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Versatile Trityl Spin Labels for Nanometer Distance Measurements on Biomolecules In Vitro and within Cells

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Abstract: *Structure determination of biomacromolecules under in-cell conditions is a relevant yet challenging task. Electron paramagnetic resonance (EPR) distance measurements in combination with site-directed spin labeling (SDSL) are a valuable tool in this endeavor but the usually used nitroxide spin labels are not well-suited for in-cell measurements. In contrast, triarylmethyl (trityl) radicals are highly persistent, exhibit a long relaxation time and a narrow spectral width. Here, the synthesis of a versatile collection of trityl spin labels and their application in in vitro and in-cell trityl–iron distance measurements on a cytochrome P450 protein are described. The trityl labels show similar labeling efficiencies and better signal-to-noise ratios (SNR) as compared to the popular methanethiosulfonate spin label (MTSSL) and enabled a successful in-cell measurement.*

In the last two decades, EPR based dipolar spectroscopy has become an increasingly effective tool for the measurement of nanometer distances in biomolecules.[1] Up to date, the most frequently applied kind of spin labels are nitroxides, which have been successfully used for a large variety of different distance measurements.[2] However, it is well-known that nitroxides are sensitive to reducing environments such as encountered within cells[3] and exhibit generally short relaxation times at room temperature in aqueous solutions.[4] To overcome these limitations, alternative spin labels are currently under development. Among them are derivatized nitroxides,[5] Gd^{III}-complexes[6] and triarylmethyl radical (trityl) spin labels.[7] The latter ones show microsecond relaxation times in buffer at room temperature[8] and are stable under the reducing conditions of the cell.[9] They are thus promising candidates for in-cell measurements at physiological temperatures. Furthermore, their narrow spectral width allows for a better SNR than achievable for nitroxides, helps avoiding orientation selectivity and can make single frequency distance measurements techniques like for example, relaxation-induced dipolar modulation enhancement (RIDME)[10,11] better suited methods than pulsed electron–electron double resonance (PELDOR or DEER).[12,13] Recently, trityl derivatives for labeling materials,[14] oligonucleotides[7] and proteins[15] have been reported. However, trityl labeling of biomolecules is still very limited. Oligonucleotides can only be labeled at their ends[16] and the two protein labels show either low labeling yields[17] or long linker groups.[18]

Here, a series of trityl spin labels **1**–**4** (Figure 1) with different bioconjugation moieties is presented, designed for the SDSL of proteins, DNA, and RNA by means of disulfide bridging, thioether bonding, Sonogashira coupling or copper-(I)-catalyzed alkyne–azide cycloaddition (CuAAC) reactions. Their labeling efficiencies are tested on the *Pseudomonas putida* cytochrome P450 CYP101 mutant C58 for labels **1**–**3**, and on the membrane protein FeoB mutant R152 from *E. coli* BL21 for label **4**. Labels **1** and **3** were then used to demonstrate first applications of a trityl–Fe^{III} distance measurement on cytochrome P450 in vitro and within cells. Previous in-cell measurements focused on interlabel distances

