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Fast and Cost-Efficient ^{17}O-Isotopic Labeling of Carboxylic Groups in Biomolecules: From Free Amino Acids to Peptide Chains

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Abstract: ^{17}O NMR spectroscopy is a powerful technique, which can provide unique information regarding the structure and reactivity of biomolecules. However, the low natural abundance of ^{17}O (0.04%) generally requires working with enriched samples, which are not easily accessible. Here, we present simple, fast and cost-efficient ^{17}O-enrichment strategies for amino acids and peptides by using mechanochemistry. First, five unprotected amino acids were enriched under ambient conditions, consuming only microliter amounts of costly labeled water, and producing pure molecules with enrichment levels up to ~40%, yields ~60-85%, and no loss of optical purity. Subsequently, ^{17}O-enriched Fmoc/*t*Bu-protected amino acids were produced on a 1 g/day scale with high enrichment levels. Lastly, a site-selective ^{17}O-labeling of carboxylic functions in peptide side-chains was achieved for RGD and GRGDS peptides, with ~28% enrichment level. For all molecules, ^{17}O ssNMR spectra were recorded at 14.1 T in reasonable times, making this an important step forward for future NMR studies of biomolecules.

Introduction

Peptides and proteins are present in every cell and tissue of the human body. They are involved in numerous essential biological processes serving, for example, as messengers (peptide hormones such as insulin), structure-forming agents (e.g. collagen, fibrin) or as immunity defense lines in the form of antibodies (immunoglobulins). Their biological functions are determined by a specific sequence of amino acids (AA) interconnected via peptide bonds and by the 2D and 3D structure of the long protein chains.[1] A lot of effort has thus been invested in exploring their structure and better understanding their reactivity, as a crucial prerequisite for elucidating biological processes, and for developing drugs or biomimetic materials.[2,3]

A possible way to address some of the key questions regarding the structure and functional properties of peptides and proteins, is to prepare them in an isotopically labeled version, starting from amino acids labeled in stable isotopes, such as ^{2}H, ^{13}C, ^{15}N, or ^{17}O/^{18}O.[4] Such labeled species have been involved in many studies where both qualitative and/or quantitative information were extracted from MS,[5] IR[6] or NMR analyses.[7] Producing peptides/proteins labeled in ^{17}O/^{18}O is particularly interesting, with oxygen atoms being frequently involved in hydrogen bonds stabilizing complex macromolecular protein structures. ^{18}O-labeled proteins have been used in high-resolution FTIR (Fourier transform infrared) studies providing side-specific structural information and/or allowing to follow rapid structural changes and protein dynamics,[6b,8] or as MS standards for quantitative proteomics.[5b,9] For such applications in IR and MS, it is required to work with almost quantitatively ^{18}O-labeled products (with ^{18}O-enrichment level $> 90\%$).

The great potential of ^{17}O-labeled products can be exploited through high-resolution solution and solid-state NMR studies.[10] Indeed, multinuclear NMR spectroscopy has proven to be a versatile analytical technique capable of providing information on the structure, function and dynamics of peptides and proteins by probing the local environment of a given nucleus, typically ^{1}H, ^{13}C or ^{15}N.[11] In this context, ^{17}O NMR spectroscopy appears as particularly advantageous, due to the very high sensitivity of ^{17}O NMR parameters to the oxygen local environment, with chemical shifts spanning over a ~1000 ppm range.[7b,12] For example, a transmembrane protein WALP23 containing ^{17}O-labeled alanine was characterized by ^{17}O NMR (at 14.1 and 18.8 T) and the $C=O$ bond lengths at the labeled sites were estimated from these analyses.[13] In a more recent study of ion channel polypeptide Gramicidine A, high-resolution ^{17}O NMR experiments performed at 35.2 T enabled to detect

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selective hydrogen bonds between protons belonging to a "water wire" and isotopically labeled carbonyl oxygens of glycine and leucine lining the channel pore.[14] Furthermore, in terms of larger biomolecules, a recombinant protein, yeast ubiquitin (made of 76-residues), was prepared via expression with *E.coli* and analyzed by ^{17}O NMR spectroscopy, including measurements at 35.2 T.[15] Both side-chain signals and carbonyl backbone signals were identified in the solution and solid-state ^{17}O NMR spectra, respectively, and parameters were extracted for each oxygen site.

Compared to the other NMR active nuclei, ^{17}O NMR studies are still rare due to the constraints associated with performing ^{17}O NMR analyses. This is mainly because of the very low natural abundance of the only NMR active oxygen isotope (0.04%), which requires working with ^{17}O-enriched biomolecules to obtain high-resolution data with sufficient sensitivity. In contrast with the applications of ^{18}O-labeled products, the ^{17}O-enrichment levels of the amino acids within peptides are typically in the 15–60% range, which is sufficient for performing 1D and 2D ^{17}O NMR experiments at high magnetic fields. For most peptides involved in high-resolution NMR analyses, the ^{17}O-label was introduced in the sequence during a solid-phase peptide synthesis (SPPS) using ^{17}O-labeled fluorenylmethyloxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) orthogonally protected amino acids as building blocks. This approach enables to label selectively only the chosen amino acid positions. In contrast, in the recently reported study of ubiquitin, the recombinant protein expression method employed (using free, unprotected amino acids, labeled in ^{17}O), resulted in labeling of all the glycine, phenylalanine or tyrosine units within the protein.[15]

With only two ^{17}O-enriched *free* amino acids currently available on the market (L-glutamic acid and L-tyrosine enriched on the phenolic group,[16] the laboratory synthesis of ^{17}O-labeled *free* and/or Fmoc/*t*Bu-*protected* amino acids is inevitable for nearly all studies of ^{17}O-enriched peptides and proteins. Nevertheless, their preparation is not straightforward. Indeed, most oxygen labeling schemes available in the literature are developed and applied for ^{18}O-enrichment using a large excess of ^{18}O-enriched water. However, due to the considerably higher cost of ^{17}O-enriched water (1 mL of 90% ^{17}O-enriched H_2O^* costs 1800–2900 € compared to 60–150 € for 1 mL of $>97\%$ ^{18}O-enriched H_2O^*), the application of these protocols for ^{17}O-enrichment would be prohibitively expensive. In a typical labeling procedure, the ^{18}O-enrichment of *free* amino acids is based on acid-catalyzed oxygen exchange between the amino acid and isotopically enriched water (introduced in excess, typically ~30–100 equiv) leading to highly enriched molecules (with enrichment level $\geq 90\%$, using 95–98% $H_2{}^{18}O$).[17] In addition to using a large excess of enriched water, this labeling scheme suffers from other drawbacks, such as very long reaction times (up to several days) at elevated temperatures ($>$ 60 °C), and the necessity to use a strong acid for catalysis, which is incompatible with some functional groups. In many cases, toxic and corrosive HCl gas is bubbled through the reaction medium for this purpose, which adds experimental constraints regarding the safety of the labeling process. Furthermore, most of the time, the *free* amino acids thus enriched are isolated under their hydrochloride salt form, and an additional anion-exchange procedure is then required to provide the pure zwitterionic form of the labeled product.[15] Therefore, it is desirable to develop more cost-efficient and user-friendly procedures for ^{17}O-enrichment of *free* amino acids.

