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A convenient and rapid method for the selective oxygen-17 enrichment of aspartyl peptides during solid-phase synthesis

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Abstract—In this work we describe, for the first time, a rapid and efficient method for ^{17}O selective labeling on the β-carboxyl group of an aspartic acid residue already incorporated into a peptide sequence anchored on a solid-phase support. The β-*O*-benzyl ester of the Asp residue of the Ac-RGD-benzydrylamine resin was successfully saponified using $Na^{17}OH$ in a methanol/dichloromethane mixture. The ^{17}O selective enriched peptide was then released from the solid support by acidic cleavage. The ^{17}O NMR spectrum confirmed the ^{17}O labeling of the Asp β-carboxylate.

^{17}O NMR spectroscopy has been widely applied to the study of peptides in order to gain insight into their structural features.[1–7] It provides a sensitive tool for searching the conformational interactions in solution, such as inter- and intramolecular hydrogen bonding, the nature of solvent interactions and details of the secondary structure. The Asp and Glu side chains are involved in many biological processes either by stabilizing the active conformation of the peptide or protein through ionic interactions, or by participating directly as interacting sites in several biological systems, e.g. receptor–ligand interactions, enzyme active sites, etc. The use of ^{17}O NMR spectroscopy in these cases can provide valuable information about their mode of interaction. Amino acid α-carboxyl groups are usually labeled with ^{17}O by saponification of the corresponding N^{α}-Boc amino acid-*O*-methyl esters[1,3–6] with $Na^{17}OH$ in methanol or by acid-catalyzed ^{17}O exchange of the free amino acid in highly enriched $H_2^{17}O$. The latter requires vigorous reaction conditions (e.g. high temperature), which are associated with racemization problems resulting in restrictions of its application, while specific labeling on α-, β- or γ-COOH cannot be achieved by this method. On the other hand, labeling with ^{17}O by saponification of the corresponding β- or γ-esters of Asp or Glu amino acids, before their use in peptide synthesis, presents the following difficulties: (i) multistep side chain protection procedures, which result in losses in yield and in the percentage of ^{17}O enrichment due to the re-esterification step and (ii) a threefold excess of pure protected labeled amino acid is required for its incorporation into the peptide sequence.

Our effort is focused on the development of an easy and efficient procedure for selective ^{17}O chemical enrichment of β- or γ-COOH groups of Asp or Glu residues, respectively, which are already incorporated in the peptide. The tripeptide Ac-Arg-Gly-Asp-NH_2 (Ac-RGD-NH_2) was selected for performing direct ^{17}O enrichment of the Asp-β-COOH. This sequence is of great importance as it is involved in a variety of physiological processes (e.g. platelet aggregation).[8] We succeeded in a very simple and effective way in the aspartic acid ^{17}O side chain enrichment after its incorporation in the peptide sequence on the solid-phase support.

In order to establish the optimum conditions for the saponification of two Asp-β-esters (Boc-Asp(OR)-OH(I), where R = All or Bzl, Scheme 1), suitable for orthogonal protection in solid-phase peptide synthesis (SPPS), we carried out several mini-scale experiments in solution with unlabelled NaOH, varying the solvent, the time of the reaction and the ratio of the reactants. The solvent mixtures were methanol/dichloromethane and methanol/dioxane, which are compatible with SPPS. In each case the reaction was monitored by TLC and ES-MS after quenching by acidification with 1 M $NaHSO_4$ (pH 3–4) and extracting with ethyl acetate.

Analyzing the mass spectra, recorded using several reaction conditions (Table 1), we observed that under the examined conditions: (a) in the presence of 0.5 M

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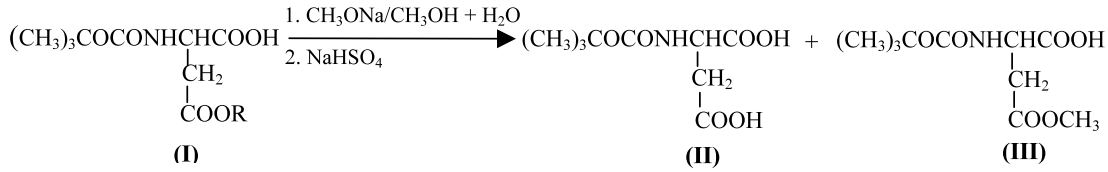

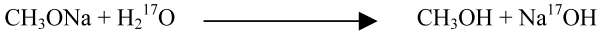

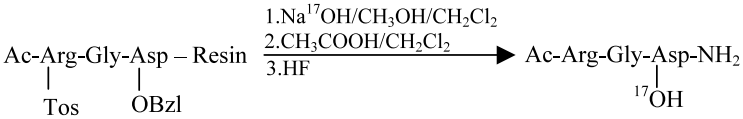

Scheme 1.