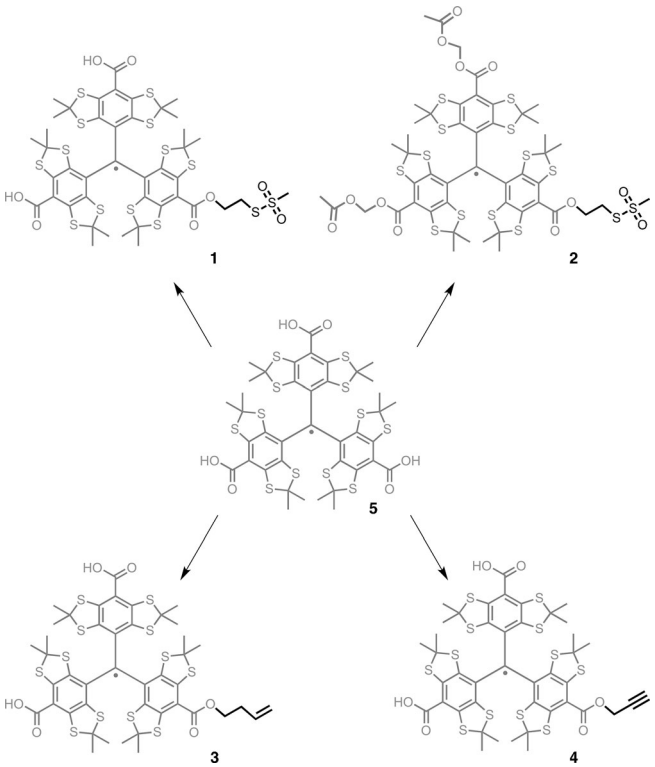

Figure 1. The synthesized trityl spin labels **1**–**4** and the trityl precursor **5** (bioconjugating moieties set in bold).

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Supporting information, including synthesis protocols, cw EPR simulations, and original RIDME time traces, and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201609085.

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involving gadolinium[6] and nitroxide[19,20] spin pairs. Recently, a trityl/nitroxide spin pair was investigated in native membrane proteins.[15] The report here shows distance measurements between a trityl spin label and a native metal cofactor in vitro and within cells.

The precursor compound for all presented spin labels is the Finland Trityl **5** (Figure 1), which was synthesized following a combination of known protocols.[21–24] Slight modifications led to a noticeable increase of the overall yield for **5** from 40% to 56% compared to the latest benchmark.[23]

The idea was to attach the bioconjugating moieties to **5** using a mild and viable esterification reaction. The use of conventional esterification reagents such as DCC and HOBt/BOP results in amidification products of **5** or no conversion at all. However, using 2-chloro-1-methylpyridinium iodide (CMPI) as activating reagent,[25] compounds **1**–**4** were obtained in high-yielding statistical esterification reactions (details see the Supporting Information). The monofunctionalization is essential, since it prevents label-mediated bridging between investigated sample compounds. In addition, the two carbon acid groups in **1**, **3** and **4** support the water solubility of the labels while the fully esterified and therefore more hydrophobic label **2** can be advantageous for the labeling of membrane proteins[15] and of oligonucleotides during synthesis.[26]

All four labels were characterized by high-resolution mass spectrometry (see the Supporting Information) and continuous-wave (cw) EPR measurements (see Figure 2). The ^{1}H and ^{13}C hyperfine coupling constants, linewidths and g-values are typical for trityl radicals (see the Supporting Information).[18]

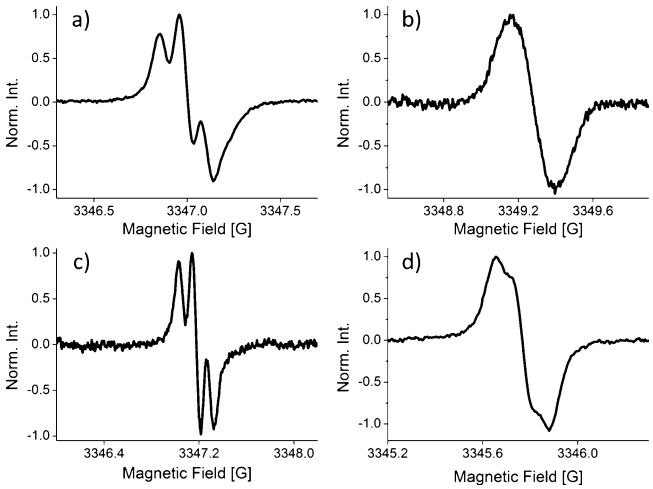
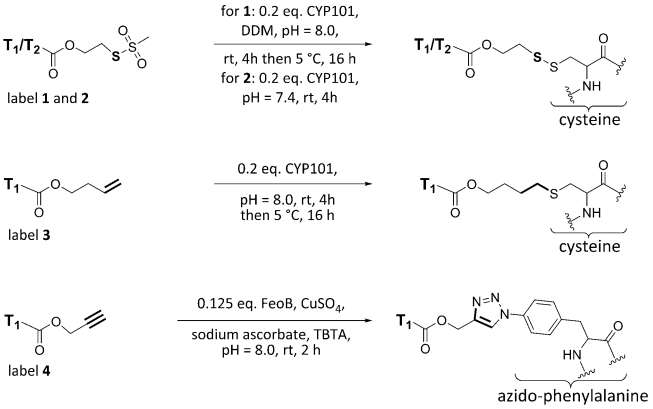


