Alternative and Simple Normal-Phase HPLC Enantioseparation of a Chiral Amino Acid-Type Spin Label Derivative


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In this work an alternative chromatographic process was developed for fractionating the (+)-(3R,4R) and (−)-(3S,4S) enantiomers of the chiral β-amino acid trans-2,2,5,5-tetramethylpyrrolidine-3-amino-4-carboxylic acid (POAC), which was protected at its amine group for further coupling to a peptide, polymer or other macromolecule. The HPLC enantioseparation was achieved using a chiral cellulose-based normal stationary phase and isocratic elution. The n-hexane:isopropanol system, always with greater amount of the former solvent, was used as mobile phase as revealed by improved fractionation property of both components, demonstrated by the separation factor and resolution index values. These parameters presented values of 3.7 and 18.4 and of 2.0 and 6.7 when in 90:10 (v/v) and 80:20 (v/v) n-hexane:isopropanol solutions, respectively. These findings indicate that the one-step chromatographic purification strategy using normal-phase is feasible, thus opening the perspective of a fast large-scale production this paramagnetic spin probe.

Keywords: spin label, peptide, column chromatography, chiral compound

Introduction

In the early eighties, a chemical strategy for specifically and covalently coupling the paramagnetic amino acid derivative 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) to the N-terminal end of a peptide using the solid phase method was reported in the literature. About one decade later, an improvement in this experimental approach allowed the insertion of this spin probe into any internal position of the peptide backbone using the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc)-Nα temporary protecting group. Since the first synthesis of the Fmoc-TOAC derivative was described, a progressive increase in the use of this nitroxide-based...
achiral Cαα-tetrasubstituted α-amino acid in electron paramagnetic resonance (EPR) analysis has been observed in the literature as recently reviewed. As expected, most uses of TOAC are related to peptides, although also to macromolecules such as polymers.

Although TOAC has a great potential, the low reactivity of its α-amine function is indeed a serious drawback when the subsequent amino acid of the peptide sequence has to be coupled. In an attempt to solve this problem, the 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amino-4-carboxylic acid (POAC) spin probe, a β-amino acid described earlier and derived with the Fmoc group, was also proposed by us as an alternative paramagnetic probe for labeling peptide sequences. With the POAC probe, the coupling of the subsequent amino acid residue proceeded much faster than with TOAC, which comparatively required larger excess of reagents, repeated coupling reactions and use of more severe acylating conditions such as the increase in the reaction temperature. Unfortunately, POAC and its Fmoc-POAC derivative are chiral compounds due to the presence of two asymmetric carbon atoms (C3 and C4) in their pyrrolidine structure. On the other hand, previous X-ray diffraction studies have shown that POAC is composed of only trans isomers, thus indicating that the main target is to develop a fast and practical chromatographic separation of its (+)-(3R,4R) and (−)-(3S,4S) enantiomers (Figure 1).

To date, this problem has been solved either by direct chromatographic separation of these enantiomers in an analytical chiral column (Chiralcel OD-RH) with application of a reversed-phase elution mode [0.1 mol L−1 aqueous KPF6/acetonitrile (80:20, v/v)] or indirectly, by first synthesizing a POAC derivative with binaphthyl temporary group esterified to its carboxyl function and then chromatographically separating the derivatized POAC molecule. After this step, both enantiomeric POAC derivatives are saponified to recover the parent Fmoc-POAC enantiomers. After this sequence of procedures, it is finally possible achieving complete enantioseparation of these two compounds to further use them in labeling of a peptide sequence.

Despite this successful result, the need for a fast and simple purification strategy that allows large-scale production of both Fmoc-POAC enantiomers for use in peptide synthesis still remains. The present study thus describes an alternative and practical strategy for enantioseparation of a racemic mixture of the Fmoc-POAC derivative.

**Experimental**

**Materials**

All solvents and reagents and chemicals were of analytical grade and met the ACS standards. They were purchased from Aldrich or Sigma Co. (St. Louis, MO, USA).

