaction is fully compatible with standard Fmoc, Boc, Trt, and O*t*Bu protecting groups and provides a means to selectively label the α-carboxylic acids of functionalized amino acids with stable oxygen isotopes.

Isotopically labeled amino acids have found wide application in structure elucidation of peptides and proteins. In addition to ^{17}O NMR studies,[1−5] 2D-IR techniques are emerging as important new tools for probing protein folding and ligand binding.[6−10] Due to its time resolution in the subpicosecond range, time-resolved 2D-IR spectroscopy can report the rapid evolution of nonequilibrium conformational states to reveal folding pathways.[7−10] These techniques rely upon site-specific incorporation of ^{18}O-labeled amino acids into peptides and proteins to serve as spectroscopic probes.[8−10] To date, only aliphatic, aromatic, and sulfur-containing amino acids have been utilized in such studies because currently

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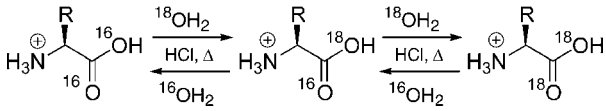
used methods for preparing ^{18}O-labeled amino acids result in uniform ^{18}O-labeling of side chain and α-carboxylic acids.[11−13]

^{18}O-labeling is normally conducted by heating the carboxylic acid with $^{18}OH_2$ in the presence of a strong acid to catalyze exchange (Scheme 1). By using an excess of ^{18}O

Scheme 1. Typical Equilibrium-Based ^{18}O Labeling Reaction

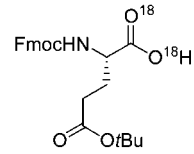
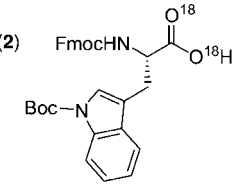
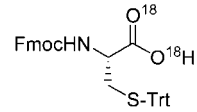


water, the reported yields for isotopic enrichments are typically 75−85%.[6,11−13] A labeling procedure for Fmoc-protected amino acids with multiple rounds of acid-catalyzed $^{18}OH_2$ equilibration recently reported ^{18}O enrichments of 90−96% for Phe, Ala, Gly, and Phe.[6] This method can be very efficient with respect to $^{18}OH_2$ consumption, but the protecting groups typically used for functionalized side chains (Boc, Trt, *t*Bu, etc.) are not stable under the strongly acidic conditions used to catalyze exchange. These conditions can therefore cause uniform labeling of Asp, Glu, Asn, and Gln residues.[13]

Single-turnover $^{17}OH_2$ saponification reactions of pentafluorophenyl esters have recently been reported.[4] These reactions tolerate acid labile (*t*Bu) protecting groups and can selectively label α-carboxylic acids with isotopic enrichments ranging from 20% to 85% for products containing a single ^{17}O atom.[4]

We are interested in developing mild and selective reactions for the isotopic labeling of amino acids, peptides, and proteins to provide highly enriched materials for NMR, IR, and MS studies. Here we report a nonequilibrium multiple-turnover reaction where a carboxylic acid is reacted with a carbodiimide to form an *O*-acylisourea that is hydrolyzed by ^{18}O water (abstract). After one round of activation and hydrolysis, the mixed ^{16}O/^{18}O carboxylic acid will be reactivated and hydrolyzed again and again (Scheme 2). Since carbodiimides react more rapidly with carboxylates than with water,[14] one-pot reactions containing an excess of coupling reagent and $^{18}OH_2$ result in multiple rounds of *O*-acylisourea formation and hydrolysis to provide isotopic enrichments of approximately 92−95% for both oxygen atoms of the carboxylic acid (Table 1).

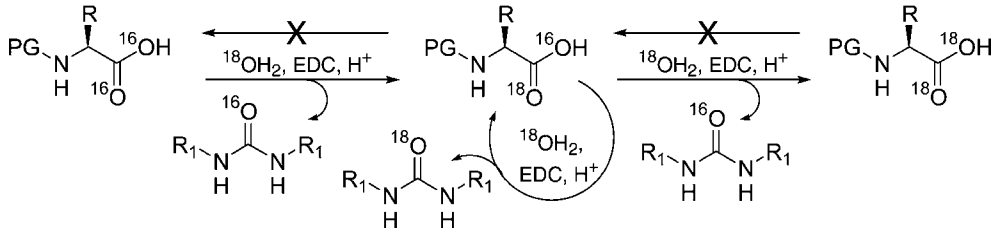
Table 1. ^{18}O-Enrichment and Isolated Yields[a]

entry	product	% enrichment[a]	isolated yields
(1)		93%	94%
(2)		92%	88%
(3)		95%	95%

[a] Total enrichment for both ^{18}O oxygen atoms is indicated.

