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A Spirocyclohexyl Nitroxide Amino Acid Spin Label for Pulsed EPR Distance Measurements

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Abstract

Site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy offer accurate, sensitive tools for the characterization of structure and function of macromolecules and their assemblies. A new rigid spin label, spirocyclohexyl nitroxide α -amino acid and its *N*-(9-fluorenylmethoxycarbonyl) (Fmoc) derivative, has been synthesized that exhibit slow enough spin echo dephasing to permit accurate distance measurements by pulse EPR at temperatures up to 125 K in 1:1 water:glycerol and at higher temperatures in matrices with higher glass transition temperatures. Distance measurements in the liquid nitrogen temperature range are less expensive than those that require liquid helium, which will greatly facilitate applications of pulsed EPR to the study of structure and conformation for peptides and proteins.

Keywords

spin labels; EPR; radicals; spiro compounds; spin relaxation

Introduction

Accurate and sensitive methods to probe structure and function of proteins, nucleic acids, and their assemblies are important for understanding disease processes and interventions, and contribute to drug design. Conformations and interactions of these macromolecules can be probed using nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), fluorescence spectroscopy, and small angle scattering (SAS).

An exciting and important development is site-directed spin labeling (SDSL) of biomacromolecules, mostly proteins, but recently also RNA and DNA, and the use of pulsed EPR spectroscopy to measure distances between labels separated by 2–8 nm.^[1–11] The spin-labeling/pulsed EPR technique is one of the best methods for accurate measurement of conformational changes and characterization of flexible regions of biomacromolecules, with minimum perturbation by relatively small labels. Applications are however impeded by the electron spin-spin relaxation properties of spin labels. In contrast to advances in SDSL and pulsed EPR techniques, improvements in spin labels have lagged behind.

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Supporting information for this article is available on the WWW under <http://www.chemeurj.org/> or from the author.

Double electron resonance (DEER) and double quantum coherence (DQC) methods for interspin distance determination are based upon detection of an electron spin echo. The time constant for decay of the echo as a function of the time between pulses is the phase memory time T_m . The longer T_m , the longer the distance one can measure, and the more precisely the distribution of distances can be defined.^[10, 12] Most spin labels, including the widely used 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-(methyl)methanethiosulfonate (MTSSL) (Figure 1), are nitroxide radicals in which the N-O radical moiety is sterically shielded by two gem-dimethyl-substituted quaternary carbons to provide kinetic stability. At temperatures above about 70 K rotation of these methyl groups averages inequivalent couplings of the unpaired electron to the protons on the EPR timescale, which decreases T_m ,^[13–15] and imposes a 50–65 K upper limit on the temperature at which distance measurements can be made with optimum performance.^[12] To achieve these temperatures liquid helium is required. The gemdimethyl structure motif in spin labels has remained unchanged since McConnell's pioneering studies.^[16] Recently, Velavan et al. showed that nitroxides such as 7-aza-4,12,15-trihydroxydispiro[5.1.5.3]hexadecane-7-oxyl (trihydroxy-DICPO) (Figure 1), which are stabilized by spirocyclohexyl groups at the 2-and 6-positions of the piperidine ring exhibit values of T_m in 1:1 water:glycerol that are long enough for pulsed EPR distance measurements up to about 125 K.^[17] New spin labels with cyclohexyl groups instead of gem-dimethyls would make it possible to perform distance measurements with less expensive liquid nitrogen.

In spin labels such as MTSSL, the linkage between the protein and the nitroxide N-O group is relatively flexible and the distance between the protein backbone and the N-O moiety is about 0.7 nm. Another spin label is 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) (Figure 1) – first synthesized by Rassat and Rey.^[18] This unnatural α -amino acid greatly improves EPR distance measurements because it can be rigidly built into a polypeptide chain, though it poses more strict restraints on backbone geometry than natural amino acids. Although incorporation of TOAC into peptides and proteins is more difficult compared to MTSSL, TOAC has been used to study protein folding, backbone dynamics, and peptide aggregation.^[8, 19–29]

Here we report the synthesis and characterization of spirocyclohexyl nitroxide α -amino acid **1** (7-aza-dispiro[5.1.5.3]hexadecane-7-oxyl-15-amino-15-carboxylic acid and its *N*-(9-fluorenylmethoxycarbonyl) (Fmoc) derivative, Fmoc-**1**, for incorporation into a peptide via solid-phase synthesis, analogous to that for TOAC (Figure 1).^[33] The bulky spirocyclohexyl groups may make incorporation of **1** into a peptide more challenging than of TOAC. These bulky groups may also pose greater constraints on conformations of the peptide backbone, as well as on packing of the side groups, however, the significance of these constraints is likely to depend on the peptide. To distinguish between the impact of intramolecular motions and molecular librations,^[13, 30, 31] the temperature dependence of T_m for **1** in 1:1 water:glycerol is compared with that in a poly(vinyl alcohol)–borate glass (PVA/borate)^[32] that has a glass transition temperature well above ambient. This new spin label has long enough T_m at 125 K to perform DEER with liquid nitrogen in 1:1 water:glycerol, and at higher temperatures in PVA/borate.

