http://dx.doi.org/10.5935/0103-5053.20130229

J. Braz. Chem. Soc., Vol. 24, No. 11, 1840-1845, 2013.
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0103 - 5053 $6.00+0.00

Article

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

Joao P. F. Vieira,[a] Erick F. Poletti,[b] Renata F. F. Vieira,[b] Vinicius Veredas,[a] Cesar C. Santana[c] and Clovis R. Nakaie*[,b]

[a]*Faculdade de Engenharia Química, Universidade Estadual de Campinas (UNICAMP), Av. Albert Einstein, 500, 13083-852 Campinas-SP, Brazil*

[b]*Departamento de Biofísica, Escola Paulista de Medicina (EPM), Universidade Federal de São Paulo (UNIFESP), Rua 3 de Maio, 100, 04044-020 São Paulo-SP, Brazil*

[c]*Núcleo de Estudos em Sistemas Coloidais do Instituto de Tecnologia e Pesquisa (NUESC/ITP), Universidade Tiradentes (UNIT), Av. Murilo Dantas, 300, 49032-490 Aracaju-SE, Brazil*

Neste trabalho desenvolveu-se um processo alternativo para separação cromatográfica dos enantiômeros (+)-(3*R*,4*R*) e (–)-(3*S*,4*S*) do β-aminoácido quiral *trans*-2,2,5,5-tetrametilpirrolidina-3-amino-4-carboxílico (POAC), que estava protegido no grupo amínico para posterior ligação a um peptídeo, polímero ou outra macromolécula. A enantioseparação foi obtida por HPLC usando uma fase estacionária normal à base de celulose quiral e eluição isocrática. O sistema *n*-hexano:isopropanol, sempre com maior quantidade do primeiro solvente, foi usado como fase móvel, pois forneceu os melhores resultados na separação dos dois componentes, constatado pelos valores mais elevados de fator de separação e de índice de resolução cromatográfica. Estes parâmetros apresentaram valores de 3,7 e 18,4 e de 2,0 e 6,7 nas soluções com proporção 90:10 (v/v) e 80:20 (v/v) de *n*-hexano:isopropanol, respectivamente. Estes dados indicam que a estratégia de purificação cromatográfica em uma única etapa usando fase normal é viável, abrindo assim a perspectiva de uma produção rápida e em grande escala desta sonda paramagnética.

In this work an alternative chromatographic process was developed for fractionating the (+)-(3*R*,4*R*) and (–)-(3*S*,4*S*) enantiomers of the chiral *trans* 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 component, 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)[1] to the *N*-terminal end of a peptide using the solid phase method[2] was reported in the literature.[3,4] 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)-N^{α} temporary protecting group.[5] Since the first synthesis of the Fmoc-TOAC derivative was described, a progressive increase in the use of this nitroxide-based

*e-mail: cnakaie@unifesp.br

achiral $C^{\alpha\alpha}$-tetrasubstituted α-amino acid in electron paramagnetic resonance (EPR) analysis[6] has been observed in the literature as recently reviewed.[7] As expected, most uses of TOAC are related to peptides,[8-11] although also to macromolecules such as polymers.[12-14]

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.[4,15] In an attempt to solve this problem, the 2,2,5,5-tetramethylpirrolidine-1-oxyl-3-amino-4-carboxylic acid (POAC) spin probe, a β-amino acid described earlier[1] and derived with the Fmoc group,[16] was also proposed by us as an alternative paramagnetic probe for labeling peptide sequences.[17] 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.[5,15] Unfortunately, POAC and its Fmoc-POAC derivative are chiral compounds due to the presence of two asymmetric carbon atoms (C_3 and C_4) in their pyrrolidine structure. On the other hand, previous X-ray diffraction studies[17] 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 (+)-(3*R*,4*R*) and (–)-(3*S*,4*S*) enantiomers (Figure 1).

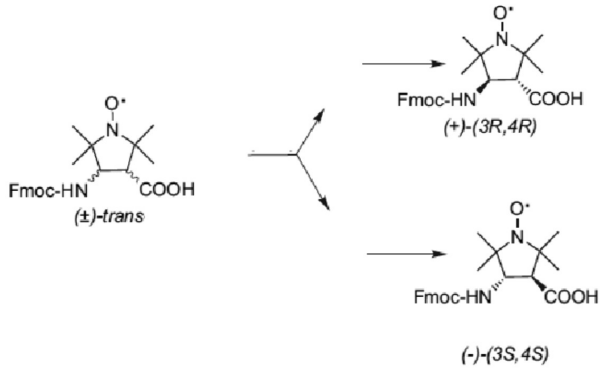

Figure 1. Fmoc-POAC enantiomeric structures.

