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Semi-rigid Nitroxide Spin Label for Long-Range EPR Distance Measurements of Lipid Bilayer embedded β-Peptides

Janine Wegner,[a] Gabriele Valora,[b,c] Karin Halbmair,[b] Annemarie Kehl,[a,b] Brigitte Worbs,[a] Marina Bennati[a,b] and Ulf Diederichsen*[a]

Abstract: β-Peptides are an interesting new class of transmembrane model peptides based on their conformationally stable and well-defined secondary structures. Herein, we present the synthesis of the paramagnetic β-amino acid β^3-hTOPP (4-(3,3,5,5-tetramethyl-2,6-dioxo-4-oxylpiperazin-1-yl)-D-β^3-homophenylglycine) that enables investigations of β-peptides by EPR spectroscopy. This amino acid adds to the so far sparse number of β-peptide spin labels. Its performance was evaluated by investigating the helical turn of a 3_{14}-helical transmembrane model β-peptide. Nanometer distances between two incorporated β^3-hTOPP labels in different environments were measured using PELDOR/DEER (pulsed electron-electron double resonance) spectroscopy. Due to the semi-rigid conformational design, the label delivers reliable distances and sharp (one-peak) distance distributions even in the lipid bilayer. The results indicate that the investigated β-peptide folds into a 3.25_{14} helix and maintains this conformation in the lipid bilayer.

The specific biological function of proteins is intrinsically related to their structure, which in case of integral proteins is strongly influenced by the lipid environment. To investigate the complex interactions between integral proteins and their lipid environment, new classes of transmembrane model peptides are of interest. β-Peptides are promising candidates since they fold into various conformationally stable helical structures resembling those formed by α-peptides. The 3_{14}-helix is the most comprehensively investigated β-peptide secondary structure, mainly by X-ray and NMR spectroscopy.[1] Most investigations were performed in solution. However, these techniques suffer from limitations within lipid environment fostering the development of complementary and sensitive analytical methods, such as electron paramagnetic resonance (EPR) techniques.[2] In recent years pulsed electron double resonance (PELDOR, also called DEER) has gained more significance in studies of transmembrane peptides and proteins since distances between ~ 2 nm up to 10 nm can be measured.[3] In combination with site-directed spin labeling (SDSL) important structural information can be received also about biomolecules that lack intrinsic paramagnetic centers.[4] In this case, the quality of the experimental data and reliability of their interpretation strongly relies on the properties of the chosen paramagnetic label. The most established class of EPR spin probes are nitroxide based labels, most notably the frequently used MTSSL.[5] However, MTSSL is highly flexible which leads to low defined distance distributions especially in a lipid bilayer.[6] We have recently reported that distances measured in the lipid bilayer are affected by interactions of the label within the lipid environment.[7] Therefore, interpretation of observed distances based on the MTSSL label is often challenging. For α-peptides we introduced the semi-rigid nitroxide label TOPP (4-(3,3,5,5-tetramethyl-2,6-dioxo-4-oxylpiperazin-1-yl)-D-phenylglycine) that enables structural investigation of transmembrane α-peptides in their natural environment.[7,8] Despite its sterical demand, the label did not show influence on the respective secondary structure and delivered sharp distance distributions. However, spin probes for β-peptides are scarce which aggravates the search for proper labels to study β-peptide structures.[9] β-TOAC was designed as a spin label with reduced mobility, as required for the investigation of β-peptide structures in solution.[10] Nevertheless, like the α-TOAC the β-analogue is likely to influence the natural secondary structure formation.[10,11] Therefore, we report the synthesis of the conformationally semi-rigid 4-(3,3,5,5-tetramethyl-2,6-dioxo-4-oxylpiperazin-1-yl)-D-β^3-homophenylglycine (β^3-hTOPP) spin label for β-peptides. In a structural study of doubly-labeled 3_{14}-helical transmembrane β-peptides in solution and in lipid bilayers the β^3-hTOPP label provided well defined distances determined by PELDOR/DEER spectroscopy allowing to conclude on the conformation of the investigated membrane-incorporated β-peptide.