Results and Discussion

Synthesis

The synthesis of nitroxide α -amino acid **1** and its Fmoc-protection is outlined in Scheme 1. Nitroxide **2** was prepared by the modified method of Bobbit and coworkers,^[34] and then converted to hydantoin nitroxide **3** in high yield. Using the method for preparation of TOAC,^[18] direct hydrolysis of hydantoin nitroxide **3** under forcing conditions, provided α -amino acid nitroxide **1** in about 50% yield. However, it was difficult to isolate **1** with 100%

spin purity and free from the intermediate hydrolysis product (hydantoic acid).^[35] The increased steric shielding of the 15-position by the spirocyclohexane rings of **1**, compared to the methyl groups of TOAC, may contribute to the incomplete conversion of the intermediate hydantoic acid to **1**. The synthesis was modified by following Rebek's method for conversion of hydantoin-nucleosides to the corresponding amino acid-nucleosides,^[36] in two steps. Therefore, hydantoin nitroxide **3** was converted to the di-Boc derivative **4**, followed by hydrolysis under relatively mild conditions to provide **1** in the zwitterionic form, as precipitate from water.

The use of an amino acid as spin label is best implemented using Fmoc as an *N*-protecting group, to enable incorporation of the spin label in the peptide chain via solid-phase synthesis.^[37, 38] Therefore, **1** was *N*-protected to provide Fmoc-**1** in ~50% yield and ~100% spin purity (Scheme 1).

Spectroscopic data for **1** and Fmoc-**1** are similar to those reported for their TOAC analogues (Supporting Information).^[37, 38] In particular, ¹H NMR spectrum for a 0.18 M lithium salt of **1** in D₂O shows a broad resonance with a shoulder at about -1 ppm, compared to the single resonance at -5.7 ppm reported for a saturated solution of TOAC in D₂O at pH 12.^[39] Also, ¹H NMR spectra for **1** and Fmoc-**1** indicate that these nitroxide radicals possess high spin purity (Figures S17 and S18, Supporting Information).

X-ray crystallography

Although a single crystals of **1** could not be grown, the structure and conformation of hydantoin nitroxide **3** was determined by X-ray crystallography using synchrotron radiation (Figure 2).^[40] Two different crystal structures were observed, designated as structure A and B, and one of them is the solvent polymorph of the other. Both structures have space group P-1 with two unique molecules (molecule A and B) per asymmetric unit; the asymmetric unit of structure B includes half of a solvent molecule (ethyl acetate). The two structures differ in the connectivity of hydrogen bonds between hydantoin moieties (Figures S4 and S5, Supporting Information).

In both structures A and B, the piperidine ring of molecule A adopts a twist-boat conformation and that of molecule B adopts a chair conformation with an approximately *C*_s symmetry (Figure 2). In both unique molecules with chair conformations, the carbonyl group of the hydantoin is in the "equatorial" position. The spirocyclic cyclohexane rings of all unique molecules are in chair conformations, in which the nitroxide moiety is in an equatorial position. In peptides, the TOAC piperidine ring adopts a twist-boat conformation.^[41, 42]

The co-existence of unique molecules with twist-boat and chair piperidine rings in single crystals of **3** suggests that the energy difference between the two conformations is small. This energy difference was calculated using the B3LYP density functional method with the triple- ζ basis set 6-311+G(d,p).^[43] Optimization of the structures starting from the X-ray determined geometries for molecule A and molecule B in structure A gives a preference for chair over twist-boat by 0.9 kcal mol⁻¹ (corrected for zero-point energies).^[44]

Spin Echo Dephasing

The dephasing rates, $1/T_m$ (Figure 3), for **1** in 1:1 water:glycerol are similar to those for trihydroxy-DICPO,^[17] but they are very different from methyl-containing MTSSL which is commonly used for DEER experiments, for which rotation of the gem-dimethyls dominates dephasing between about 80 and 250 K.^[45] The relatively slow dephasing rates for **1** up to about 125 K will make it possible to perform DEER experiments in this liquid-nitrogen accessible temperature range. In 1:1 water:glycerol which has a glass transition temperature

about 175 K,^[46, 47] the dephasing rates for both MTSSL and **1** increase rapidly above about 125 K (Figure 3), which is attributed to increasing molecular motion as the glass softens.

The dramatic impact of the changes in $1/T_m$ from MTSSL to **1** on the spin echo intensity at the interpulse spacings (τ) of 1 to 2 μ s that are commonly used for DEER and DQC experiments is evident from the plots shown in Figure 4. The echo intensity at the τ values used for the DEER or DQC experiments determines the signal-to-noise. Longer T_m provides adequate signal-to-noise at longer τ values, which permits measurement of longer interspin distances and more accurate definition of distributions of interspin distances.

