Spin Labels

DOI: 10.1002/anie.201103315

# TOPP: A Novel Nitroxide-Labeled Amino Acid for EPR Distance Measurements**

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Electron paramagnetic resonance (EPR) spectroscopy is a well-established technique for the structural study of biomolecules.[1] In particular, over the past decade distance measurements between methanethiosulfonate spin labels (MTSSL) attached to cysteine residues of proteins or peptides have been successfully established.[2] Recently, not only the distance but also the relative orientation of essential amino acid radicals rigidly oriented in proteins were measured by pulsed electron-electron double resonance (PELDOR or DEER) spectroscopy.[3] To determine the relative orientation of topological units together with their intermolecular distance, spin labels with restricted mobility are required.[4] Since the MTSSL-modified cysteine contains single bond flexibility in the linker between the backbone and the nitroxide, the amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxyl (TOAC) is often used as an alternative for a conformationally rigid label in peptide studies (Figure 1). In TOAC the nitroxide group is incorporated in a six-membered ring attached to the backbone α carbon and it provides distance measurements with higher accuracy.[5] However, TOAC is an achiral amino acid with a tetrasubstituted α carbon affecting the peptide secondary structures.[6] Furthermore, the determination of the relative orientation of two spin-labeled units is hampered by various TOAC conformations.[7] Thus, for distance measurements and investigations of the relative orientation of peptide secondary structures or domains, a spin label is desirable that can be incorporated into peptides as regular chiral α-L-amino acid and provides conformational rigidity such that the nitroxide N–O unit is located at a defined position in space. However, a chiral amino acid has at least the Cα–Cβ bond as an axis of rotation, a fact that must be considered in the design of a new spin label with a defined nitroxide orientation. Furthermore, to facilitate the interpretation of distances it would be advantageous to locate the nitroxide bond as an elongation of the Cα–Cβ axis.

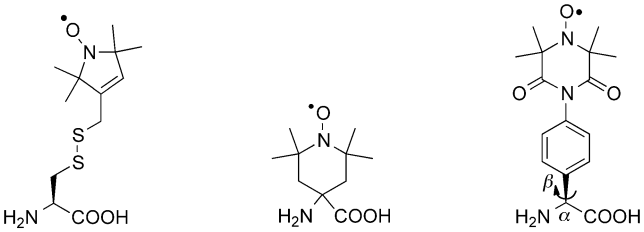

***Figure 1.*** Spin labels for EPR structural studies in proteins: MTSSL-modified cysteine (left), TOAC (middle), and TOPP (right).

Herein, we report the design and synthesis of the chiral amino acid 4-(3,3,5,5-tetramethyl-2,6-dioxo-4-oxylpiperazin-1-yl)-L-phenylglycine (TOPP), in which the nitroxide bond and the Cα–Cβ bond are aligned on the same axis (Figure 1). Further, we describe the incorporation of TOPP into an alanine-rich peptide and report EPR distance measurements in a doubly labeled peptide. The spectroscopic data are compared with those for the same peptide marked with conventional MTSSL. The design of TOPP takes advantage of a planar phenyl ring connected to Cα and *para* substitution by a dioxopiperazine that is also kept nearly planar by the amide functionalities and the geminal methyl residues. Therefore, a continuous axis from Cα to the nitroxide bond is expected.[8] The nearly collinear alignment of the nitroxide and Cα–Cβ bond was confirmed by analysis of the *N*-acetyl methylamine amino acid derivative of TOPP by means of DFT calculations (see the Supporting Information).

The key step in the synthesis of the spin-labeled amino acid Fmoc-TOPP-OH (**1**) was the copper(II)-catalyzed Chan–Lam coupling of 3,3,5,5-tetramethylpiperazine-2,6-dione (**2**) and boronic acid **3** (Scheme 1). Imide **2** was synthesized in three steps according to a published synthesis.[9] Starting from acetone, 2-amino-2-methylpropionitrile was generated and dimerized under reduced pressure to provide amine **4**. Cyclization of the biscyano amine **4** yielded imide **2** for the Chan–Lam coupling. The synthesis of amino acid Fmoc-TOPP-OH (**1**) is based on the functionalization of L-hydroxyphenylglycine (**5**). With the introduction of the carboxybenzyl (Cbz) and benzyl (Bn) protecting groups phenylglycine derivative, **6** is prone to racemization (Scheme 1). The reaction conditions throughout the synthesis were carefully adjusted in this regard. Treatment of **6** with triflic anhydride delivered enantiomerically pure aryl triflate **7**. In order to avoid racemization in subsequent steps, the *N*-Cbz group was replaced by two *N*-Bn protecting groups generating compound **8**. The arylboronic ester **9** was obtained by borylation of the aryl triflate **8** under Miyaura conditions.