The synthetic route to the Fmoc-β^3-hTOPP-OH (**1**) started with commercially available D-4-hydroxyphenylglycine (**2**) (Scheme 1). To avoid side reactions during the homologation of **2** to the β^3-amino acid via the Arndt-Eistert reaction, the amino group was protected with benzyl chloroformate (Cbz) and the hydroxyl function with *tert*-butyldimethylsilyl (TBDMS), respectively. In the two step Arndt-Eistert homologation the α-amino acid **3** was converted into the diazo ketone **4** using diazomethane and then into the benzyl (Bn) protected β^3-amino acid **5** via silver(I)-catalyzed Wolff rearrangement. It is known that phenylglycine undergoes epimerization during the activation step of the carboxyl group.[12] However, determination of the enantiomeric excess (ee) demonstrated that β^3-amino acid **5** was synthesized in sufficient

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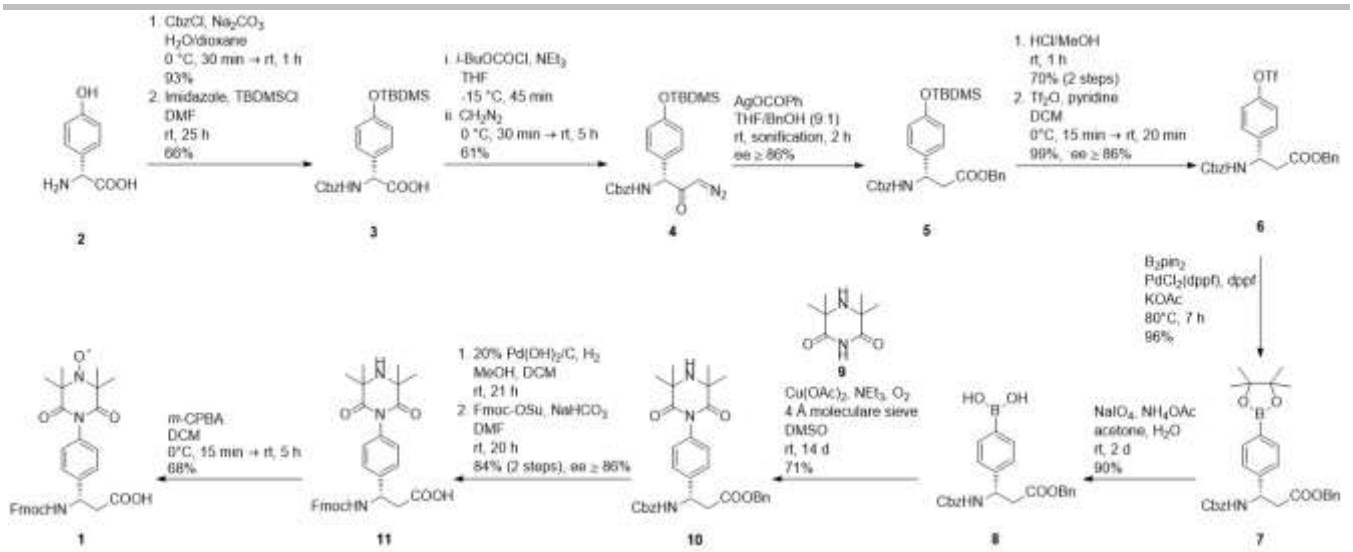

Scheme 1. Synthesis route of Fmoc-β^3-hTOPP-OH (**1**). AgOCOPh = silver(I)-benzoate; B_2pin_2 = bis(pinacolato)diborone; dppf = 1,1'-bis(phenylphosphino)ferrocene; Fmoc-OSu = *N*-(9-fluorenylmethoxycarbonyloxy)succinimide; *i*-BuOCOCl = *iso*-butyl chloroformate.