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 KPF_6/acetonitrile (80:20, v/v)][18] or *indirectly,* by first synthesizing a POAC derivative with binaphthyl temporary group esterified to its carboxyl function[19-21] 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.[22]

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 recemic 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.[17] Briefly, equimolar amounts of POAC in water/ Na_2CO_3.10 H_2O (prepared accordingly to earlier work)[1] 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 Na_2SO_4 overnight. After filtration and evaporation, the product was crystallized in $CHCl_3$/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 $C_{24}H_{27}O_5N_2$: C, 68.08; H, 6.28; N, 6.62; found: C, 67.9; H, 6.35; N, 6.60; IR (KBr) ν_{max} / cm^{-1}: 3444-3338 (νNH and OH); ~3030 (νArCH); 3000-2700 (νCOOH); 1723 (νCOOH and R–O–C–O–N); 1543 (δNH and νCN); 1450 (δCH_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.

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,

manual injector (Rheodyne), detector (model 486), and data module (model 746). The analytical chiral column (Lux Cellulose-2, 250 mm length, 4.6 mm i.d., 5 μm particle size) containing tris-(3-chloro-4-methylphenylcarbamate) cellulose as stationary phase was purchased from Phenomenex, and used in a normal-phase elution mode with a mixture of *n*-hexane and isopropanol (different proportions, 27 ± 2 ºC). Other analytical chiral column (Chiralcel OD-RH, Daicel, Tokyo, Japan) column was used as a control in comparative assays.

LC/ESI-MS experiments

Electrospray ionization (ESI) operating in the positive ion detection mode was used for mass determination of enantiomers. The LC/ESI-MS experiments are performed on a system consisting of a separation module Waters Alliance model e2695 and a 2489 UV/Visible detector, both from Waters Systems (Milford, USA). This equipment is coupled to a mass detector Waters model 3100 and is controlled by a workstation ThinkCentre. The samples are automatically injected onto a Waters narrow bore Nova-Pak column C18 (2.1 x 150 mm, 60 Å pore size, 3.5 μm particle size). The elution is carried out with solvents A (0.1% TFA/H_2O) and B (60% acetonitrile/0.1% TFA/H_2O) at a flow rate of 0.4 mL min^{-1} using a linear gradient of 5-95% B in 30 min and UV detection at 220 nm.

EPR experiments

EPR spectra were acquired on a Bruker spectrometer (model ER 200D-SRC, room temperature). Fmoc-POAC samples (5 x 10^{-5} mol L^{-1}) were diluted in phosphate buffer (0.02 mol L^{-1}, pH 7.0; 22 ± 2 ºC) and placed in flat quartz cells for aqueous solutions (J. Scalon, Costa Mesa, CA, USA). Values of 0.5 G, 5 mW, 0.05 s and 9.5 GHz were used for modulation amplitude, microwave power, time constant and frequency parameters during EPR experiments. The spectra were collected with a field range of 100 G, centered approximately at 3450 G. The rotational correlation time (τ_C) values[23] were calculated as described elsewhere.[24]

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

The racemic *trans*-Fmoc-POAC probe was synthesized and characterized accordingly to above detailed protocols as earlier reported.[17] HPLC separation of the (+)-(3*R*,4*R*) and (–)-(3*S*,4*S*) 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*-hexane and isopropanol in different proportions) was used in a normal-phase mode isocratic-elution protocol. A constant flow rate (1 mL min^{-1}) was used for elution of sample components.

Table 1 shows the values for retention time (t_R), separation factor (α), and resolution index (R_s) of both enantiomers[25,26] obtained with different proportions of the *n*-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*-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 (–)-(3*S*,4*S*) enantiomer eluting faster when the amount of *n*-hexane becomes higher than that of isopropanol. Optimized separation, as detected by the highest values for the chromatographic parameters α and R_s, was observed with the 90:10 (v/v) proportion of *n*-hexane:isopropanol (Table 1 and Figure 2).

