A Novel Spin-Labeled Amino Acid Derivative for Use in Peptide Synthesis: (9-Fluorenylmethyloxycarbonyl)-2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic Acid

Reinaldo Marchetto,† Shirley Schreier,‡ and Clovis R. Nakaie*

Department of Biophysics
Escola Paulista de Medicina, Rua 3 de Maio, 100
04044-020 São Paulo, SP, Brazil
Department of Biochemistry
Institute of Chemistry, UNESP
14800-060 Araraquara, São Paulo, SP, Brazil
Department of Biochemistry
Institute of Chemistry of University of São Paulo
CP 20780, 01498-970 São Paulo, SP, Brazil

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Essentially all previous reports on use of the electron spin resonance (ESR) method[1] for the study of proteins and peptides involved coupling of the spin label either to amino acid side chains or to the N- or C-terminal residues.[2] When solid-phase peptide synthesis[3] was employed, coupling of spin-labeled amino acids was done exclusively at the amino terminal position.[4-6] The synthesis of peptides containing spin labels in residues at internal positions of their sequences has not yet been described[7] because of the lability of the nitroxide moiety, which decomposes during the repeated deprotection step required to remove the *tert*-butyloxycarbonyl (Boc) N^{α}-protecting group of each coupled amino acid. During this step, treatment with trifluoroacetic acid (TFA)/CH_2Cl_2 leads to protonation followed by disproportionation and decomposition of the nitroxide group due to the strong oxidizing properties of the resulting intermediate.[8] For this reason, labeling has been limited to the N-terminal residue, which avoids subjecting the nitroxide group to the repeated acidic deprotection steps. The final single-step cleavage reaction (in anhydrous HF) to remove the peptide from the resin causes only nitroxide protonation, which is reverted in basic conditions.[4-6]

The present communication describes a paramagnetic amino acid derivative which, in principle, allows the labeling of peptides, via peptide synthesis, at any position in the sequence. Such labeling could provide valuable information on conformational properties of peptides and on their interactions with macromolecules and membranes of biological interest.

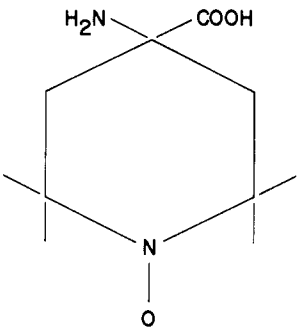

Figure 1. 2,2,6,6-Tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC).

The stable free radical 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC, Figure 1) was synthesized as previously reported[9] and derivatized[10] with the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) N^{α}-protecting group,[11] yielding 3.4 g of pure Fmoc-TOAC[12] as a yellow powder (62% yield). To test the usefulness of this derivative for labeling peptides, the Pro[7] residue of the octapeptide angiotensin II (AII: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was chosen to be replaced by Fmoc-TOAC. The Fmoc peptide synthesis protocol[13] was employed to synthesize TOAC[7]-AII on the 0.2 mmol scale. Fmoc-TOAC was easily coupled to the Phe resin, and kinetic studies of amino group deprotection of the resulting Fmoc-TOAC-Phe-resin in 20% piperidine/*N*,*N*-dimethylformamide showed that the removal of the Fmoc group occurred in a few seconds. Introduction of the next amino acid (His) was possible only after three coupling steps, probably due to the low nucleophilicity of the TOAC amino group when bound to the peptide chain ($pK_a < 6.0$).[5,6] No difficulties were met with during the assembly of the remainder of the peptide chain.

The Fmoc and Boc protocols differ in that the former usually uses 82.5% TFA-containing reagent K,[14] whereas the latter employs anhydrous HF for cleavage of the peptide from the resin. To compare both procedures, two portions (0.1 mmol each) of the peptide-resin were submitted either to reagent K or to anhydrous HF. Whereas reagent K decomposed the paramagnetic group, inducing loss of the ESR signal, which was not recovered

* Author to whom correspondence should be addressed at Escola Paulista de Medicina. Phone: 55-11-575.9617. FAX: 55-11-571.5780.
† Institute of Chemistry, UNESP.
‡ Institute of Chemistry of University of São Paulo.