To determine whether piperidine ring dynamics impact spin echo dephasing at higher temperatures, echo decays were studied in PVA/borate, which has a glass transition temperature well above ambient. In this glass, $1/T_m$ for spirocyclic nitroxides **1** and trihydroxy-DICPO, which have 6-membered piperidine rings, exhibits negligible temperature dependence up to 125 K, then increases gradually with increasing temperature up to 350 K (Figure 5). In contrast, methyl rotation dominates echo dephasing between about 80 and 250 K for both MTSSL which has a 5-membered pyrroline ring and for 1-oxy-2,2,6,6-tetramethyl-4-hydroxypiperidine (Tempol), which has a 6-membered piperidine ring. For MTSSL and Tempol the temperature dependence of $1/T_m$ above 300 K is similar to that for **1** and for trihydroxy-DICPO. At 350 K T_m for trihydroxy-DICPO and for **1** are 200 ns and 70 ns longer, respectively, than T_m for MTSSL or Tempol.

Conclusion

The synthesis of **1** demonstrates that it is possible to prepare a spin label with a short rigid linker analogous to that of TOAC and the favorable electron spin relaxation properties of spirocyclohexyl nitroxides. The shorter distance between the α -carbon and the paramagnetic center for **1** than for MTSSL will decrease the range of conformations present in spin-labeled samples. The negligible temperature dependence of $1/T_m$ for **1** in 1:1 water:glycerol facilitates EPR distance measurements up to 125 K with liquid nitrogen. The spin lattice relaxation rates ($1/T_1$) for **1** at 125 K are about 4 times faster than for MTSSL at 65 K (Figure S2, Supporting Information). The differences in Boltzmann populations, which determine echo intensity, decrease approximately linearly with increasing temperature. The loss in signal intensity due to the decrease in the difference in populations of the spin states on increasing temperature from 65 K to about 125 K is approximately compensated by the faster pulse repetition rates that can be used at 125 K. Thus, incorporation of **1** into peptides and proteins should permit EPR distance measurements using liquid nitrogen at temperatures up to about 125 K with sensitivity similar to that which has previously been possible only by using liquid helium.

Spin echo dephasing experiments as a function of temperature in rigid PVA/borate demonstrate that the faster dephasing rate for MTSSL and **1** above about 150 K in 1:1 water:glycerol is due to the onset of motion as the glass softens. MTSSL and Tempol have different ring structures, but have similar $1/T_m$ rates in PVA/borate above room temperature, and $1/T_m$ of **1** is similar to that for MTSSL at 350 K, which suggests that piperidine ring dynamics do not dominate dephasing at temperatures up to 350 K. These results demonstrate that the use of rigid matrices with high glass transition temperatures should permit pulsed EPR distance measurements with **1** as a spin label at temperature well above 125 K. Slower dephasing for trihydroxy-DICPO than for **1** above about 200 K could be attributed to hydrogen-bonding between the three hydroxyl groups and the PVA/borate matrix, which would decrease librational motion.^[13, 31] The dependence of T_m on motion above about 200 K suggests that when **1** is incorporated into a peptide, T_m may increase.

Experimental Section

Experimental details on the synthesis and characterization of amino acid nitroxide and its Fmoc-derivative, Fmoc-1, as well as description of pulsed EPR experiments, may be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Nitroxide spin labels and related radicals.

Figure 2. Molecular structure and conformation for hydantoin nitroxide **3**. (a) Structure A. (b) Structure B. ORTEP plots with thermal ellipsoids set at 50% probability show one of the two unique molecules, without solvent of crystallization.

Figure 3. Temperature dependence of spin echo dephasing rates, $1/T_m$, in 1:1 water:glycerol for **1** (■), and MTSSL (●).

Figure 4. Intensity of spin echo in 1:1 water:glycerol at 105 K as a function of the time between pulses (τ) for **1** (solid red line), and MTSSL (dashed green line).

Figure 5. Temperature dependence of spin echo dephasing rates, $1/T_m$, in 1:1 PVA/borate for **1** (□), trihydroxy-DICPO (▶), Tempol (Δ), and MTSSL (○)

Scheme 1.

Synthesis of spirocyclic nitroxide α -amino acid **1** and its *N*-protected derivative Fmoc-**1**. (i) $(\text{NH}_4)_2\text{CO}_3$, NaCN, H_2O , 75 °C, 35 h; (ii) $\text{Ba}(\text{OH})_2$, H_2O , then $(\text{NH}_4)_2\text{CO}_3$, H_2O , 140–170 °C; (iii) Boc_2O , DMAP, THF, 25 °C, 3 h; (iv) LiOH , 40–45 °C, 12 h; (v) HCl , 0 °C, pH 6.5; (vi) Fmoc-succinimidyl carbonate (Fmoc-OSu), triethylamine, acetonitrile/water, pH 8–9, 25 °C, 45 min, then 20% aqueous citric acid, 0 °C, 15 min