Table 1. Chromatographic parameters determined for the (+)-(3*R*,4*R*) and (–)-(3*S*,4*S*) enantiomers of Fmoc-POAC when submitted to HPLC normal-phase separation. Chromatographic conditions were as follows: sample: 1 mg mL^{-1}, 25 μL; column: Lux Cellulose-2, 250 mm length, 4.6 mm i.d.; mobile phase: *n*-hexane:isopropanol (different proportions; isocratic elution); temperature: 27 ± 2 ºC; flow rate: 1.0 mL min^{-1}; and detector wavelength: 265 nm

Enantiomers	(–)-(3*S*,4*S*)	(+)-(3*R*,4*R*)		
n-hexane:isopropanol (v/v)	t_{R1} / min[a]	t_{R2} / min[a]	α[b]	R_s[c]
10:90	12.0	10.1	1.5	2.9
20:80	11.7	10.1	1.4	2.8
30:70	11.2	9.9	1.3	2.4
70:30	5.3	6.2	1.4	2.9
80:20	5.3	7.6	2.0	6.7
90:10	6.1	14.0	3.7	18.4

[a]Retention time; [b]separation factor; [c]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 mail goal of this study lies upon the search of an efficient separation of both

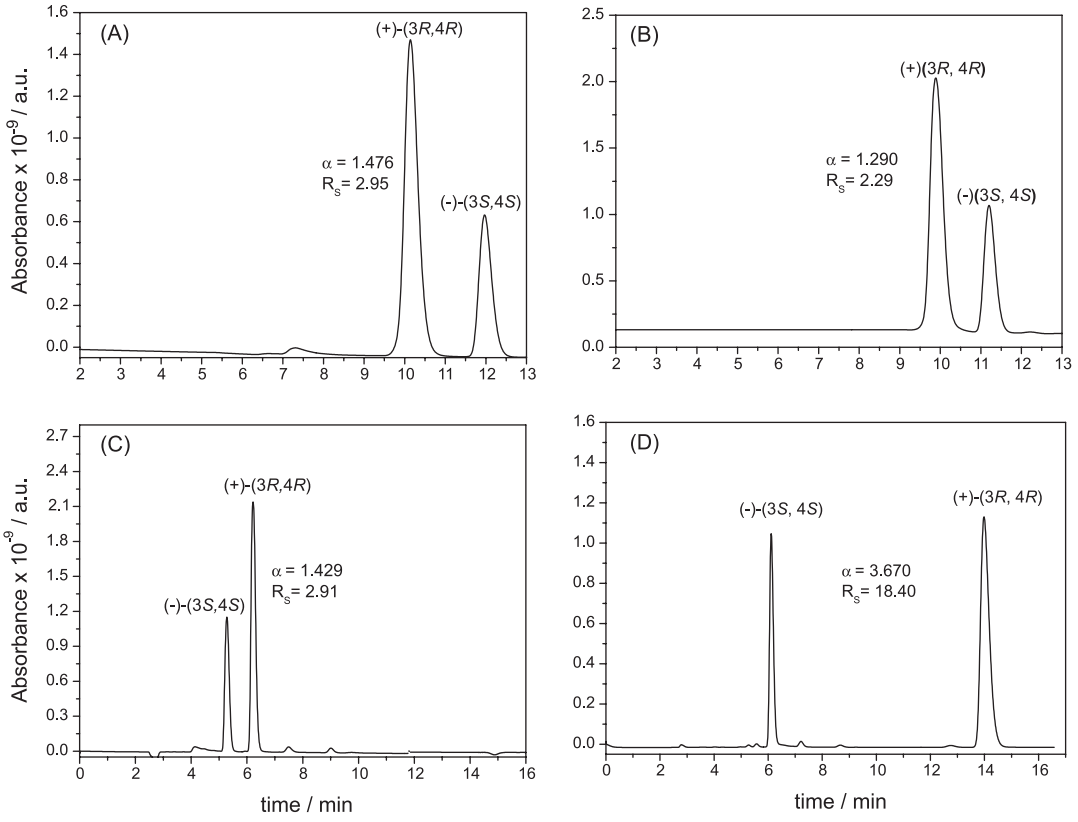

Figure 2. HPLC chromatograms of (+)-(3*R*,4*R*) and (–)-(3*S*,4*S*) *trans*-Fmoc-POAC enantiomers. Chromatographic conditions were as follows: sample: 1 mg mL^{-1}; column: Lux Cellulose-2, 250 mm length, 4.6 mm i.d.; mobile phase: *n*-hexane:isopropanol, v/v, [(A) 10:90; (B) 30:70; (C) 70:30; (D) 90:10], isocratic elutions; temperature: 27 ± 2 ºC; flow rate: 1.0 mL min^{-1}; detector wavelength: 265 nm.

