# Fmoc-POAC: [(9-Fluorenylmethyloxycarbonyl)-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl-3-amino-4-carboxylic Acid]: A Novel Protected Spin Labeled β-Amino Acid for Peptide and Protein Chemistry

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**The stable free radical 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC) is the only spin labeled amino acid that has been used to date to successfully label peptide sequences for structural studies. However, severe difficulty in coupling the subsequent amino acid has been the most serious shortcoming of this paramagnetic marker. This problem stems from the low nucleophilicity of TOAC's amine group towards the acylation reaction during peptide chain elongation. The present report introduces the alternative β-amino acid 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl-3-amino-4-carboxylic acid (POAC), potentially useful in peptide and protein chemistry. Investigations aimed at addressing the stereochemistry of this cyclic molecule through X-ray diffraction measurements of crystalline and bulk samples revealed that it consists only of the *trans* conformer. The 9-fluorenylmethyloxycarbonyl group (Fmoc) was chosen for temporary protection of the POAC amine function, allowing insertion of the probe at any position in a peptide sequence. The vasoactive octapeptide angiotensin II (AII, DRVYIHPF) was synthesized by replacing Pro[7] with POAC. The reaction of Fmoc-POAC with the peptidyl-resin occurred smoothly, and the coupling of the subsequent amino acid showed a much faster reaction when compared with TOAC. POAC[7]-AII was obtained in good yield, demonstrating that, in addition to TOAC, POAC is a convenient amino acid for the synthesis of spin labeled peptide analogues. The present findings open the possibility of a wide range of chemical and biological applications for this novel β-amino acid derivative, including structural investigations involving its differentiated bend-inducing characteristics.**

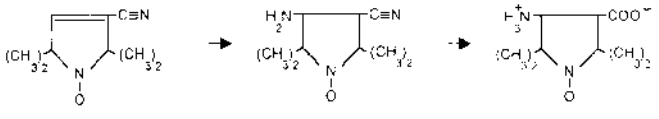
**Key words** peptide; spin label; electron paramagnetic resonance; amino acid; TOAC; POAC

TOAC,[1] the stable free radical 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid,[2] acylated at the amine function with the *tert*-butyloxycarbonyl group (Boc-TOAC), was the first protected spin-labeled amino acid derivative successfully introduced[3,4] for labeling peptides through the solid phase method.[5,6] However, due to partial decomposition[3,7] of the nitroxide moiety during the repeated trifluoroacetic acid (TFA) treatments necessary for Boc group[5] removal, labeling with this paramagnetic marker was restricted to the N-terminal position of the sequence. To overcome this shortcoming, the 9-fluorenylmethyloxycarbonyl (Fmoc) group[6] was used for protection of the amine function. Moreover, the conjugation of both Fmoc (for peptide chain elongation) and Boc (for peptide cleavage from the resin) chemistries allowed us to propose a procedure for TOAC insertion at any internal position of a peptide sequence.[8]

Since its use for the synthesis of spin labeled peptides was first described, this $C^{\alpha}$-tetra-substituted cyclic α-amino acid was employed in a great variety of investigations. For instance, the first example of a labeled peptide hormone (α-melanocyte stimulating hormone) that retained full biological activity was recently reported.[9] Furthermore, a large sampling of mono- or doubly-labeled model peptides have been studied in terms of structural features.[10—14]

As a further development in our research on spin labeled amino acids, here we describe the convenience of using the β-amino acid derivative 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl-3-amino-4-carboxylic acid (POAC), protected with the Fmoc group, for peptide labeling. Besides the inherent interest in using this alternative spin label for a broad range of chemical and biochemical purposes, an additional objective for introducing POAC in peptide chemistry is to overcome the serious difficulty in coupling the subsequent amino acid residue after TOAC incorporation.[8] This is due to the very low nucleophilicity of its amine function when bound to a peptide sequence ($pK_a<6$)[3,4] which can be overcome when a more efficient coupling procedure is applied.[8] In contrast, the amine function linked to the POAC pyrrolidine β-carbon should confer higher nucleophilicity than that in TOAC, facilitating the coupling reaction during peptide synthesis. The steps for POAC (**3**) synthesis are shown in Fig. 1 and have been described in detail elsewhere.[2,15]

Owing to the presence of two asymmetric carbons in POAC's β-cyclic structure, its synthesis yields a racemic mixture. Crystals suitable for X-ray diffraction were crystals obtained by slow evaporation from an ethanol-water solution. Single crystal structure determination showed that a *trans*