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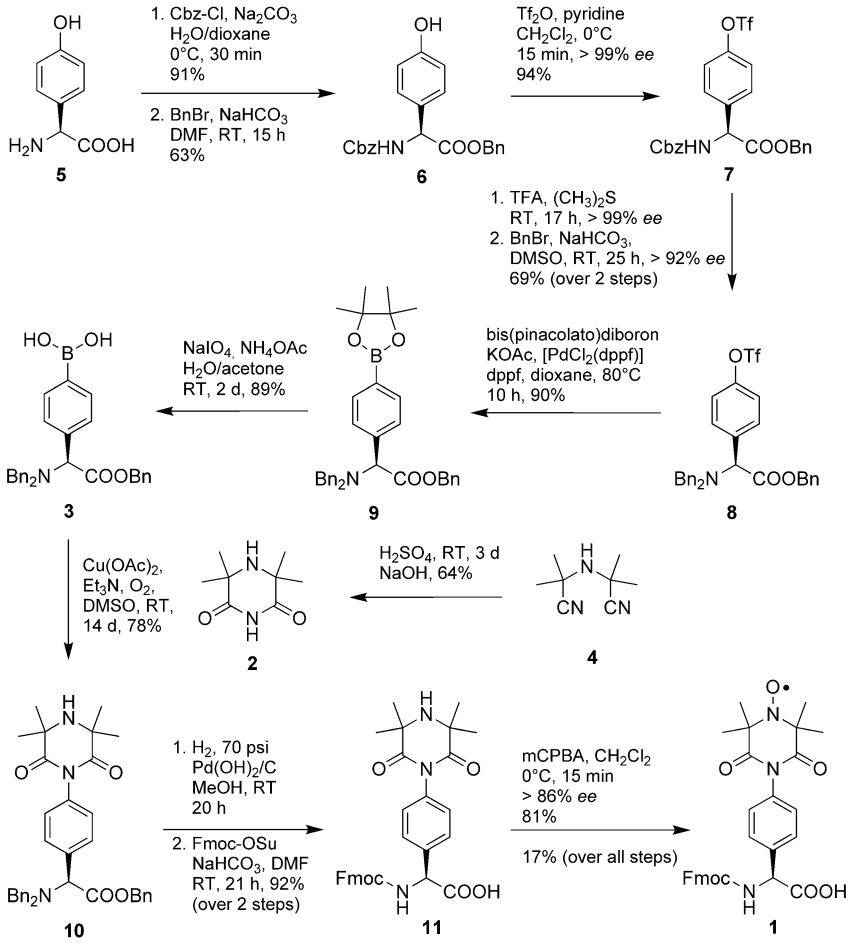
[**] We thank the Deutsche Forschungs-gemeinschaft DFG (SFB 803) for generous support.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201103315.




***Scheme 1.*** Synthesis of Fmoc-TOPP-OH (**1**). TFA = trifluoroacetic acid, DMSO = dimethyl sulfoxide, dppf = 1,1′-bis(diphenylphosphanyl)ferrocene, Fmoc-OSu = *N*-(9-fluorenylmethoxycarbonyloxy)succinimide.

The oxidation of compound **9** with $NaIO_4$ yielded the free boronic acid **3**, which was directly treated with 3,3,5,5-tetramethylpiperazine-2,6-dione (**2**) in a copper-mediated Chan–Lam coupling to assemble the amino acid core structure **10**. The benzyl groups were removed by hydrogenolysis at 70 psi using Pearlman's catalyst. The crude product was submitted to 9-fluorenylmethoxycarbonyl (Fmoc) protection yielding amino acid **11** with a good optical purity (> 86 % *ee*, determined by preparation and HPLC analysis of dipeptides generated by coupling with resin-bound D-alanine). Oxidation with mCPBA provided the target compound **1** in an overall yield of 17 % over 11 steps.

Also for Fmoc solid-phase peptide synthesis (SPPS) conditions were required that avoid racemization during coupling of the activated phenyl glycine derivative **1**. HBTU/HOBt activation was applied except for the spin-labeled Fmoc-TOPP-OH (**1**), which was coupled using 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT) and $NaHCO_3$ in THF. Cleavage and deprotection were achieved with TFA/TIS/$H_2O$ (90:5:5) and the respective peptide containing the spin label TOPP was obtained in its hydroxylamine form. In order to regenerate the nitroxide group, the peptide was treated with $Cu(OAc)_2$ prior to HPLC purification. The oligomers were characterized by high-resolution ESI mass spectrometry, CD spectroscopy, and EPR measurements.