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:isoproponal 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,[18] using the chiral OD-RH Chiralcel column in HPLC reversed-phase mode. In this elution protocol, the (–)-(3*S*,4*S*) 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 τ_C values determined for these purified paramagnetic compounds (in methanol) were about 7×10^{-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[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 binaphtol (2,2'-dihydroxy-1,1'-binaphthyl) chiral auxiliary moiety to enhance HPLC enantioseparation and further saponification.[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[18] with use of

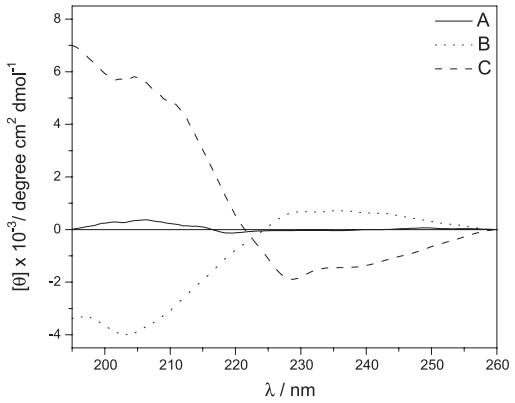

Figure 3. CD spectra (195-260 nm) of *trans*-Fmoc-POAC (**A**) and its purified (+)-(3*R*,4*R*) (**B**) and (–)-(3*S*,4*S*) (**C**) enantiomers.

n-hexane:isopropanol mixture, the same mobile phase that was used in the present study with the Lux Cellulose-2 column.

A possible explanation for the successful use of normal-phase separation of the Fmoc-POAC enantiomers with a Lux Cellulose-2 column, in comparison with the Chiralcel OD-H column, might due to the presence of a chorine atom in the resin matrix [(tris-(3-chloro-4-methylphenylcarbamate) cellulose] of the former column. The high electronegativity of this atom would maximize selectivity due to a specific interaction between each enantiomer and the column stationary phase, thus favoring a better chromatographic resolution in the apolar environment provided by the *n*-hexane:isopropanol mixtures. Accordingly, the best resolution was observed with the 90:10 (v/v) and 80:20 (v/v) *n*-hexane:isopropanol mixtures, which yielded greater values of separation factor and resolution parameter (Table 1).

Indeed, besides the search for large-scale production strategy of Fmoc-POAC enantiomers, additional objectives also of this study comprise the improvement in the enantioseparation strategy of other β-amino acid-type compounds. Large amount of recent reports have shown that the incorporation of this type of organic compounds may generate relevant oligomers in the chemical and biological fields.[27,28] In this sense, the investigation for alternative chromatographic purification strategies applicable specially for chiral products has resulted in publication of several studies[25,29,30] mainly targeting the isolation of chiral drugs with great potential for therapeutic purposes.[31,32]

Conclusions

This study showed that enantioseparation of the racemic *trans*-Fmoc-POAC spin probe for application in the chemistry of peptides and other macromolecules is feasible. A simple and one-step normal-phase mode chromatography using a Lux Cellulose-2 column and *n*-hexane:isopropanol as mobile phase in appropriate proportion allowed easy isolation of the (+)-(3*R*,4*R*) and (–)-(3*S*,4*S*) 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.

Acknowledgements

Grants from Grants from FAPESP (2007/56480-0) and CNPq are gratefully acknowledged. JPFV and RFFV are, respectively, recipients of PhD and postdoctoral fellowships from FAPESP. CRN and CCS are research fellows from CNPq.

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Submitted: April 8, 2013
Published online: September 18, 2013

FAPESP has sponsored the publication of this article.

J. Braz. Chem. Soc., Vol. 24, No. 11, S1-S2, 2013.
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Supplementary Information

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[a]Faculdade de Engenharia Química, Universidade Estadual de Campinas (UNICAMP), Av. Albert Einstein, 500, 13083-852 Campinas-SP, Brazil

[b]Departamento de Biofísica, Escola Paulista de Medicina (EPM), Universidade Federal de São Paulo (UNIFESP), Rua 3 de Maio, 100, 04044-020 São Paulo-SP, Brazil

[c]Núcleo de Estudos em Sistemas Coloidais do Instituto de Tecnologia e Pesquisa (NUESC/ITP), Universidade Tiradentes (UNIT), Av. Murilo Dantas, 300, 49032-490 Aracaju-SE, Brazil