Regarding the synthesis of enriched Fmoc-*protected* amino acids (Fmoc-AA-OH), acid-catalyzed oxygen isotope exchange schemes, like the one described above, have been typically employed, providing the labeled species in one-step starting from non-labeled Fmoc-AA-OH.[8c,18] However, these protocols are only applicable to residues that do not require the presence of acid-labile *t*Bu-based side-chain protection groups for subsequent SPPS, thereby limiting the scope of accessible ^{17}O/^{18}O-labeled protected amino acids. More problematically, a large excess of enriched water is generally employed, making these procedures expensive. Concerning the ^{17}O/^{18}O-enriched Fmoc/*t*Bu *orthogonally protected* amino acids, their preparation is more challenging, due to the simultaneous presence of the Fmoc-group protecting the α-NH_2 functions that is labile under basic conditions, and the side-chain protection group (*t*Bu) that is labile under strongly acidic conditions. Therefore, the development of alternative labeling schemes working under more mild reaction conditions has been a focus of attention.[19]

For both *free* and Fmoc-*protected* amino acids, another important caveat is that for many of the protocols reported, the synthetic procedures are not properly described, missing some important details, such as the number of water equivalents, the work-up conditions, isolated yield, enrichment level, or the purity of the isolated enriched compounds. This makes it difficult for other researchers to use or adapt the labeling schemes for their needs. The development of cost-efficient, reliable, broadly applicable, and detailed labeling schemes is therefore of outmost importance.

In order to facilitate the access to ^{17}O-labeled amino acids and peptides, we recently turned our attention to mechanochemistry, a rapidly developing green synthetic methodology with increasing numbers of applications in organic synthesis.[20] The mechanochemical reactions are performed in reaction vessels (jars) containing reagents and milling beads vigorously agitated by a ball-mill, without needing a bulk solvent. During ball-milling, the mechanical energy is transferred to the reagents by impact and shear forces, which most often result in a highly efficient mixing of the reagents, a decrease of solid particles' size, and the creation of fresh surfaces for subsequent reaction.[21] The lack of solvent presence has a beneficial impact not only on decreasing the solvent waste generated, but also on the reaction time which can be significantly reduced due to the high "concentration" of the reaction medium.[22] In some cases, the beneficial effect of adding a small amount of liquid (less than 1 μL per 1 mg of substrates) was observed.[23] This is referred to as "liquid-assisted grinding" (LAG).[24] In our previous studies, we have shown that ^{17}O-enriched water can be used for LAG, by playing the role of both a "liquid assistant" and a ^{17}O-labeled reagent at the same time, producing labeled carboxylic acid-containing molecules (including fatty acids) via either a two-step CDI-activation/hydrolysis protocol or a one-pot saponification reaction.[25] It is both the rapidity and the minimal

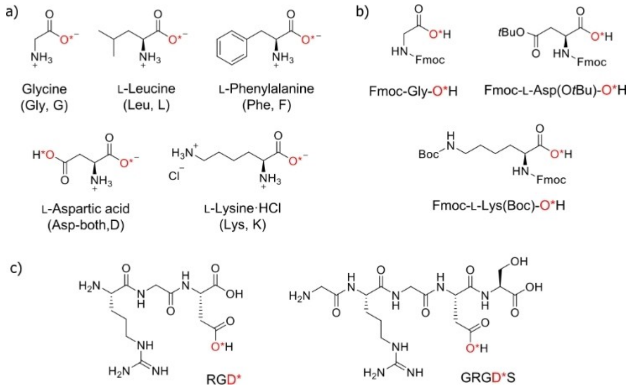


Figure 1. Scope of ^{17}O-labeled molecules prepared in this work: a) *free* amino acids, b) Fmoc/*t*Bu-*protected* amino acids and c) peptides. Only one oxygen is labeled per carboxylic function using mechanochemistry, but globally, the label is distributed between both oxygen sites in the isolated phases (e.g. C=O* and C–O*H, 50:50), as evident from ^{17}O solid state NMR analyses.

consumption of ^{17}O-enriched water that makes the use of ball-milling advantageous compared to the traditional solution-based labeling schemes.

In this work, we present at first how ^{17}O-enriched *free* amino acids could be obtained easily in a highly efficient manner using mechanochemical saponification, consuming only microliter amounts of labeled water. The versatility of this approach is demonstrated in the enrichment of diverse amino acids selected based on their different physicochemical properties and reactivity, related to their respective side-chains. In particular, we focused on: Glycine (Gly, G), as the simplest and the most studied amino acid; L-Leucine (Leu, L) and L-Phenylalanine (Phe, F) as representatives of amino acids with non-polar side-chains; L-Aspartic acid (Asp, D) with an additional carboxylic group in the side-chain; and finally L-Lysine (Lys, K) having a basic side-chain (Figure 1a). This study will show how the labeled amino acids could be isolated in their zwitterionic forms reproducibly with high enrichment levels and enantiomeric excesses. Comparison to procedures adapted from the previously reported acid-catalyzed oxygen exchange will also be proposed.

Subsequently, a two-step protocol for synthesizing ^{17}O-enriched Fmoc/*t*Bu *orthogonally protected* amino acids will be introduced, combining mechanochemistry with classical solution Fmoc-protection synthesis, and leading to highly enriched, essentially pure Fmoc-AA-O*H (Figure 1b). Furthermore, for the first time, the application of mechanochemical saponification for the site-selective ^{17}O-enrichment of a whole peptide chain will be described, in the case of two short peptides widely encountered in biological systems, namely RGD and GRGDS (Figure 1c).[2b] Following the synthetic part, ^{17}O solid-state NMR spectra of thus ^{17}O-enriched biomolecules will be presented and discussed.

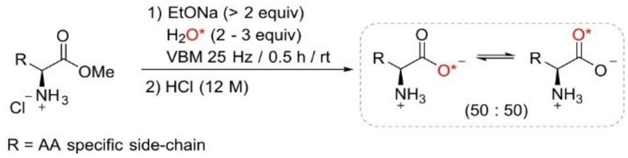
Results and Discussion

Synthesis of ^{17}O-enriched free amino acids by mechanochemistry

The ^{17}O-enriched *free* amino acids were prepared by a mechanochemical saponification route (Scheme 1), as developed previously in our laboratory for the ^{17}O/^{18}O-enrichment of fatty acids.[25c] Here, the saponification reactions were performed using a vibratory ball-mill (VBM) Retsch MM400 equipped with a stainless-steel jar (10 mL inner volume) containing two stainless-steel beads (1 cm diameter), and the following reagents: an amino acid methyl ester hydrochloride salt (H-AA-OMe·HCl, 1 equiv), sodium ethoxide (NaOEt, 2.5–3.5 equiv), and labeled water (H_2O^*, 2–3 equiv. per carboxylic group to be labeled) (Scheme 1). After 30 min of milling at 25 Hz under ambient conditions, complete conversion was confirmed by ATR-IR analyses by looking at the shift of carbonyl stretching vibration band (from ~1740 to ~1560 cm^{-1}, see Supporting Information).