optical purity (≥ 86%, determined by HPLC on a chiral column, Figure S1). Subsequently, the hydroxyl group was deprotected and then functionalized with a triflate (Tf) group. Then, the β^3-amino acid **6** was converted into a boronic ester via a Miyaura borylation, and the resulting boronic ester **7** was hydrolyzed to the corresponding boronic acid **8**. The following Chan-Lam coupling between **8** and 3,3,5,5-tetramethylpiperazine-2,6-dione (**9**) is one of the key steps, since in this reaction the core structure **10** of the label is formed. In order to use the label in a fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) the Cbz and Bn group were cleaved through hydrogenation using the Pearlman's catalyst and the primary amine was re-protected with Fmoc using Fmoc-OSu. β^3-Amino acids are not prone to epimerization,[13] yet the optical purity of amino acid **11** was evaluated by HPLC using a chiral column and indeed the optical purity (ee ≥ 86%, Figure S2) did not change in the course of the synthesis. The oxidation of amino acid **11** using *m*-CPBA led to the desired Fmoc-β^3-hTOPP-OH (**1**) in a yield of 9% over 12 steps. We then employed the transmembrane β^3-peptide **P1** consisting of homologated proteinogenic amino acids as model system for the structural investigation of the 3_{14}-helix (Figure 1 A). Similar to other transmembrane model peptides, **P1** is composed of a hydrophobic stretch, in this case a β^3-hVal sequence, flanked by β^3-hTrp and β^3-hLys. Trp is known to serve