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(12) Synthesis and characterization of Fmoc-TOAC. Synthesis. TOAC (2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid) was synthesized as previously reported.[9] TOAC (12.56 mmol, 2.7 g) was dissolved in 20 mL of water in the presence of 1 equiv of triethylamine. A solution of Fmoc-succinimidyl carbonate (12 mmol, 4.05 g) in 20 mL of acetonitrile was added in one portion to this mixture. The pH was maintained at 8.5–9.0 by the addition of triethylamine. After being stirred for 30 min, the yellow mixture was filtered and concentrated *in vacuo*, and the residue was poured into 50 mL of cold 20% citric acid with stirring. The Fmoc-TOAC was isolated with five extractions of ethyl acetate, and the combined extracts were washed with water and with saturated NaCl and then dried in anhydrous Na_2SO_4. The organic extract was evaporated, and the product was crystallized with ethyl acetate:petroleum ether (1:1) to provide 3.4 g of Fmoc-TOAC as yellow crystals (62% yield). Characterization: mp 161–163 °C; ^1H NMR (200 MHz, $CDCl_3$) 1738 (2 H, $CH_2OC{=}O$), 2540 (1 H, ring H), 3059 (1 H, N–H), 3877 (12 H, ring CH_3), 4594, 4900 (4 H, ring CH_2), 7969, 8448 (Ar–H), and 13435 (COOH) ppm; FAB-MS calcd $[M + H]^+$ = 438.52, found 438.24; λ_{max} (ϵ M^{-1} cm^{-1}) 267 (18 950), 290 (5280), 301 (6200) nm; IR (KBr) 3349 (s), 2981–2949 (s), 1953 (w), 1725 (s), 1525 (s) cm^{-1}. Anal. Calcd for $C_{25}H_{29}N_2O_5$: C, 68.65; H, 6.64; N, 6.41. Found: C, 68.30; H, 6.80; N, 6.64.

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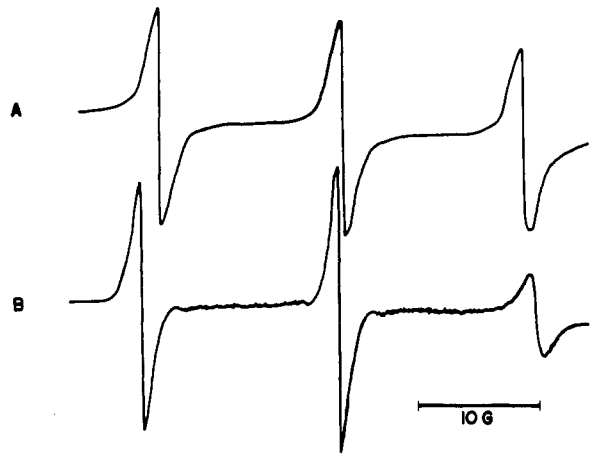

Figure 2. ESR spectra of 5×10^{-4} M Fmoc-TOAC in DMF (A) and of 5×10^{-4} M TOAC^7-AII in 0.02 M ammonium acetate, pH 5.0 (B).

in basic solution, the HF treatment induced only N–O protonation, which was fully reverted upon treatment with 0.02 M ammonium acetate at pH 9.0 for 3 h, in agreement with our previous studies.[5,6] These results indicate that HF cleavage is more appropriate to remove spin-labeled peptides from the resin. The crude peptide obtained after HF cleavage and alkaline treatment was purified by preparative HPLC, yielding 37 mg of pure TOAC^7-AII with a molecular ion m/z = 1147.46 with FAB-MS (calculated 1147.36).

ESR spectra were obtained for Fmoc-TOAC and TOAC^7-AII (Figure 2). Although the motional narrowing formalism is only strictly applicable to small spherical molecules tumbling isotropically,[15] calculation of rotational correlation times[16] yielded 2.3 $\times 10^{-10}$ and 0.8×10^{-10} s·rad^{-1} for TOAC^7-AII and Fmoc-TOAC, respectively. Structure–activity studies with the labeled peptide are currently in progress.

In conclusion, our results demonstrate that by conjugating Fmoc N$^{\alpha}$-protection and HF cleavage, it is possible to introduce a spin-labeled amino acid at an internal position of the peptide chain. Additionally, the usefulness of Fmoc-TOAC may be extended to the labeling of selective nucleophilic sites in previously purified peptides and proteins.

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Supplementary Material Available: Synthetic procedures for TOAC^7-AII and mass spectra for Fmoc-TOAC and TOAC^7-AII (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(16) ESR spectra were obtained at 9.5 GHz with a Bruker ER200 spectrometer at room temperature (22 ± 2 °C) using flat quartz cells. The magnetic field was modulated with amplitudes less than one-fifth the line widths, and the microwave power was 5 mW to avoid saturation effects. The rotational correlation times (τ) were calculated according to the equation (Cannon et al.; *Arch. Biochem. Biophys.* **1975**, *167*, 505) $\tau = (6.55 \times 10^{-10})\Delta H_0[(h_0/h_{+1})^{1/2} + (h_0/h_{-1})^{1/2} - 2]$, where ΔH_0 is the width of the central resonance and h_{+1}, h_0, and h_{-1} are the heights of the low-, center-, and high-field resonances, respectively.