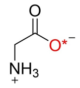
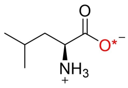
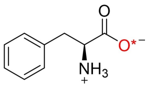
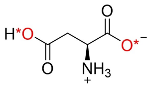
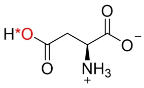
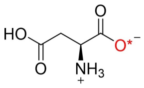
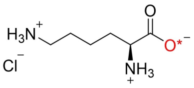
This one-pot synthesis was performed with minimal excess of reagents (especially H_2O^*) with respect to the amino acid substrate. The excess of NaOEt (> 2 equiv) was required due to the substrate form (used here as a hydrochloride salt), hence, one NaOEt equivalent was consumed simply by reacting with the hydrochloride. Enriched hydroxide anions were formed in situ by an acid-base reaction between H_2O^* (2-3 equiv excess per ester-derivatized carboxylic function) and the remaining NaOEt, and enabled to hydrolyze the ester function in the substrate producing the labeled molecule (in a carboxylate form), together with small amounts of ethanol and methanol (side-products, easy to eliminate by evaporation). The reaction mixture was recovered from the jar using minimal amounts of non-labeled water, and the pH was adjusted accordingly to obtain the labeled amino acids in their zwitterionic form (except for Lys, which was obtained as the hydrochloride salt, see Table 1). The work-up procedures were optimized to isolate each of the labeled species free of the NaCl side-product, while maintaining the highest yields possible in a reproducible manner. No loss of enrichment was observed upon the work-up conditions applied. Their detailed descriptions can be found in the Supporting Information. For each amino acid, the labeling schemes were first optimized using ^{18}O-enriched water due to its lower cost, before producing the ^{17}O-labeled molecules.

The synthetic and enrichment yields, as well as the enrichment levels and enantiomeric excesses for each free amino acid ^{17}O/^{18}O-enriched by this mechanochemical saponification pro-



Scheme 1. Mechanochemical saponification procedure used to enrich free unprotected amino acids.

Table 1. Summary of results for ^{17}O/^{18}O-labeled *free* amino acids prepared by using mechanochemical saponification.

Enriched amino acid		Low scale[a] Mass [mg]/ Yield [%]	Scale-up[b] Mass [mg]/ Yield [%]	EY[c] [%]	^{18}O-EL[c] [%]	^{17}O-EL[c] [%]	Enantiomeric excess (*ee*) [%]
	Gly (G)	58 ± 3 64 ± 3	133 ± 3 73 ± 1	89 ± 2[d]	44 ± 1[d]	41[d]	–
	Leu (L)	86 ± 3 78 ± 3	N. P.	82 ± 1	41 ± 1	29	> 99
	Phe (F)	71 ± 3 61 ± 2	N. P.	92 ± 2	46 ± 1	32	> 99
	Asp-both[e] (D)	80 ± 2 76 ± 1	174 ± 5 84 ± 3	88 ± 1	44 ± 1	31	> 99
	Asp-side[e] (D)	77 ± 7 70 ± 6	N. P.	85 ± 1	42	31	N. D.
	Asp-main[e] (D)	113 ± 12 83 ± 11	N. P.	95 ± 1	47	34	N. D.
	Lys·HCl[f] (K)	88 ± 5 62 ± 4	221 ± 6 78 ± 2	90 ± 2	46 ± 1	31	> 99

[a] Low scale corresponds to a reaction starting from 150 mg of H-AA-OMe·xHCl substrate. [b] Scale-up corresponds to a reaction starting from 300 mg of H-AA-OMe·xHCl. [c] EY corresponds to ^{17}O/^{18}O-enrichment yield, ^{18}O-EL and ^{17}O-EL correspond to the enrichment level of oxygen-18 and oxygen-17 isotopes per carboxylic oxygen using 99 % ^{18}O-enriched or 70 % ^{17}O-enriched water, respectively, and were calculated from HRMS analyses. [d] EL estimated from measurement of protected Fmoc-Gly-O*H,[26] 90 % ^{17}O-enriched water was used here for the ^{17}O-labeling. [e] Asp-both, Asp-side and Asp-main correspond to L-Asp labeled either on both carboxylic functions, or only on the side-chain or main-chain. [f] L-Lysine isolated as monohydrochloride dihydrate. Amino acids were enriched using 2 or 3 equiv. excess of ^{17}O/^{18}O-enriched H_2O^* per ester-derivatized carboxylic function. The synthetic and enrichment yields correspond to the average values of both ^{18}O and ^{17}O-enriched products (when available), the enrichment levels correspond to the products produced at the low-scale. Error bars are calculated from repeated experiments (n = 2–4); further details on error bars can be found in Supporting Information (section I-D). One labeled oxygen is introduced per carboxylic function, but globally, the label is distributed between both oxygen sites in the isolated phase (e.g. C=O* and C–O*H). N. P. = Not Performed, N. D. = Not Determined.

cedure are summarized in Table 1. Here, as a starting point, the initial mass of amino acid methyl ester reagents was set to 150 mg (0.6–1.2 mmol), referred to as a "low scale" in this text. With this scale, ~60–110 mg of pure oxygen labeled free amino acids could be produced in one reaction while consuming only ~25–55 μL of labeled H_2O^*. The corresponding isolated yield varied from around 60 % for the highly water-soluble amino acids (Gly, Lys), to approximately 80 % for the less soluble molecules (Asp, Leu) (see Table 1). An increase in isolated yield of ~11 % was observed, on average, when the reaction was scaled-up, producing up to ~200 mg of labeled amino acid in a single run. The purity of labeled compounds was confirmed by ^{1}H and ^{13}C solution NMR and powder XRD analyses. Moreover, the enantiomeric excess of all optically active enriched amino acids was verified by chiral HPLC analysis of their Fmoc-protected derivatives, and no loss of enantiomeric excess upon the mechanochemical saponification was observed (see the Supporting Information).

Regarding the labeling efficiency of the proposed protocols, the enrichment yield of the labeling scheme (EY), and average enrichment level per carboxylic oxygen (EL), were calculated from high-resolution mass spectrometry analyses (HRMS) of isolated products (see Supporting Information for calculation details). Due to the reaction mechanism of saponification, only one labeled oxygen can be introduced per carboxylic function, which results in maximal theoretical average enrichment level per carboxylic function ≤50 %. For the mechanochemical saponification, the EY was typically around 90 % using only a small excess of labeled water (2–3 equiv. per carboxylic function), which enabled to obtain the ^{17}O-labeled amino acids with high EL (29–34 % using 70 % ^{17}O-enriched H_2O^*, see Table 1). This allowed ^{17}O solid-state NMR spectra to be recorded with good signal-to-noise ratios in short times (details provided later in this text and Supporting Information). In the case of L-aspartic acid (Asp), which contains two carboxylic functions, the site-selective labeling could also be achieved, producing Asp enriched either on both carboxylic sites or only on one, by varying the substrate used for the saponification (dimethyl ester or a specific mono-methyl ester derivative, respectively).

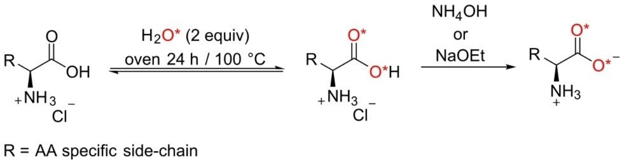
Up to date, only a few examples of ^{17}O-labeling schemes based on saponification have been reported in the literature.[17c,27] These saponification reactions were performed in solution on the Boc-protected amino acid esters and also *N*-unprotected esters. In general, only a small excess of labeled water (2–5 equiv) was used, and the ester function was hydrolyzed in the presence of sodium hydroxide within several hours of stirring at room temperature (up to 12 h). However, the isolated yields were typically not reported, and an additional Boc-removal step was required to access the *free* labeled amino acids, thereby prolonging the overall reaction time.[17c,27b] Here in this work, mechanochemistry enables to access highly enriched molecules (up to 41 % in ^{17}O here), under ambient conditions, rapidly (30 min reaction time, followed by work-up), without a need for aggressive reagents, and consuming only microliter quantities of costly labeled water (~25–55 μL per reaction at the low scale).