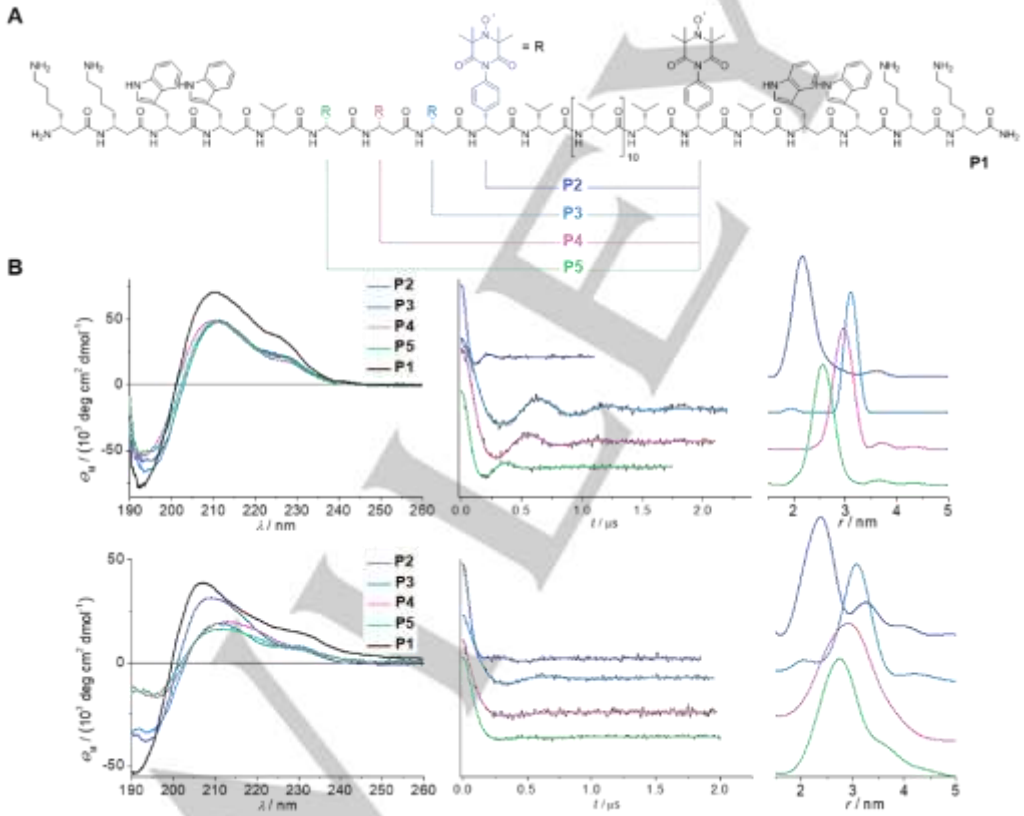

Figure 1. Peptide sequences and results of CD and PELDOR spectroscopy. **A**: Positions of β^3-hTOPP label (R) within the respective peptide sequence. **B**: Top: Measurements in MeOH. Left: CD spectra (*c* = 10 µM at 10 °C). Right: PELDOR results: Background corrected time traces (black lines), DEER analysis fit (coloured lines) and resulting distance distributions (*c* = 50 µM at 50 K, experimental modulation depths Δ were 15-20% (see Figure S7)). Bottom: Measurements in lipid bilayer. Left: CD spectra (SUVs of POPC, P/L = 1/20, *c* = 20 µM, phosphate buffer (50 mM, pH 7.5) at 20 °C). Right: PELDOR results: Background corrected time traces (black line), DEER analysis fit (coloured line) and resulting distance distributions (MLVs of d31-POPC, P/L = 1/3000, *c* ≈ 20 µM, Tris-HCl buffer (20 mM, pH 7.4) at 50 K, experimental modulation depths Δ were 15-20% (see Figure S7)). For detailed analysis of PELDOR raw data see Supplementary Information and Figs. S7a & S7b.

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as membrane anchor and orientates proteins in the lipid bilayer.[14] Recently, it was demonstrated that this peptide motif is incorporated into a lipid bilayer preserving a well-defined and conformationally stable 3_{14}-helical structure.[15,16] As indicated by FRET experiments, this type of peptide has a low tendency to aggregate.[15] In order to investigate the more detailed structure of the 3_{14}-helix in solution and in a lipid bilayer, the β^3-hTOPP labels were incorporated at various inter-residual separation resulting in four β^3-hTOPP double-labeled β^3-peptides **P2**–**P5**. The peptide sequences and labeling positions within the transmembrane domain are illustrated in Figure 1 A. The β^3-amino acids hVal, hTrp and hLys were synthesized via the Arndt-Eistert homologation. The labeled peptides **P2**–**P5** and the reference peptide H-hLys$_2$-hTrp$_2$-hVal$_{19}$-hTrp$_2$-hLys$_2$-NH$_2$ (**P1**) lacking β^3-hTOPP labels were synthesized using microwave-assisted Fmoc SPPS. All amino acids including the β^3-hTOPP were coupled using HATU/HOAt as coupling reagent and a mixture of 2,6-lutidine/DIEA as base dissolved in a mixture of LiCl/NMP/DMF/DMSO. After acidic cleavage (TFA/H$_2$O/TIS) from the resin, the β^3-hTOPP containing peptides were obtained as the reduced hydroxyl species. The peptides were oxidized using Cu(OAc)$_2$ dissolved in MeCN/MeOH, purified by HPLC and characterized by high-resolution ESI, CD and EPR spectroscopy. CD experiments were performed in MeOH and TFE and small unilamellar vesicles (SUVs) composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). According to the basic structural parameter of an ideal 3_{14}-helix, which is three amino acids per turn with a pitch of 0.5 nm,[17] the hydrophobic stretch of the peptides matches the hydrophobic thickness of POPC ($2D_C \approx 2.71$ nm) and DOPC ($2D_C \approx 2.68$ nm).[18] The CD spectra recorded in solution and lipid bilayer show the typical pattern of a right-handed 3_{14}-helix (Figure 1 B, left and Figures S3–4) with a minimum at 195 nm, a zero-crossing at 202 nm and a maximum at 210 nm.[19,15,16] A small shoulder around 225 nm results from absorption of the four hTrp.[20] Intensity differences in the spectra recorded with the β^3-hTOPP labeled peptides and the reference peptide can be explained by the different extinction coefficients of the peptides. Also, it is assumed that the small variations of the CD intensity and bands in the lipid bilayer occur due to concentration differences and the inhomogeneous lipid environment (cf. CD experiments in DOPC, Figure S4). The results indicate that the β-TOPP label does not disturb the β-peptide secondary structure in solution and within the lipid bilayer.