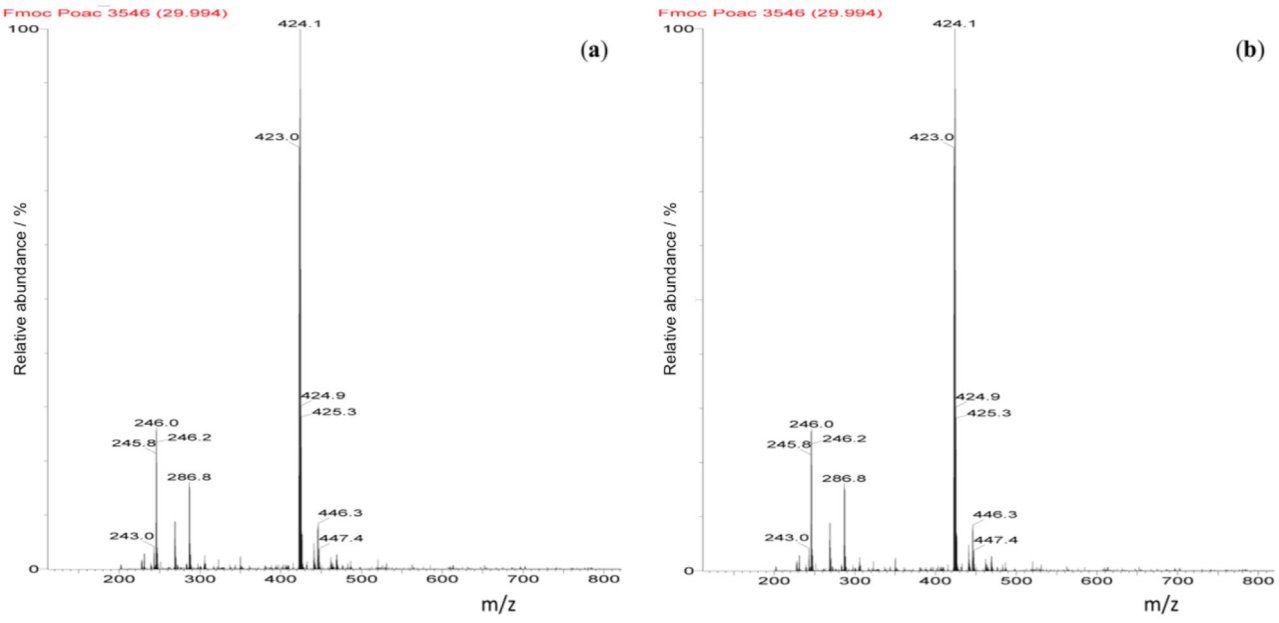

Figure S1. ESI/MS spectra of (**a**) (+)-(3*R*,4*R*) and (**b**) (–)-(3*S*,4*S*) Fmoc-POAC enantiomers. Characterization: see details in the Experimental Section.

*e-mail: cnakaie@unifesp.br

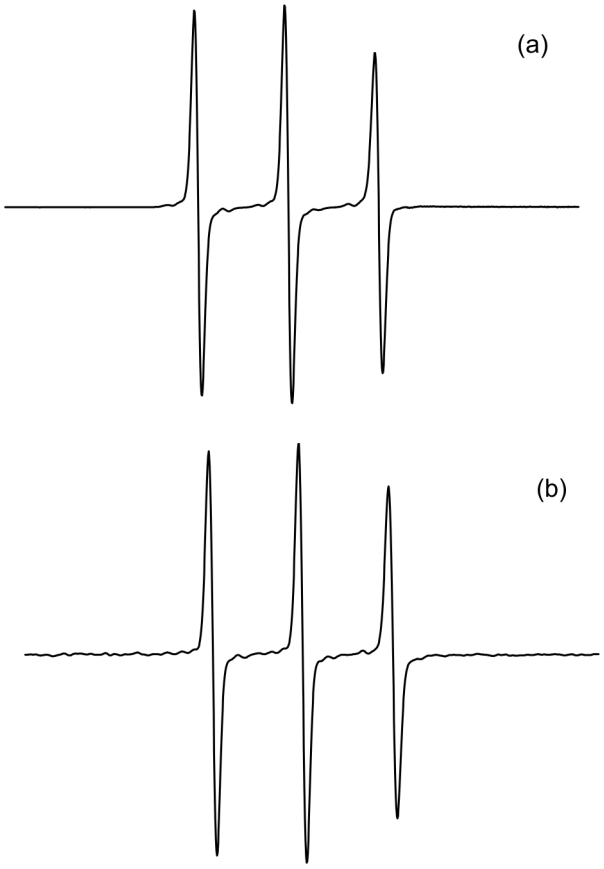

Figure S2. EPR spectra of (**a**) (+)-(3*R*,4*R*) and (**b**) (–)-(3*S*,4*S*) Fmoc-POAC enantiomers. Characterization: see details in the Experimental Section.