Synthesis of ^{18}O-enriched free amino acids by revisiting acid-catalyzed oxygen exchange procedures

To evaluate the efficiency of the mechanochemical saponification approach, a comparison was made with an acid-catalyzed oxygen exchange enrichment procedure, which, as mentioned previously, is the one used the most in the literature for enriching amino acids. More specifically, we adapted a procedure reported by Mears in 1938 for producing ^{18}O-enriched glycine, who had started from the glycine hydrochloride salt (Gly·HCl) and had heated it in the presence of ^{18}O-enriched H_2O^* for 24 h (Scheme 2).[28] The use of Gly·HCl salt as a substrate is indeed particularly interesting, as it avoids having to produce and engage HCl gas, which is experimentally constraining, or having to add aqueous HCl to the amino acid, which would reduce the labeling efficiency due to the presence of additional non-labeled water.

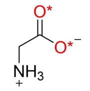
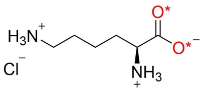
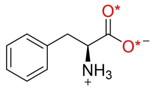
Here, for comparison purposes, the molar amounts and stoichiometry of reagents in the acid-catalyzed oxygen exchange procedure of glycine were adjusted to be identical to the low scale conditions used for the mechanochemical saponification, and the work-up conditions were likewise adapted to provide the same forms of labeled products. All tests were performed here using ^{18}O-enriched water only. The reaction was carried out in a vial containing the Gly·HCl substrate and ^{18}O-enriched H_2O^* (2 equiv), which was closed with a screw cap and placed in an oven heated at 100 °C for 24 h (i.e. using the same heating time as initially reported by Mears).[28] The results are presented in Table 2. The EL achieved for Gly was estimated to be around 49 %,[26] which is slightly higher compared to the EL achieved using saponification (44 %), and corresponds to almost quantitative conversion (the maximum EL expected was ~50 %, see Supporting Information for calculation details). To further approach the experimental times used in the mechanochemical saponification, the heating duration was reduced to 2 h. Under these conditions, the conversion determined in the isolated phase was already at 91 %, which corresponds to an enrichment level of 46 %, and suggests that the kinetics of the oxygen isotopic exchange are, in fact, very fast for this substrate under the conditions applied.

Due to the simplicity of this labeling scheme for glycine, its extension for oxygen enrichment of other amino acids commercially available as HCl salts, namely L-lysine and L-phenylalanine, was further investigated. First, in the case of L-lysine (Lys), Lys·2HCl was used as a substrate as no enrichment was observed when starting from the monohydrochloride salt. Using 2 equiv. of H_2O^*, a 45 % average enrichment level per carboxylic oxygen was determined after 24 h of heating at 100 °C, which is equivalent to what could be reached by VBM saponification (46 %) within 30 min of milling at room temperature. Just like for glycine, when the reaction time was reduced to 2 h, only a small decrease in the final enrichment level was detected (42 %) (see Table 2). Second, in the case of L-phenyl-



Scheme 2. Acid-catalyzed oxygen exchange procedure used for the enrichment of *free* amino acids. Both oxygen atoms per carboxylic function can become labeled by this procedure.

Table 2. Summary of results for ^{18}O-labeled *free* amino acids prepared via acid-catalyzed oxygen exchange.

Enriched amino acid	Reaction time [h]	EY[a] [%]	^{18}O-EL[a] [%]	Enantiomeric excess (*ee*) [%]
 Gly (G)	2	91 ± 3[b]	46 ± 1[b]	–
	24	97 ± 1[b]	49 ± 1[b]	–
 Lys·HCl (K)	2	84 ± 3	42 ± 1	N. D.
	24	90 ± 1	45 ± 1	>99
 Phe (F)	24	77 ± 5	39 ± 2	N. D.

[a] EY corresponds to ^{18}O-enrichment yield, ^{18}O-EL corresponds to enrichment level of oxygen-18 using 99 % ^{18}O-enriched water, calculated from HRMS analyses. [b] EL estimated from measurement of protected Fmoc-Gly-O*H.[26] All amino acids were enriched using 2 equiv. of ^{18}O-enriched H_2O^* per carboxylic function. Error bars are calculated from repeated experiments (n = 2); further details on error bars can be found in Supporting Information (section I-D). N. D. = Not Determined. Only lysine was isolated here as HCl salt (dihydrate form) after the work-up.

alanine hydrochloride (Phe·HCl), a first study of its reactivity towards oxygen exchange was performed (Table 2). Despite the lower solubility of the Phe·HCl substrate in water, which remained substantially non-dissolved after 24 h of heating at 100 °C (compared to Gly·HCl and Lys·2HCl, which were completely dissolved), an average 39% enrichment level was achieved. Overall, these results suggest that this labeling scheme, starting from HCl salts, may be applicable to other amino acid hydrochlorides.

As not all amino acids are commercially available as HCl salts (notably aspartic acid), an alternative related approach was also tested as part of this work. Here, the enrichment of ʟ-Asp was performed by adding to the free amino acid LiCl, to catalyze the oxygen isotopic exchange using Li^+ as Lewis acid. After 24 h of heating at 100 °C in the presence of 4 equiv of ^{18}O-enriched H_2O* (i.e. 2 equiv. per carboxylic function), and 1 equiv of LiCl, a minor enrichment level of around 5% was detected in the isolated product. Reaction conditions were not further optimized at this stage. Yet, most importantly, it is worth highlighting that no enrichment was observed when the same reaction was performed in the absence of LiCl. As far as we know, this test is the first application of Lewis acid catalysis for oxygen isotope exchange of carboxylic groups in amino acids, which could offer an interesting alternative for some substrates.

Overall, the acid-catalyzed isotopic exchange presented here enabled obtaining ^{18}O-enriched amino acids easily without the need for toxic and corrosive HCl gas. Moreover, the reaction time could be significantly reduced to only 2 h (compared to the initially reported 24 h),[28] still providing highly enriched molecules (up to 46%, using 99% ^{18}O-enriched H_2O*, and engaging 2 equiv. of enriched water). Therefore, this can be seen as an attractive cost-efficient alternative to the VBM saponification. Moreover, if needed, higher enrichment levels could be reached in these acid-catalyzed schemes, by simply adding more than 2 equiv. of enriched water (as shown for glycine in Supporting Information, section III–A). On the other hand, the weakness of this approach lies in the commercial availability of starting hydrochloride salts, which are not as easily accessible as the amino acid esters used for saponification. Furthermore, unlike in the VBM approach, the acid-catalyzed oxygen exchange protocol does not allow straightforward site-selective labeling of amino acids containing additional carboxylic groups in their side-chains (e.g. aspartic or glutamic acid).

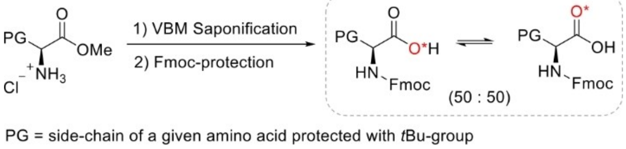
Synthesis of ^{17}O/^{18}O-enriched Fmoc-protected amino acids

In contrast to the recombinant protein synthesis, which uses ^{17}O/^{18}O-labeled *free* amino acids, SPPS enables to selectively enrich only the chosen carbonyl positions within a peptide chain using ^{17}O/^{18}O-labeled Fmoc/*t*Bu *orthogonally protected* amino acids as building blocks. This approach is particularly convenient for subsequent ^{17}O NMR studies where direct information from the specific oxygen environment can be obtained. Since none of the oxygen labeled *protected* amino acids is commercially available (to the best of our knowledge), their in-house preparation is an indispensable prerequisite.