Since alanine-rich peptides display a high propensity for forming α helices that even increases with decreasing temperature,[10] the alanine-rich peptide **P1** (Ac-AAAAK-**TOPP**-AKAAAAAKAAKA-**TOPP**-KAAAA-$NH_2$) containing two TOPP spin labels was used as a model system for the EPR distance measurements (Figure 2). The corresponding peptide **P2** (Ac-AAAAK-**Y**-AKAAAAAKAAKA-**Y**-KAAAA-$NH_2$) was prepared as reference in which the TOPP labels are replaced by tyrosine. Finally, a third peptide **P3** (Ac-AAAAK-MTSSL-AKAAAAAKAAKA-MTSSL-KAAAA-$NH_2$) was synthesized which contains cysteine for attaching the MTSSL label instead of the TOPP amino acid.

The α-helical content of peptide **P1** twice labeled with TOPP was characterized by circular dichroism (CD) and compared to that of peptide analogues **P2** and **P3**. The minimum at 208 nm and the shoulder at 222 nm are typical for an α-helical peptide conformation.[11] All peptides provide quite similar CD spectra in trifluoroethanol (TFE), indicating the expected helical content (Figure 3). The TOPP spin label can be incorporated in peptide helices without conformational distortion of the helix and without any indication for epimerization of the TOPP amino acids during oligomer synthesis.

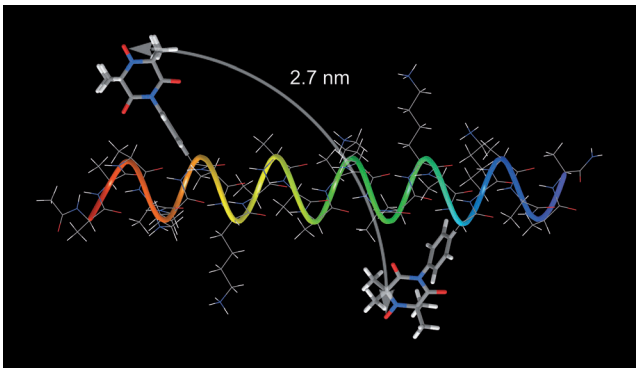



***Figure 2.*** Model representation of the α-helical structure of peptide **P1** (Maestro, version 9.1, Schrödinger, LLC, New York, 2010).

Since the α-helical content of alanine-rich peptides increases with decreasing temperature, the TOPP-labeled peptide **P1** was studied at various temperatures between 20 and −40 °C by means of continuous-wave (CW) EPR

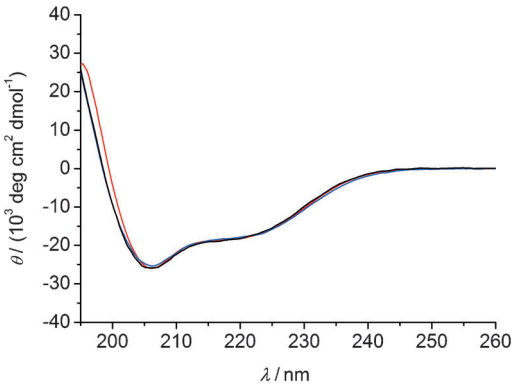



***Figure 3.*** CD spectra of peptides **P1** (blue), **P2** (red), and **P3** (black) in TFE at 20 °C.

spectroscopy.[10,11] Peptide **P1** was dissolved in TFE/glycerol (90:10) to obtain a cryogenic-type medium. According to the CW-EPR spectra, decreasing temperature results in reduced mobility of the spin label, which is caused by the increase in the α-helical fraction and not only by an increase in viscosity, as indicated by a control experiment with pure TOPP in the same medium (see the Supporting Information).

Peptide **P1** was dissolved in TFE/EtOH/$H_2O$ as well as in TFE/EtOH/MeOH and slowly cooled down to −80 °C (before flash-freezing in liquid nitrogen) in order to increase the fraction helicity. Analogous results were obtained in different mixtures (Supporting Information). The EPR distance measurements at the X-band (9 GHz) gave rise to well-defined dipolar oscillations characterized by several oscillation periods (Figure 4, blue curve). Furthermore, some Pake pattern deformation was observed for selected pump and detection frequencies (see the Supporting Information) indicating the contribution of orientational selectivity owing to the rigidity of the label.