**1** **2** **3**

Fig. 1. Scheme of the Synthesis of the POAC Spin Label

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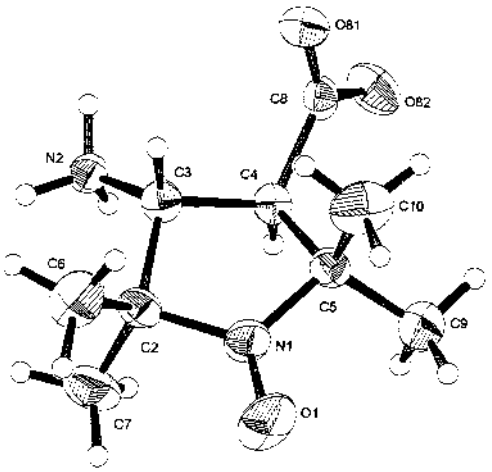


Fig. 2. ZORTEP Representation of POAC

Thermal ellipsoids of non-H atoms are drawn at the 50% probability level. H atoms are at an arbitrary scale.

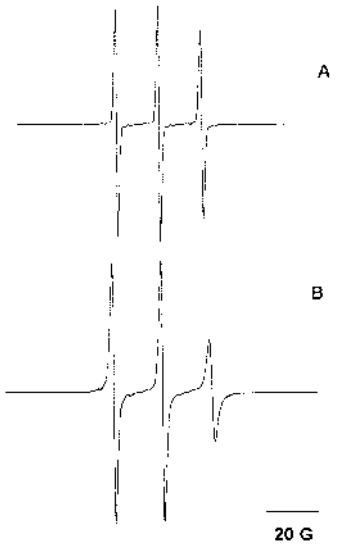


Fig. 3. EPR Spectra of 0.05 mM POAC (A) and POAC$^7$-AII (B) in Aqueous Solution, pH 5.0

conformer was obtained (Fig. 2).[16] The presence of only one conformer was established by X-ray powder analysis of the bulk sample. Since both (*R*) and (*S*) isomers are obtained during the synthesis of POAC, work is in progress aimed at complementing the stereochemical analysis. For this purpose, we are making use of different standard procedures in order to achieve stereospecific isolation and synthesis.

The synthesis of Fmoc-POAC was carried out as previously reported,[8] and yielded 380 mg of pure compound as a bright yellow powder (see the Experimental section). The feasibility of using Fmoc-POAC for peptide chemistry was evaluated by substituting it for the Pro$^7$ residue of the vasoactive peptide angiotensin II (AII, DRVYIHPF). The POAC$^7$-AII analogue was assembled in a 0.15 mmol scale starting from Phe-Wang resin (0.41 mmol/g) and following the Fmoc chemistry procedure.[6] Couplings were done with 2.5-fold excess using diisopropylcarbodiimide and 1-hydroxybenzotriazol as activators. After POAC incorporation, the coupling of the next amino acid residue [Fmoc-(trityl) His] occurred smoothly in only 1 h. This result confirmed the facilitated acylation of the subsequent amino acid residue to the POAC amine group when compared to TOAC. In the latter case, the recoupling procedure was repeated three times for quantitative insertion of the His residue using the same experimental conditions.[8] Peptide cleavage from the resin, making use of HF, lead to protonation of the nitroxide group. Recovery of the electron paramagnetic resonance (EPR) signal of the crude peptide was achieved upon treatment with 0.02 M ammonium acetate, pH 10, for 10 h. The main peak obtained in the analytical HPLC chromatogram of the crude material was purified by a preparative reverse phase-HPLC column yielding 29 mg of desired POAC$^7$-AII. The characterization of the sample was confirmed through amino acid analysis, analytical HPLC, and MALDI-MS $(M+H)^+=1132.55$.

EPR spectra of POAC and POAC$^7$-AII in aqueous solution (pH=5.0) are given in Fig. 3. Both compounds displayed narrow lines, as expected for small molecules tumbling freely in a non-viscous solution, but the rotational correlation times ($\tau$)[17,18] calculated for POAC and POAC$^7$-AII ($0.6\times 10^{-10}$ and $5.3\times10^{-10}$ s$^{-1}$, respectively) reflect the fact that the molecular weights of both compounds differed by nearly one order of magnitude.