In this work, three Fmoc-protected amino acids were enriched in ^{17}O (Figure 1b): the Fmoc-Gly-O*H molecule with no side-chain protection group, and the two orthogonally protected molecules Fmoc-ʟ-Asp(O*t*Bu)-O*H and Fmoc-ʟ-Lys(Boc)-O*H. All were obtained in two steps combining mechanochemistry (used for enrichment) with classical solution-based chemistry (used for Fmoc-protection) (Scheme 3). For each substrate, the reaction conditions were first optimized using ^{18}O-enriched water due to its lower purchasing price before producing the ^{17}O-labeled equivalents. In the first step, the amino acids were enriched by mechanochemical saponification starting from their methyl ester derivatives (as described in the previous section). However, in order to increase the overall isolated yield, the labeled amino acid intermediates were not isolated. Instead, the reaction medium after saponification was dissolved in non-labeled water, and recovered from the jar. After a minor pH adjustment, the subsequent Fmoc-protection step was directly performed in solution following published procedures (for synthetic details, see Supporting Information). No loss of enrichment was observed upon the Fmoc-protection steps. Notably, the *tert*-butyl group at the β-carboxylate of Asp as well as the *tert*-butyloxycarbonyl (Boc) protection group at the side-chain of Lys were found to be stable under the basic VBM-saponification conditions when using exactly 2 equiv of NaOEt, with only limited side-chain deprotection observed in the case of Asp.

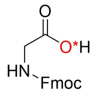
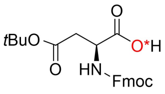
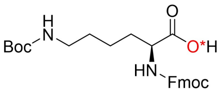
Yields and enrichment levels of ^{17}O/^{18}O-enriched Fmoc/*t*Bu-*protected* amino acids are reported in Table 3. Because SPPS synthesis is generally more efficient when using large excesses of Fmoc-protected amino acids, the production of Fmoc-Gly-O*H and Fmoc-ʟ-Asp(O*t*Bu)-O*H was also scaled-up as part of this work, providing up to ~1 g of labeled products within 1 day (work-up included).[29] Importantly, the final products were isolated essentially pure, in high yields, and with high enrichment levels (up to ~40% using 90% ^{17}O-enriched water).

The two-step procedure described above was then compared to the protocols previously reported in the literature for the ^{17}O-enrichment of Fmoc/*t*Bu-orthogonally protected amino acids. To our knowledge, only two methods working under mild conditions compatible with the presence of side-chain protection groups have been described so far.[19a,b] In the work of Yamada et al.,[19b,d] Fmoc/*t*Bu protected amino acids were enriched via hydrolysis of their pentafluorophenyl esters at room temperature and nearly neutral pH, using a small excess of ^{17}O-labeled H_2O* (5 equiv.), but with long reaction times (up



Scheme 3. Two-step procedure used for the synthesis of ^{17}O/^{18}O-enriched Fmoc/*t*Bu-*protected* amino acids.

Table 3. Summary of results for ^{17}O/^{18}O-labeled Fmoc/*t*Bu-*protected* amino acids.

Enriched Fmoc/*t*Bu-protected amino acid	Low scale[a] Mass [mg]/ Yield [%]	Scale-up[b] Mass [mg]/ Yield [%]	^{18}O-EL[c] [%]	^{17}O-EL[c] [%]
 Fmoc-Gly-O*H	225 ± 13 75 ± 4	496 ± 7 83 ± 1	43 ± 1	38 ± 1
 Fmoc-L-Asp(O*t*Bu)-O*H	165 ± 7 71 ± 3	342 ± 3 74 ± 1	43 ± 2	40 ± 1[d]
 Fmoc-L-Lys(Boc)-O*H	180 ± 24 76 ± 10	N. P.	46 ± 1	32[e]

[a] Low scale corresponds to a reaction starting from 150 mg of H-AA-OMe·xHCl substrate. [b] Scale-up corresponds to a reaction starting from ~300 mg of H-AA-OMe·xHCl. [c] ^{18}O-EL and ^{17}O-EL correspond to enrichment level of oxygen-18 and oxygen-17 isotopes per carboxylic oxygen, using 99% ^{18}O-enriched or 90% ^{17}O-enriched water, calculated from HRMS analyses. [d] ^{17}O-EL of the products prepared under scaled-up conditions. [e] 70% ^{17}O-enriched water was used. The synthetic yields correspond to the average values of both ^{18}O and ^{17}O-enriched products (when available), the enrichment levels correspond to the products produced at the low-scale. Error bars are calculated from repeated experiments (n = 2-5); further details on error bars can be found in Supporting Information (section I–D). One labeled oxygen is introduced per carboxylic function, but globally, the labeled is distributed between both oxygen sites in the isolated phase (i.e. C=O* and C–O*H). N. P. = Not Performed.

to several days). In another approach developed by Seyfried et al.,[19a] the protected amino acids were enriched based on multiple turnover in situ activation/hydrolysis cycles in the presence of coupling reagent (EDC·HCl) and ^{18}O-labeled H_2O*. The final molecules were isolated with high enrichment levels (up to 95% using 95% ^{18}O-enriched H_2O*) but to the expense of using a large excess of labeled water (50 equiv) and long reaction times (up to 40 h).[19a] Recently, this method was adapted and optimized to be suitable for ^{17}O-enrichment.[19c,30] The reaction was performed within 8 h, and the labeled Fmoc-AA-O*H products were isolated with enrichment level around 40% (using 40% ^{17}O-enriched H_2O*), which is sufficient for most high-resolution ^{17}O NMR studies.[19c,30a] Nevertheless, despite the optimizations, the excess of ^{17}O-enriched water involved remained rather high (30 equiv). Moreover, neither the synthetic yields nor the purity of isolated ^{17}O-labeled Fmoc-AA-O*H were reported.

Here, our methodology provides ^{17}O-enriched Fmoc-protected products (up to ~40% enrichment here) in high isolated yields (up to ~85%) in just one day, consuming only 2–3 equiv. of ^{17}O-enriched water per amino acid. As a result, the labeling scheme presented is very efficient in terms of the labeling cost. In cases when up to 1 g quantities of Fmoc-protected amino acid were produced within 1 day, the overall water consumption per 100 mg of *isolated product* was only ~18 μL (90% ^{17}O-enriched H_2O*). This corresponds to a labeling cost of 50 €/100 mg of *isolated* ^{17}O-labeled Fmoc-AA-O*H with ~40% ^{17}O-enrichment level (see Supporting Information for full details on the calculation of the enrichment cost).[31] Compared to the very recent work from Ha et al.,[30a] where the labeling cost was calculated for 100 mg of *starting Fmoc-AA-OH* based on kinetics experiments,[32] a different approach for calculating the labeling cost was reported here, which, we believe, brings a more straightforward and informative estimation for other researchers interested in ^{17}O-enrichment of biomolecules.

Site-selective ^{17}O-labeling of peptide side-chains using mechanochemistry

Using ^{17}O-enriched Fmoc/*t*Bu-*protected* amino acids (like those prepared as described in the previous section), it is possible to produce ^{17}O-labeled peptides by SPPS. When starting from the precursors reported in Table 3, only the carbonyl oxygen in the peptide back-bone chain will be labeled. As the ^{17}O-labeling of peptide side-chains is also important to gain information on their three-dimensional structure and/or reactivity, we looked into the possibility of enriching the β-carboxylic function in aspartic acid side-chain by performing the mechanochemical saponification on a whole peptide chain. As model substrates, a short peptide sequence Arginyl-glycyl-aspartic acid (Arg-Gly-Asp, RGD) and its longer derivative Gly-Arg-Gly-Asp-Ser (GRGDS) were selected. The RGD sequence serves indeed as a key structural recognition motif for cell transmembrane protein receptors (integrins), which facilitate cell-cell and cell-extracellular matrix adhesion,[33] and the RGD-related peptides are involved in many biological applications (such as drug delivery systems or coatings of implants).[34] Moreover, both peptides are commercially available, which highlights their general interest for the research community.