The chiral Fmoc-POAC was synthesized as previously reported. Briefly, equimolar amounts of POAC in water/Na2CO3·10 H2O (prepared accordingly to earlier work) and Fmoc-succinimidyl carbonate in acetone were mixed 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 mol L−1 HCl. The desired product was extracted with ethyl acetate, washed with small portions of water, and dried over anhydrous Na2SO4 overnight. After filtration and evaporation, the product was crystallized in CHCl3/petroleum ether yielding Fmoc-POAC as light yellow crystals in 90% yield. The homogeneity of the sample was determined by analytical HPLC, ESI-MS: (M+) = 423; elemental analysis calculated for C24H27O5N2: C, 68.08; H, 6.28; N, 6.62; found: C, 67.9; H, 6.35; N, 6.60; IR (KBr) νmax/cm−1: 3444-3338 (νNH and OH); ~3030 (νArCH); 3000-2700 (νCOOH); 1723 (νCOOH and R–O–C–O–N); 1543 (dνH and νCN); 1450 (dCH3); 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.

**Methods**

**Analytical HPLC**

HPLC separations were carried out in either a Shimadzu (Tokyo, Japan) equipment, consisted of two pumps (models LC-20 AT and LC-10AD) and a detector (model SPD-20A), or a Waters (Milford, USA) system, consisted of two pumps (model 510), automatic gradient controller,
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The racemic \textit{trans}-Fmoc-POAC probe was synthesized and characterized accordingly to above detailed protocols as earlier reported.\textsuperscript{17} HPLC separation of the \((+)-(3R,4R)\) and \((-)-(3S,4S)\) enantiomers was achieved using a chiral analytical column (Lux Cellulose-2, 250 mm length x 4.6 mm i.d.) containing tris-(3-chloro-4-methylphenylcarbamate) cellulose-2 matrix as stationary phase. The mobile phase \((n\text{-hexane and isopropanol in different proportions})\) was used in a normal-phase mode isocratic-elution protocol. A constant flow rate \((1 \text{ mL min}^{-1})\) was used for elution of sample components.

Table 1 shows the values for retention time \((t_\alpha)\), separation factor \((\alpha)\), and resolution index \((R_s)\) of both enantiomers obtained with different proportions of the \(n\text{-hexane:isopropanol mixture as mobile phase. In addition, Figure 2 shows the elution profiles obtained in different isocratic runs in which the proportion of \(n\text{-hexane:isopropanol in the mobile phase varied from 10:90 (v/v) to 90:10 (v/v). It is noteworthy that an inversion occurs in the order of the eluting peaks. The \((-)-(3S,4S)\) enantiomer eluting faster when the amount of \(n\text{-hexane becomes higher than that of isopropanol. Optimized separation, as detected by the highest values for the chromatographic parameters \(\alpha\) and \(R_s\), was observed with the 90:10 (v/v) proportion of \(n\text{-hexane:isopropanol (Table 1 and Figure 2).}\n
CD experiments

CD spectra of the purified enantiomers (in methanol) were acquired in a Jasco spectropolarimeter (model 2095 Plus, Tokyo, Japan). Cylindrical quartz cells (0.1 mm path length) were used.

Results and discussion

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\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Enantiomers} & \textbf{\((-)-(3S,4S)\)} & \textbf{\((+)-(3R,4R)\)} & \textbf{\(n\text{-hexane:isopropanol (v/v)}\)} \\
\hline
\textbf{\(10:90\)} & 12.0 & 10.1 & 1.5 & 2.9 \\
\textbf{\(20:80\)} & 11.7 & 10.1 & 1.4 & 2.8 \\
\textbf{\(30:70\)} & 11.2 & 9.9 & 1.3 & 2.4 \\
\textbf{\(70:30\)} & 5.3 & 6.2 & 1.4 & 2.9 \\
\textbf{\(80:20\)} & 5.3 & 7.6 & 2.0 & 6.7 \\
\textbf{\(90:10\)} & 6.1 & 14.0 & 3.7 & 18.4 \\
\hline
\end{tabular}
\caption{Chromatographic parameters determined for the \((+)-(3R,4R)\) and \((-)-(3S,4S)\) enantiomers of Fmoc-POAC when submitted to HPLC normal-phase separation. Chromatographic conditions were as follows: sample: 1 mg mL\textsuperscript{-1}; 25 \mu L; column: Lux Cellulose-2, 250 mm length, 4.6 mm i.d.; mobile phase: \(n\text{-hexane:isopropanol (different proportions; isocratic elution); temperature: 27 \pm 2^\circ\text{C}; flow rate: 1.0 mL min}^{-1}; and detector wavelength: 265 nm.}
\end{table}

\textsuperscript{Retention time; \textsuperscript{a}separation factor; \textsuperscript{b}resolution index.}