EPR experiments were performed with peptides **P2**–**P5** in multilamellar vesicles (MLVs) composed of POPC and deuterated POPC (D31-POPC) as well as in MeOH for comparison. To minimize intermolecular spin-spin interactions low peptide/lipid (P/L) ratios around 1/3000 were employed. The peptide incorporation into the lipid bilayer was verified first by continuous wave (CW)-EPR experiments in solution and in the lipid bilayer (Figure S5). Compared to the measurements in MeOH, the CW EPR signals of the nitroxide within the lipid bilayer are consistently broadened, indicating a decrease of mobility of both labels within a peptide due to the lipid environment. Furthermore, electron spin-echo envelope modulation (ESEEM) experiments showed a strong dipolar interaction between the nitroxide radical and the nearby ^{2}H from D31-POPC in H$_2$O (Figure S6). Instead, a strongly reduced ^{2}H-ESEEM signal was observed when the interaction of

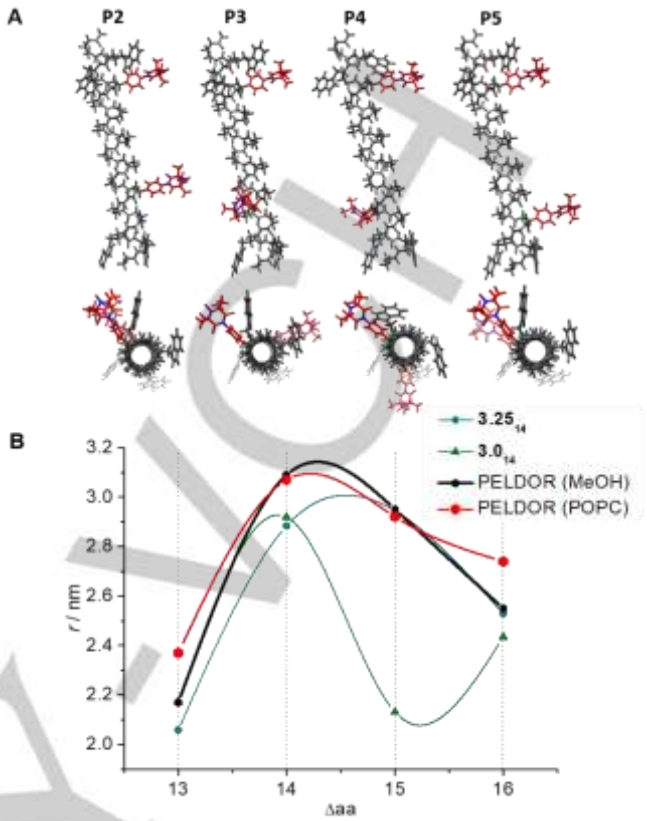

Figure 2. A: Simplified models of the labeled β^3-peptides created according to backbone angles of set **3.25$_{14}$**. **B:** PELDOR peak distances in MeOH (black) and POPC (red) in comparison with inter-spin distances extracted from β^3-peptide models. β^3-peptide models are generated from two different torsions angle sets named **3.25$_{14}$** (turquoise) and **3.0$_{14}$** (green). The curves illustrate the inter-spin distances dependence on the number of amino acids separating the two spin labels (Δaa).

the peptide in fully protonated POPC in D$_2$O was probed. This indicated that both spin labels were incorporated in the bilayer.[21] Thus, CW-EPR and ESEEM experiments are both consistent with the β^3-peptides located in the lipid environment.

PELDOR/DEER distance measurements performed at Q-band frequencies (34 GHz/1.2 T) allow for a more detailed discussion about the β^3-peptide structure in solution and in the lipid bilayer. To elongate spin relaxation times all PELDOR/DEER experiments presented here were performed in deuterated lipids (d31-POPC). Background corrected time traces and resulting distance distributions are illustrated in Figure 1 B (for detailed analysis see Figure S7). In frozen MeOH solution, time traces of all peptides show visible oscillations arising from one predominant distance, as displayed by the well-defined single-peak distance distributions. Peak distances and half widths of the distributions are listened in Table S2. The dipolar modulation of the time traces recorded in lipid environment is visible but slightly dampened as compared to solution. Analysis of each trace results in a broadened but, apart from **P2**, still single-peak distance distribution (Table S3). The peptides` structure is known to be very stable in the lipid environment.[16] However, Interactions with the lipid bilayer can increase the label`s conformational space resulting in broadening of the resulting distance distributions. According to our results, this effect is more pronounced in P4 and P5 where labelling positions are closer to the lipid head group tail

interface when compared to P2 and P3. The origins of an additional distance in **P2,** confirmed by a validation analysis (Fig. S7b), is still unclear but do not interfere with the overall interpretation of the results.