Here, the RGD tri-peptide and GRGDS penta-peptide were site-selectively ^{17}O-labeled at the β-carboxylic group of the aspartic acid unit, by mechanochemical saponification of the corresponding peptides containing an aspartic acid side-chain initially protected with methyl ester. The latter were prepared by SPPS as part of this study, with each synthetic and purification step carefully selected and carried out, as detailed in Supporting Information, to be able to isolate a sufficient amount of pure compound. The mechanochemical saponification was then performed on 8 mg of the methyl ester protected peptides in a stainless-steel jar (1.5 mL inner volume) containing two stainless steel beads (5 mm diameter) in the presence of NaOEt (3.5 equiv) and ^{17}O-labeled water (7 equiv, 90% in ^{17}O) (Scheme 4). After 15 min of milling at 25 Hz, the content of the jar was recovered using non-labeled water and then acidified to pH ~3. Finally, ^{17}O-labeled peptides were isolated in a mixture with NaCl side-product via lyophilization (~15 mg of a peptide-NaCl mixture), and characterized by LCMS, ^{1}H solution NMR and ^{17}O solid-state NMR analyses (for details, see Supporting Information). No degradation of the peptide chains upon the milling treatment was observed.

Scheme 4. Synthesis of site-selectively ^{17}O-enriched RGD* tri-peptide by VBM saponification of methyl ester protected peptide (prepared by SPPS, for synthetic details see Supporting Information). Only one oxygen is labeled per ester-derivatized carboxylic function using mechanochemistry, but globally, the labeled oxygen is distributed between both oxygen sites (i.e. C=O* and C–O*H, 50:50).

The enrichment levels of the ^{17}O-labeled aspartic side-chains in RGD* and GRGD*S peptides, as calculated from HRMS analyses of the isolated phases, were 29 and 27%, respectively, which enabled to record the ^{17}O MAS (magic angle spinning) NMR spectra at 14.1 T (Figure 2b). The fact that a spectrum could be recorded on only ~3 mg of peptide-NaCl mixture underscores the high labeling efficiency of VBM saponification producing molecules with enrichment levels high enough for ^{17}O NMR analyses, when incorporated in more complex biological systems. Overall, the mechanochemical saponification approach enables a straightforward and cost-efficient access to side-chain ^{17}O-labeled peptides, which may be easily adapted for enrichment of other peptides containing aspartic or glutamic acid residues that are key to biological processes, due to the presence of their pending carboxylic functions.

^{17}O Solid-state NMR analyses of the ^{17}O-enriched amino acids and peptides

As developed in more detail in previous sections, using mechanochemical saponification enabled obtaining ^{17}O-enriched free amino acids, their Fmoc/*t*Bu protected derivatives and also selectively side-chain labeled short peptides, all with high enrichment levels (up to ~40%, when using 90% ^{17}O-enriched H_2O*). Notably, the high labeling efficiency of VBM saponification allowed ^{17}O MAS NMR spectra of amino acids to be recorded in just a few hours at 14.1 T (see Supporting Information for acquisition details).

The ^{17}O-labeled free amino acids prepared in this work were characterized in their zwitterionic form by ^{17}O solid-state NMR spectroscopy, some of which for the first time. Indeed, the ^{17}O MAS NMR spectra reported in the literature generally correspond to the amino acid HCl salts.[17e,35] Some of the ^{17}O MAS

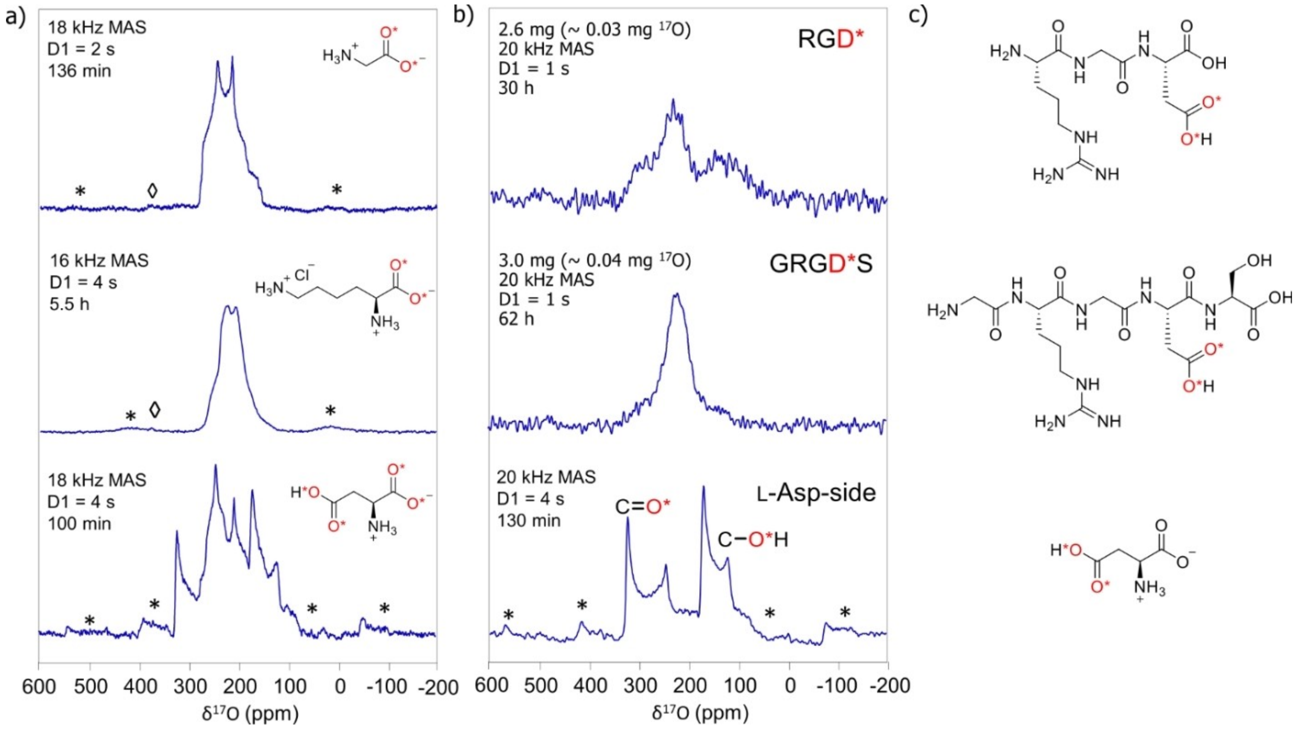


Figure 2. ^{17}O MAS NMR spectra recorded at 14.1 T with temperature regulated at 0 °C of ^{17}O-labeled a) Gly (β-form), L-Lys·HCl (isolated as dihydrate phase) and L-Asp-both (labeled at both carboxylic groups). b) RGD* tri-peptide and GRGD*S penta-peptide labeled at Asp side-chain (isolated via lyophilization, spectra recorded using DFS Hahn echo sequence, see Supporting Information for acquisition parameters) compared to L-Asp labeled selectively only at the side-chain (L-Asp-side, crystalline phase). c) Chemical structures of ^{17}O-labeled RGD* tri-peptide, GRGD*S penta-peptide and L-Asp labeled at the side-chain. For Gly and L-Asp-both, samples were physically mixed with silica before packing the rotor, to prevent preferential orientation of the crystallites (leading to line shape distortions in the initial analyses performed). Symbol "*" corresponds to the spinning side-bands, symbol "◇" corresponds to the ^{17}O natural abundance signal of the zirconia rotor. Only one oxygen was labeled per carboxylic function using mechanochemistry, but globally, the label is distributed between both oxygen sites in the isolated products (e.g. C=O* and C–O*H, 50:50), which is why both carboxylic oxygen atoms are shown here as labeled in red. The acquisition conditions and parameters extracted from the fits can be found in the Supporting Information.