Table 1. Saponification of Boc-Asp(OR)-OH with 0.1–0.5 M NaOH in various solvent mixtures

R	t (min)	Solvent (ml)[a]			% Compounds[b]			Ratio[c]
		Dichloromethane	Methanol	Dioxane	**I**	**II**	**III**	0.1:1.5
Allyl	1	1.5	1.5	–	<1	~6	94	0.1:1.5
	10	1.5	1.5	–	0	19	81	0.1:1.5
	20	1.5	1.5	–	0	44	56	0.1:1.5
	30	1.5	1.5	–	0	51	49	0.1:1.5
	120	1.5	1.5	–	0	81	19	0.1:1.5
	1	2.25	0.75	–	0	60	40	0.1:1.5
	5	2.5	0.5	–	0	100	0	0.1:1.5
	15	2.7	0.3	–	0	100	0	0.1:0.6
	10	2.85	0.15	–	0	100	0	0.1:0.3
	10	–	0.75	2.25	<1	27	~73	0.1:1.5
Benzyl	5	2.5	0.5	–	0	100	0	0.1:1.5
	10	2.85	0.15	–	0	100	0	0.1:0.3

[a] The total volume of the reaction solution was 3 ml.
[b] The percentages were estimated from the relative intensities of the corresponding peaks in the ES-MS spectra.
[c] The ratio of the reactants refers to mmol of ester/mmol of NaOH.

NaOH in methanol/dichloromethane (1:1 v/v), transesterification to the corresponding methyl ester (**III**) occurred initially (within 1 min) followed by slow saponification (Scheme 1); (b) as the ratio of methanol/dichloromethane decreased, the desired product (**II**) increased at the expense of methyl ester (**III**), and the completion of the reaction was accelerated; (c) when methanol/dioxane was used, the methyl ester (**III**) was favored over the saponification product (**II**); and (d) excess of NaOH relative to the ester does not seem to play an important role.

Based on the above results, we conclude that the optimum conditions for rapid and complete saponification of the β-allyl and β-benzyl aspartates are: 1 equiv. of the ester with 0.1 M NaOH (2–4 equiv.) in CH_3OH/CH_2Cl_2 (1:6), for 10–15 min, at rt (yield: 100%). These conditions were subsequently applied for the ^{17}O selective labeling of the aspartic acid β-carboxylate of the RGD peptide anchored on the solid support.

The tripeptide Ac-Arg-Gly-[C_4-^{17}O]-Asp-NH_2 (0.3 mmol) was synthesized by solid-phase methodology on a benzydrylamine resin using the Boc-strategy. Arginine was introduced as Boc-Arg(Tos)-OH and aspartic acid as Boc-Asp(OBzl)-OH. After the last *N*-α-Boc-deprotection followed by *N*-α-acetylation, the peptide resin was washed with absolute methanol and dry dichloromethane and then suspended in 10 ml dry CH_2Cl_2. It was then subjected, for 15 min with stirring, to treatment with a solution of $Na^{17}OH$ in methanol, prepared by the addition of 25 μl (1.4 mmol) of $H_2^{17}O$ (10% in ^{17}O) in 0.4 ml of 3 M CH_3ONa in absolute CH_3OH followed by dilution with 1.5 ml absolute methanol. After acidification with 20% CH_3COOH in CH_2Cl_2, the peptide was cleaved from the resin with HF, lyophilized, purified with RP-HPLC and identified by ES-MS (expected m/z: 388.40 unlabelled, 389.40 labeled, found m/z: 388.34 unlabeled, 389.29 labeled), and ^1H NMR spectroscopy. The ^{17}O NMR spectrum

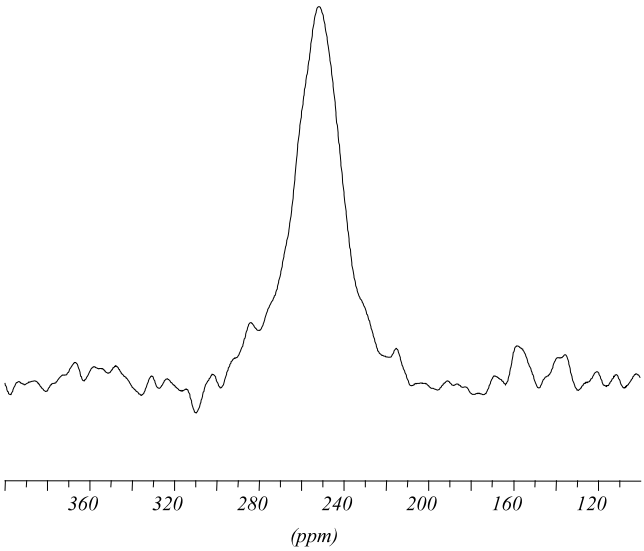

Figure 1. 54.2 MHz ^{17}O NMR spectrum of Ac-Arg-Gly-[C_4-^{17}O]-Asp-NH_2 in water.

(Fig. 1) confirmed the ^{17}O labeling of the Asp side chain.

In summary, the ^{17}O selective incorporation into the Asp-β-COOH group can be efficiently performed in protected peptides anchored on a solid support, resulting in a twofold enrichment of the Asp residue compared to the conventional methods. The application of this method in more elaborate peptides is currently in progress.

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