The determined peak distances enabled to examine the helical structure of the investigated peptide in more details. Simplified models of four doubly-labeled peptides based on 3_{14}-helices were constructed and corresponding inter-spin distances extracted (Table S5–8). Coordinates of the backbone atoms were generated from torsional angles known in literature (Table S4).[22,23] Since data for torsional angles of 3_{14}-helices differ in literature, idealized model peptides were generated according to two backbone angle sets named as **3.25_{14}** and **3.0_{14}** (models are illustrated in Figure 2 A and Figure S8–9). For the individual peptides, the inter-spin distances gained from the models were compared with the experimental ones by plotting the distances against the number of amino acids sequentially separating the two labels (Δaa) (Figure 2 B). The obtained curves indicate a better agreement of the experimental data with the structure created from the angle set **3.25_{14}** rather than **3.0_{14}**. In addition, the peak distances extracted from model **3.25_{14}** and the ones measured with the PELDOR experiment are in close agreement, in solution as well as in the lipid environment. Small deviations between predicted and experimental data most likely result from the structural simplification and the disregard of peptide-environment interactions in the model. This implies that a helical turn of the β^3-peptides is defined by approximately 3.25 amino acid residues,[23] which is supported by a NMR study of a β^3-eicosapeptide consisting of homologated proteinogenic amino acids in MeOH by Seebach and co-workers.[24] According to the results from PELDOR experiments in MLVs, the β-peptide helix conformation is preserved in the lipid environment. From the PELDOR experiments it can be concluded that the labeled β^3-peptides **P2**–**P5,** and thus the reference peptide **P1,** fold into a 3.25_{14}-helix that is conformationally stable in solution and in the lipid bilayer.

In conclusion, we have demonstrated that the combination of a new sophisticated spin label, such as the novel semi-rigid β^3-hTOPP label, with EPR and PELDOR spectroscopy provides a powerful and straightforward tool for the structural investigation of transmembrane β-peptides. Due to the semi-rigid design of the β^3-hTOPP label, site-specific distance measurements allowed for investigation of the peptide's backbone conformation with resolution at the molecular length scale even in a lipid environment. Comparison of the experimentally determined peak distances with structural models of β-peptides revealed that the oligomers fold into a 3.25_{14}-helix rather than an idealized 3.0_{14}-helical conformation. Since this method was also shown to be transferable to hydrophobic transmembrane conditions, it serves as a promising tool for the structural analysis of transmembrane β-peptides and membrane protein domains in case they are modified with β-peptide secondary structure mimics.

Experimental Section

All synthesis steps, optical purity experiments, sample preparation as well as all CD and EPR experiments are given in details in the Supporting Information.

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Keywords: β-Peptides • EPR spectroscopy • membrane proteins • nitroxide radicals • PELDOR • spin labels

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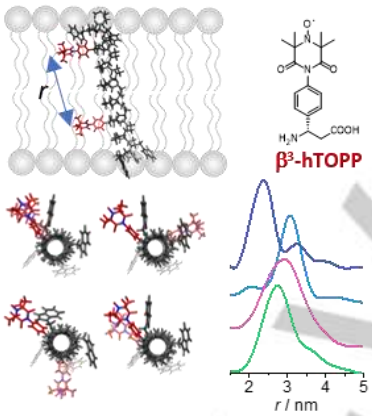
The novel semi-rigid spin label β^3-hTOPP has allowed for the investigation of a β-peptide structure by EPR spectroscopy. Well-defined inter-spin distances between two β^3-hTOPP labels in a model β-peptide have been measured in lipid bilayers using PELDOR spectroscopy. The labelling strategy has permitted to determine the peptide helical structure with resolution at molecular length scale.



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