NMR spectra we recorded are presented in Figure 2, while the rest can be found in Supporting Information (section VI). Below, a more detailed analysis of the ^{17}O NMR data obtained for Gly, Lys, Asp, and the labeled peptides is provided.

In the solid state, glycine is known to exhibit several polymorphs under ambient conditions, and their relative stability, interconversions and spectral signatures have been the subject of previous NMR studies.[36] Here, the recorded ^{17}O NMR spectrum shown in Figure 2a-top belongs to β-glycine (the least stable Gly polymorph), which was obtained due to the work-up conditions used for its isolation. The ^{17}O NMR spectrum was tentatively fitted with two oxygen sites (as expected from its crystal structure),[37] and the extracted parameters were in fair agreement with the previously published data for this polymorph (see Supporting Information).[38] It should be highlighted that our initial ^{17}O NMR analyses on this phase also revealed that careful attention needed to be paid to preferential orientation of the crystallites in the rotor (which could lead to line shape distortions), and to the possible evolution of the β-form towards a more stable polymorph.

Regarding ^{17}O-enriched Lys·HCl (dihydrate form), likewise two oxygen sites were expected based on the crystal structure.[39] However, as apparent from Figure 2a-middle, the recorded ^{17}O MAS NMR spectrum of the phase prepared here consisted of overlapping resonances situated in the middle of the chemical shift range typical for carboxylic functions, and lacked the sharp features often observed for second-order quadrupolar line shapes of carboxylic groups in crystalline phases. This featureless appearance could be due to ongoing dynamics in proximity of the carboxylic function,[40] although additional investigations would be needed to confirm this, which is out of the scope of this publication. Interestingly, in a previously published study by Pike et al.,[17e] the ^{17}O MAS NMR spectrum of a so-called "Lys·HCl" phase had been reported, which was composed of two separated oxygen signals positioned with isotropic shifts at 347 and 181 ppm (i.e. of C=O and C–OH, respectively). Considering the difference with the ^{17}O signals found here, and based on the results reported for other AA·HCl phases,[17e,35] we hypothesized that the isolated phase described by Pike et al. was a lysine dihydrochloride salt (Lys·2HCl). Indeed, based on the synthetic experimental details provided,[17e] their lysine phase had been labeled in ^{17}O using an acid-catalyzed exchange. Yet, unfortunately, no information regarding pH adjustment *prior* to its isolation had been mentioned. To verify this hypothesis, we prepared Lys·2HCl via the re-optimized acid-catalyzed exchange procedure (described in previous section), and characterized it by XRD and ^{17}O MAS NMR (data available in Supporting Information). The extracted ^{17}O NMR parameters of thus prepared phase were found to correspond to the data previously published by Pike et al.,[17e] confirming that the compound they had studied was most probably Lys·2HCl.

In the ^{17}O spectrum of L-Asp labeled at both carboxylic functions, four oxygen sites were expected based on the crystal structure.[41] Here, four overlapping oxygen resonances were observed (Figure 2a-bottom). The quadrupolar parameters for all four sites were successfully extracted taking advantage of the possibility of performing site-selective labeling (by enriching only the side-chain (β) or main-chain (α) carboxylic function), combined with multiple-field NMR analyses (details in Supporting Information). In the literature, only the ^{17}O solid state NMR parameters of the α-carboxylic group had been previously published,[42] which were found to be in agreement with the parameters obtained here.

In Figure 2b, the ^{17}O solid-state NMR spectra of RGD* and GRGD*S peptides labeled selectively at the side-chain of the aspartic acid unit are shown, together with the spectrum of L-Asp labeled likewise solely at the β-carboxylic function (recorded at 14.1 T). Compared to the crystalline L-Asp phase, the environment around oxygen atoms in peptides (obtained by lyophilization) is distributed, resulting in broad overlapping oxygen resonances situated in between the two extremes (i.e. defined C=O and C–OH sites). Further high-resolution analyses were not performed at this stage. It is important to highlight that *(i)* it is the first time that ^{17}O solid-state NMR spectra of RGD* and GRGD*S peptides were recorded, and *(ii)* only a very small amount of sample was analyzed (~3 mg of peptide/NaCl mixture), which corresponds to ~0.04 mg of ^{17}O. Altogether, owing to the high enrichment level achieved by VBM saponification, the results shown here represent a promising prerequisite for these peptides to be studied by ^{17}O NMR spectroscopy when incorporated into more complex biological systems. Such studies could then shed light, for example, on the functions of integrins in cell adhesion processes by providing unique information regarding the oxygen binding state, due to the high sensitivity of oxygen parameters to its local environment. This will be investigated as part of future work.

Conclusion

In this work, we have at first presented highly efficient ^{17}O-labeling procedures for the enrichment of *free* amino acids, namely: Gly, L-Leu, L-Phe, L-Asp and L-Lys·HCl. By using mechanochemistry, we were able to produce the labeled molecules rapidly (30 min reaction time) under ambient conditions, consuming minimal amounts of ^{17}O-labeled water (only 2–3 equiv. excess). The ^{17}O-enriched amino acids were isolated pure in their zwitterionic forms, in medium to high yields (~60–85%), with no loss of their optical purity (*ee* > 99%), and with high ^{17}O-enrichment levels (up to ~40%). This labeling scheme is currently being extended to other amino acids. The second labeling scheme proposed here is based on acid-catalyzed oxygen labeling protocol (adapted from a 1938 study by Mears),[28] and was re-optimized to serve as a comparison point for the mechanochemical saponification. The first results of ^{18}O-enriched *free* amino acids showed enrichment levels comparable with those after saponification (by using the same amount of water), after only 2 h of reaction time, making this approach an attractive and complementary alternative for the ^{17}O-enrichment of free amino acids, which are available as HCl salts, as exemplified here for L-Lys·2HCl. However, the broad application of this approach may be hindered by the lower commercial availability of amino acid hydrochloride salts compared to their ester derivatives, which are used as substrates in the mechanochemical saponification enrichment scheme.

Subsequently, the ^{17}O-enriched Fmoc/*t*Bu *orthogonally protected* amino acids, used as precursors in SPPS, were prepared under mild conditions by combining the VBM saponification with subsequent Fmoc-protection in solution. The labeled products were isolated with high purity, in high synthetic yields (~75–85%), and with high enrichment levels (~40%). Scaling-up the mechanochemical step enabled to produce up to 1 g/day quantities of ^{17}O-labeled protected amino acids cost-efficiently, consuming only ~18 μL of 90% ^{17}O-enriched water per 100 mg of isolated Fmoc-AA-O*H, which corresponds to the labeling cost of 50 €/100 mg of the labeled product. Finally, the VBM saponification was applied as a site-selective enrichment strategy for labeling solely the side-chain carboxylic functions in RGD and GRGDS peptide chains, while reaching ~28% enrichment level.