While heavy atom isotope effects are typically considered negligible for preparative-scale reactions, a modest kinetic bias should favor formation of the double-labeled ^{18}O product. The ^{16}O atom in the mixed carboxylic acid (Scheme 2) should react faster than ^{18}O to form the corresponding ^{16}*O*-acylisourea.[15] The resulting effect on efficiency depends on the kinetic isotope effect (KIE) to the exponential power of reaction turnovers (*n*), i.e., $(KIE)^n$. In theory, a very small KIA of 1.010 would result in cumulative KIA's of about 11% and 270% after 10 and 100 turnovers, respectively. This should serve to decrease $^{18}OH_2$ consumption, especially for large-scale reactions. In the current set of examples only six turnovers/equivalents of $^{18}OH_2$ (if 100% enriched) are theoretically sufficient for making amino acids with 98% isotopic enrichment, even without taking kinetic isotope effects into account.

Scheme 2. Nonequilibrium, Multiple-Turnover ^{18}O Labeling Reaction



Our protocol for the selective labeling of α-carboxyl groups of amino acids utilizes commercially available, protected amino acids and coupling reagents.[16] To activate the carbodiimide and suppress racemization of the *O*-acylisourea intermediate, an excess of 3,5-dimethylpyridinium bromide ($pK_a \approx 5$) is included as a dry proton source.[16] We conducted isotope labeling experiments with three different Fmoc-protected amino acids containing a variety of acid-labile protecting groups (Table 1). In a typical reaction, 50 equiv of $^{18}OH_2$ (95% ^{18}O), 30 equiv of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and 20 equiv of 3,5-dimethylpyridinium bromide in dry DMF were reacted overnight at room temperature.[17] While other carbodiimides (DCC, DIC, etc.) worked well for this reaction, the water solubility of EDC greatly simplified purification as the resulting ^{16}O- and ^{18}O-containing ureas were washed away with water. Isolated yields for **1** and **3** were nearly quantitative (94−95%), while the yield for Fmoc-Trp(Boc)-^{18}OH was slightly lower (88%) due to the extreme acid sensitivity of the Boc-protected indole (Table 1). Each product **1**−**3** was characterized by MS, IR, NMR, and polarimetry.[18] Selective ^{18}O labeling of the α-carboxylic acids was observed by ^{13}C NMR by using mixtures of starting materials and isolated products. Consistent with previous reports of other carboxylic acids,[11,19] the ^{18}O-labeled α-carbon of products **1**−**3** were shifted upfield by ∼0.05 ppm as compared to ^{16}O-containing starting materials (see Figure S1 of the Supporting Information). Selective labeling was also indicated by mass spectrometry as exactly two ^{18}O atoms were incorporated into each product. Quantitative ESI mass spectrometry of **1**−**3** revealed total isotopic enrichments of 92−95% (Figure S2, Supporting Information). This enrichment is very similar to the purity of the $^{18}OH_2$ (95% ^{18}O) added to each reaction. On the basis of these results, we estimate that five or more cycles of activation and hydrolysis occurred during each reaction. Similar results were also obtained for relatively simple amino acids like Fmoc-Gly-^{18}OH. To the best of our knowledge, these are the first reported examples of kinetically enhanced multiple turnover reactions used for preparative isotopic labeling.

The C═O stretching frequency in IR spectroscopy is strongly influenced by the relative mass of each atom.[20] A shift of approximately -20 cm^{-1} is observed for ^{18}O═C stretching frequencies as compared to ^{16}O═C. It has been shown that 2D-IR experiments conducted in peptides and proteins are best resolved when using $^{13}C/^{18}O$ doubled labeled amino acids.[6,8−10] Given the vast selection of commercially available ^{13}C Fmoc-protected amino acids, our mild labeling strategy can now provide a wide variety of selectively labeled $^{18}O/^{17}O$ amino acids in a single step.

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Supporting Information Available: Detailed synthetic procedures and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) Starting materials were extensively dried and mixed in DMF under N_2. EDC was added in three equal portions over 40 h. See the Supporting Information for detailed procedures.
(17) Reactions containing 18 equiv of $^{18}OH_2$ and 10 equiv of carbodiimide furnished products with only 55% enrichment.
(18) No changes in optical rotation were observed upon labeling, indicating efficient suppression of racemization.
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