In order to suppress orientational selectivity and extract a distance distribution, we performed an orientational averaging experiment at 11 field positions. However, the resulting trace (Supporting Information) did not differ significantly from the results of the single experiment recorded with standard PELDOR setup (Figure 4). Analysis of the traces indicated a narrow distance distribution centered at 2.80 nm with $\Delta r = 0.26$ nm. The distance is consistent with the estimated distance of 2.7 nm (Figure 2) for two rigid spin labels linked to a peptide with α-helical conformation ($\phi = -52°$, $\psi = -53°$). A small amount of a heterogeneous population of structures seems to be formed as indicated by a second peak (3.15 nm). This population might origin from a more poorly defined peptide.

A comparative study performed on the peptide **P3** led to an interspin distance of 2.26 nm (Figure 4, green curve). The distance distribution obtained for this sample is as narrow as that with the rigid label; this is in contrast to most reported cases, in which broad distance distributions are usually observed with MTSSL labels.[12] Nevertheless, the observed distance between the MTSSL is considerably less than that between the TOPP labels and must be related to a specific, unknown conformation of the flexible labels. Simple molecular modeling indicates that there are plausible conformations in agreement with the observed distance (Supporting Information); an unambiguous assignment of the spin–spin distance of 2.26 nm to a preferred conformation would require more sophisticated modeling. Therefore, the main advantage associated with the insertion of the TOPP spin probe as compared to MTSSL is the straightforward assignment of the distance owing to the rigid and readily predictable structure of the label.

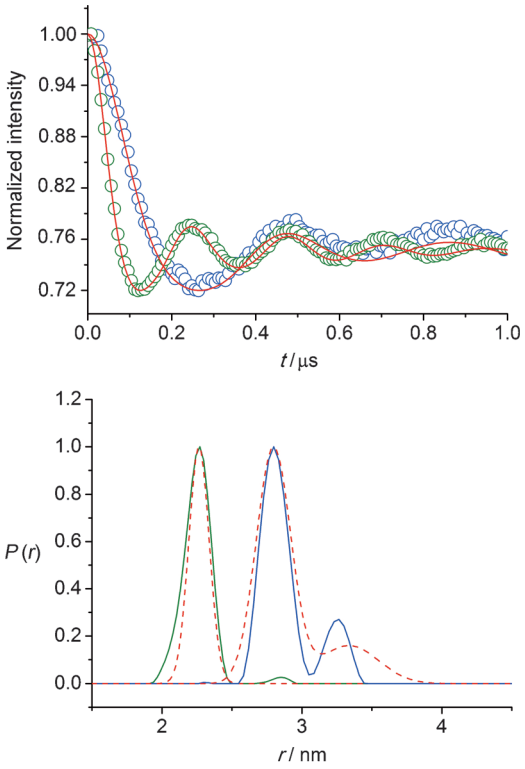



***Figure 4.*** Top: Time-domain DEER signal of double-labeled peptide **P1** in TFE/EtOH/MeOH (40:40:20). The data points (blue and green circles) represent the experimental data after background subtraction, and the red lines are the time-domain simulation of the data performed with DeerAnalysis2011.[13] Bottom: Best fits of the distance distribution obtained from Tikhonov regularization (green and blue curves) The dotted red curves correspond to the best Gaussian fit for the peptide **P3** and a two-Gaussian fit for the peptide **P1**, respectively. The DEER experiment was carried out on a Bruker ELEXYSIS 580 pulsed EPR spectrometer at 50 K; $\pi/2-\pi = 16–32$ ns; $\pi_{ELDOR} = 36$ ns; SPP = 50; SRT = 5 ms; scans = 249; acquisition time: 12 h.

In conclusion, we report the synthesis of a novel, rigid nitroxide-labeled amino acid TOPP that does not produce perturbation of the secondary structure, thus, providing a promising tool for structural studies of peptides and proteins. The design of the TOPP amino acid is based on the alignment of the nitroxide with the Cα–Cβ amino acid bond on one axis and the synthetic applicability with respect to racemization at Cα during amino acid and peptide oligomer synthesis. The present study illustrates the straightforward assignment of a spin– spin distance measured by pulsed EPR which is in contrast to the different and ambiguous result obtained with the commonly used MTSSL label. Furthermore, the predicted reduced mobility of the TOPP spin label represents a

potential advantage for its incorporation into transmembrane peptides for the structure determination of peptide arrangements at an atomic scale.

Received: May 14, 2011
Published online: September 5, 2011

**Keywords:** distance measurements · EPR spectroscopy · nitroxide radicals · peptide conformations · spin labels

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