The efficiency of the above-proposed protocols also appears through the rapidity with which the ^{17}O MAS NMR spectra of ^{17}O-labeled amino-acids were recorded in good signal-to-noise ratio at 14.1 T, several of them being reported here for the first time. Moreover, thanks to the highly efficient mechanochemical labeling scheme, the ^{17}O MAS NMR spectra of ^{17}O-labeled RGD and GRGDS peptides were recorded at 14.1 T using only ~3 mg of peptides. Such analyses represent an important prerequisite for the subsequent application of amino acids and peptides in ^{17}O NMR studies of more complex biological systems.

Lastly and most importantly, great attention was given here in providing efficient and reproducible protocols, including extensive experimental details. We believe that this will facilitate their further adoption by other researchers, in view of expanding the knowledge regarding the structure and dynamics of peptides and proteins via ^{17}O NMR studies. Moreover, because most labeling protocols reported here have been optimized to be feasible within less than a day (work-up included), we foresee that this work will also be taken up for teaching laboratories, so that the advantages of ^{17}O (and even ^{18}O) labeling for analytical purposes can be taught early on.

Experimental Section

Full details on methods, synthetic protocols and products characterization can be found in Supporting Information (Supporting Information).

Reagents and synthetic equipment: All precursors and reagents were used as received. Reagent-grade and HPLC-grade solvents were used in purification procedures (details provided in Supporting Information). ^{17}O and ^{18}O-labeled water, with different initial oxygen enrichment levels (70%, 90% ^{17}O for ^{17}O-enriched H_2O^*, and 99% ^{18}O for ^{18}O-enriched H_2O^*), was purchased from Cortecnet and Eurisotop.

Milling experiments were carried out in a Retsch Mixer Mill 400 apparatus, equipped with stainless steel jars (10 or 1.5 mL inner volume), containing two stainless steel beads (10 or 5 mm diameter, respectively). All mechanochemical protocols for enrichment of amino acids were first tested using non-labeled water and then optimized using ^{18}O-labeled water, before performing experiments with ^{17}O-labeled H_2O^*.

SPPS syntheses were performed at the SynBio3 platform of the University of Montpellier. Fritted syringes were placed in an orbital shaker using a 2-chlorotrityl chloride resin loaded at ~0.5–0.6 mmol/g, and by using the Fmoc/*t*Bu strategy. Side-chain deprotections were performed in a Carousel 12 Plus Reaction Station-Radleys apparatus.

Isotopic labeling of amino acids: *Free* amino acids were ^{17}O/^{18}O-enriched *(i)* either by mechanochemical saponification, starting from methyl ester derivatives of the targeted amino acids (1 equiv), using NaOEt (2.5–3.5 equiv) and ^{17}O/^{18}O-enriched H_2O^* (2–3 equiv) for hydrolysis, and conducting milling for 30 min at 25 Hz, or (*ii)* by acid-catalyzed procedure, starting from hydrochloride salts of the free amino acids (1 equiv), using ^{18}O-enriched H_2O^* (2–3 equiv) for oxygen exchange, and conducting heating in an oven at 100 °C for 2–24 h. The pH was afterwards adjusted for both enrichment schemes and the free amino acids were isolated in their zwitterionic forms. Full details on reagent quantities, reaction times, work-up procedures, synthetic yields, enrichment levels and enrichment yields, are provided in Supporting Information. Notably, a full section describing the general strategy for the isolation of the enriched free amino acids can be found in section II–A.

Fmoc-protected ^{17}O/^{18}O-enriched amino acids were prepared following previously published solution-based Fmoc-protection synthetic procedures, while starting from the *free* amino acids enriched in ^{17}O/^{18}O as described above (details provided in Supporting Information).

Isotopic labeling of peptides: RGD and GRGDS related peptides, with a methyl ester moiety on the aspartic side chain, were prepared by SPPS, using commercial Fmoc-L-Asp(OMe)-OH as a precursor. Reaction and purification conditions are detailed in the Supporting Information. Subsequently, the Asp side-chain was ^{17}O-enriched via mechanochemical saponification of the RGD(OMe) or GRGD(OMe)S peptides (1 equiv), using NaOEt (3.5 equiv) and ^{17}O-enriched H_2O^* (7 equiv) for hydrolysis, and conducting milling for 15 min at 25 Hz. Full details on reagent quantities, work-up procedure, synthetic yields, enrichment levels and enrichment yields, are provided in Supporting Information.

Characterization of enriched amino acids and peptides: Infrared (IR) analyses were performed to follow the course of the milling reactions. Spectra were recorded in ATR mode (attenuated total reflectance), on a Perkin Elmer Spectrum 2 FTIR instrument in the 400–4000 cm^{-1} range. Powder XRD analyses were carried out to determine the crystalline state and polymorphic form of the isolated amino acids. Analyses were performed on a X'Pert MPD diffractometer using Cu $K_{\alpha1}$ radiation; diffractograms were generally recorded between 10° and 50° in 2θ.

Mass spectrometry analyses of the labeled products (amino acids and peptides) were done by HRMS, by infusion of the sample in the electrospray source of a Synapt G2-S (Waters, SN: UEB205) mass spectrometer operating in positive or negative mode. Capillary and cone voltage were respectively 3000 V and 30 V. Source and desolvation temperatures were respectively 250 °C and 100 °C. Details on the calculation methodology for enrichment levels (EL) and enrichment yields (EY), and for the determination of error bars, are provided in Supporting Information (section I-D).

Enantiomeric excesses were determined by HPLC on chiral support with an Agilent 1260 Infinity unit (pump G1311B, autosampler G1329B, DAD G1315D) and Agilent OpenLAB Chemstation. These analyses were performed at the iSm2 laboratory in Marseille.

^{1}H and ^{13}C solution NMR spectra were recorded on an Avance III Bruker 600 MHz NMR spectrometer equipped with a TCI Prodigy cryoprobe or on an Avance III Bruker 500 MHz NMR spectrometer equipped with a BBO Helium cryoprobe, using D$_2$O or DMSO-d_6 as solvents.

^{17}O solid-state NMR experiments were acquired at multiple magnetic fields (9.4, 14.1, 18.8 and 20.0 T) at different NMR facilities (ICGM in Montpellier, IMEC-ISB-UCCS in Lille, MagLab in Tallahassee, CEMHTI in Orléans). The spectra were recorded under MAS conditions, using the acquisition conditions reported in Supporting Information (Table I-1).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Raw data can be made available upon reasonable request.

Keywords: amino acids · mechanochemistry · NMR spectroscopy · ^{17}O isotope labeling · peptides

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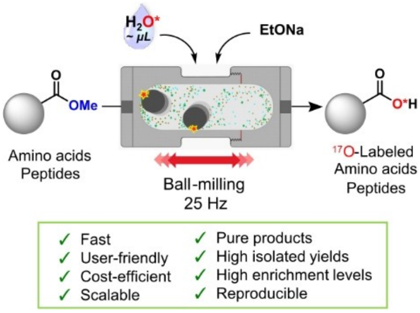
Fast, user-friendly and cost-efficient labeling strategies were developed for ^{17}O-enrichment of *free* amino acids, Fmoc/*t*Bu-*protected* amino acids (produced on 1 g/day scale) and *peptide side-chains* (site-selective labeling). ^{17}O-Labeled biomolecules were reproducibly obtained pure, in high synthetic yields (up to 85 %), and with high enrichment levels (~40 %).



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Fast and Cost-Efficient ^{17}O-Isotopic Labeling of Carboxylic Groups in Biomolecules: From Free Amino Acids to Peptide Chains