The \(80:20 (v/v)\) solvent system also yields good results in terms of fractionation data of enantiomers, mainly when the time-consuming factor is also considered. Complete elution of both components is achieved after 7 min and 15 min for \(80:20 (v/v)\) and \(90:10 (v/v)\) mixed solutions, respectively (see Figure 2c vs. 2d). Despite this finding, the latter solution should be still taking into account in terms of practical effect as the main goal of this study lies upon the search of an efficient separation of both
enantiomers, mainly focused for application in large-scale protocol. In this case, the greatest separation observed between both Fmoc-POAC enantiomers (Figure 2d) with the 90:10 (v/v) n-hexane:isopropanol solvent can minimize the occurrence of a potential overlapping effect of peaks when larger amount of Fmoc-POAC has to be fractionated in the column. In this context, preliminary analytical chromatographic experiments increasing the flow rate (from 1.5 or 2.0 mL min\(^{-1}\)) revealed problems of backpressure in the column (data not shown), possibly induced by the viscosity of the organic solvent systems.

For identification of each of the Fmoc-POAC enantiomers, they were first eluted as earlier reported,\textsuperscript{18} using the chiral OD-RH Chiralcel column in HPLC reversed-phase mode. In this elution protocol, the (\(+-\))(3\(R\),4\(R\)) enantiomer, which eluted first in the Lux Cellulose-2 column (n-hexane:isopropanol, in 30:70 or 90:10, v/v), was in contrast, more retained in the column, thus eluting later than the (\(+\)-)(3\(R\),4\(R\)) partner. Noteworthy, it was also possible to detect comparatively smaller peaks for the (\(+-\))(3\(S\),4\(S\)) enantiomer in the chromatogram. These findings should be due possibly to the observed lower solubility of this enantiomer in comparison with the (\(+-\))(3\(R\),4\(R\)) compound, inducing its precipitation/aggregation during the different steps of the entire chromatographic fractionation procedure.

Mass spectra also confirmed the similar values for molecular weight of both enantiomers, and the EPR \(\tau_c\) values determined for these purified paramagnetic compounds (in methanol) were about 7 \(\times\) 10\(^{-11}\) s\(^{-1}\). LC/ESI-MS and EPR spectra of both Fmoc-POAC enantiomers are displayed as Figure 1 and Figure 2, respectively, in the Supplementary Information. Finally, CD spectra of these compounds (in methanol) shows that the (\(+\)-(3\(R\),4\(R\)) and (\(-\)-(3\(S\),4\(S\)) enantiomers exhibit positive and negative Cotton effects in the region of 230 nm, respectively (Figure 3).

As discussed above, enantiomeric separation of racemic Fmoc-POAC mixtures were previously achieved either directly using a chiral column in a reversed-phase elution protocol\textsuperscript{18} with the need of further desalting the mobile phase (KPF\(_6\) aqueous solution) or indirectly with previous esterification of this spin probe with a binaphthol (2,2'-dihydroxy-1,1'-binaphthyl) chiral auxiliary moiety to enhance HPLC enantioseparation and further saponification.\textsuperscript{19-21} Otherwise, the use of a normal-phase elution protocol with the alternative Chiralcel OD-H column, whose selector is the same as that of the OD-RH column, failed in separating the enantiomers\textsuperscript{18} with use of...
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Figure 3. CD spectra (195-260 nm) of trans-Fmoc-POAC (A) and its purified (+)-(3R,4R) (B) and (–)-(3S,4S) (C) enantiomers.

As mobile phase in appropriate proportion allowed easy isolation of the (+)-(3R,4R) and (–)-(3S,4S) enantiomers of this amino acid-type spin label. The results obtained with the 90:10 (v/v) or 80:20 (v/v) n-hexane:isopropanol mixtures exhibited high chromatographic resolutions with good separation factors and resolution indexes. In summary, an alternative and potentially useful HPLC enantioseparation, hopefully applicable for fast and large-scale chromatographic production of these two Fmoc-POAC enantiomers, was herein proposed for further application in the broad peptide and polymer fields.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Figure S1. ESI/MS spectra of (a) (+)-(3R,4R) and (b) (−)-(3S,4S) Fmoc-POAC enantiomers. Characterization: see details in the Experimental Section.

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Figure S2. EPR spectra of (a) (+)-(3R,4R) and (b) (-)-(3S,4S) Fmoc-POAC enantiomers. Characterization: see details in the Experimental Section.