In conclusion, the present work introduces a second spin labeled amino acid POAC, and demonstrates its potential for application in peptide and protein chemistry and biochemistry. In addition to its usefulness in structure-biological activity studies, it also opens the possibility for comparative conformational investigation with proline-containing peptides due to its pyrrolidine structure. Moreover, this spin probe may be utilized in other studies, such as fluorescence quenching[19] and peptide binding to bilayer or micelles, similarly to what has been done with TOAC derivatives.[20] Furthermore, in view of the importance of examining the physico-chemical aspects of the interior of some macromolecular systems, POAC can also be used in the greatly expanding field of polymer-supported procedures in either chemical or biological areas. As an example, one may compare optimization strategies of peptide synthesis methodologies. In work done with TOAC,[21,22] EPR monitoring of peptide chain solvation throughout the resin matrix was interpreted in the light of an alternative solvent polarity parameter.[23] Research using POAC derivatives for this and related purposes is in progress.

**Experimental**

Solvents and reagents were purchased from Fluka or Aldrich and all met ACS standards. Trifluoroacetic acid was acquired from Fluka, and the anhydrous hydrogen fluoride (5 l capacity cylinder) was from Merck Co.

**Synthesis of Fmoc-POAC** According to a previously published procedure,[8] 201 mg (1 mmol) of POAC in water/$Na_2CO_3\cdot10H_2O$ was treated with Fmoc-succinimidyl carbonate (dissolved in acetone), and the pH was adjusted (8.5—9.0). After 3 h stirring, the solution was diluted with cold water and acidified to pH 2 with 1 N HCl. The desired product was extracted with ethyl acetate, washed with small portions of water, and dried over anhydrous $Na_2SO_4$ overnight. After filtration and evaporation, the product was crystallized in $CHCl_3$/petroleum ether, yielding 380 mg (90% yield) of Fmoc-POAC as light yellow crystals. The analytical HPLC profile and FAB-mass spectrum of this derivative are available in Supplementary Materials.

Characterization: $[M^+]=423$; elemental analysis calculated for $C_{24}H_{27}O_5N_2$: C, 68.08; H, 6.28; N, 6.62. Found: C, 67.9; H, 6.35; N, 6.60. IR (KBr) cm$^{-1}$: 3444 to 3338 ($\nu$NH and OH); *ca.* 3030 ($\nu$ArCH); 3000—2700 ($\nu$COOH); 1723 ($\nu$COOH and R–O–C–O–N); 1543 ($\delta$ NH and $\nu$CN); 1450 ($\delta$ $CH_3$); 1235—1150 (gem-dimethyl groups).

**Crystallographic Analysis** Data were collected on an Enraf-Nonius

CAD4-Mach 3 diffractometer. Programs used: refinement SHELXL97; graphical representation: ZORTEP.

**Mass Spectrometry** The crude peptide was analyzed either by Fast Atom Bombardment (FAB)-Mass Spectroscopy in a CG-MS HP 5988A apparatus or by Matrix Assisted Laser Desorption Ionization (MALDI) on a Micromass Spectrometer, model TofSpec SE, using $\alpha$-cyano-4-hydroxycinnamic acid as the solid matrix.

**Analytical and Semi-Prepartive RP-HPLC** The purity analyses were achieved in TFA/acetonitrile gradients using a Waters Associates HPLC system consisting of two 510 HPLC pumps, an automated gradient controller, Rheodyne manual injector, 486 UV detector and 746 data module. Solvent A: 0.1% TFA/$H_2O$ and Solvent B: 60% acetonitrile/0.1% TFA/$H_2O$ with a gradient of 5—95% of B in 30 min, at a flow rate of 1.5 ml/min. The column employed was a Vydac $C_{18}$ column (0.46×25 cm, 5 $\mu$m particle size, 300 Å pore size). The detection was at $\lambda$=210 nm. For peptide purification, a Vydac $C_{18}$ semi-preparative column (2.5×30 cm, 70 Å pore size, 7 $\mu$m particle size) was used with aqueous ammonium acetate, pH 5 and 60% (v/v) acetonitrile solutions as solvents A and B, respectively. A linear gradient of 20—65% of B in 135 min was applied.

**EPR Studies** EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 spectrometer at room temperature (22±2 °C) using flat quartz cells. Labeled peptidyl-resins were pre-swollen overnight in the solvent under study before running the spectra. The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects.

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**References and Notes**

1) Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485—2489 (1966); **6**, 362—369 (1967); **11**, 1726—1732 (1972). The symbols represent the L-isomer unless otherwise specified. The following additional abbreviations are used: Boc, *tert*-butyloxycarbonyl; $C_{18}$, octadecyl; EPR: electron paramagnetic resonance; Fmoc: 9-fluorenylmethyloxycarbonyl; POAC: 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl-3-amino-4-carboxylic acid; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; TOAC: 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid.
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