Figure 2. Room-temperature cw X-band EPR spectra of labels a) **1**, b) **2**, c) **3** and d) **4** in dry, degassed DMSO.

The stability of radical centers **1**–**4** was tested by following their EPR signal intensity at room temperature over a time span of 3 h in DMSO and in TRIS-buffer (pH 7.4) in the presence and absence of oxygen (see the Supporting Information). None of the labels showed a decay under these conditions rendering the radical center stable under the aqueous and atmospheric conditions of the labeling procedures.

The labeling procedures for trityl labels **1**–**3** were established on the C58 mutant of the cytochrome P450 CYP101 from *Pseudomonas putida*, and for trityl label **4**, on the FeoB mutant R152 carrying the unnatural amino acid 4-azido-L-phenylalanine (Scheme 1). All trityl labeling reactions where



Scheme 1. Labeling reactions with trityl labels **1**–**4**. DDM = *n*-Dodecyl-β-D-maltoside; TBTA = Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine.

performed under similar conditions as for the corresponding nitroxides but using a pH of 8 in order to increase the water solubility of the trityl labels (details see the Supporting Information). Labels **1** and **2** represent trityl analogs to the widely used nitroxide spin label MTSSL and connect to cysteine residues by disulfide bridging. The linker formed with **1** and **2** is three bonds longer than with MTSSL, but is one bond shorter than the linker of the recently published trityl label TAM-MTS.[18] However, in silico predictions show that this has only minor effects on the label volume. Compound **2** is not water soluble and the corresponding labeling procedure requires the usage of detergents. Label **3** also targets cysteine residues but forms a more stable thioether bond to the protein, thus making it suitable for in-cell experiments.[6,27] Label **4** targets commercially available non-canonical amino acids carrying an azide moiety and is bioconjugated by a CuAAC click reaction.[28] It can thus be used for orthogonal labeling strategies and in cases where cysteines are not suitable labeling sites. Additionally, compound **4** may also be attached to oligonucleotides either by CuAAC[29] or by palladium-catalyzed coupling reactions.[30] The persistence of the trityl radical against reducing agents (sodium ascorbate) is essential for the CuAAC method and the formed triazole linkage is again well suited for in-cell experiments.

The labeling efficiencies of labels **1**–**4** were determined by cw X-band EPR (see the Supporting Information) and compared to the same cytochrome P450 mutant labeled with the nitroxide MTSSL. The labeling efficiency of **1** and **2** is with ≈80% of the same order of magnitude as for MTSSL. However, it should be noted that the low polarity of **2** causes a visible amount of protein to precipitate during the labeling procedure. The labeling efficiency for **4** is with 56% noticeably lower than that of MTSSL, but is comparable with the labeling efficiencies reported for such a reaction involving

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a modified Alexa fluorophore (50%)[28] as well as with the reaction of an inversely functionalized nitroxide radical (64%).[31] The reason for the lower labeling yield as compared to MTSSL is that the in vivo protein expression leads to partial reduction of the non-canonical azide group of the amino acid.[32] The labeling efficiency for **3** is with 36% lower than what was reported for a similar reaction in the literature (70%),[33] which is attributed to the comparatively mild labeling conditions that were applied here.

Since CYP101 contains iron(III) as an intrinsic paramagnetic cofactor, labeling the protein with one trityl only is sufficient for enabling distance measurements between the trityl label and the iron center. Such measurements are desirable since metal cofactors play a critical role in many biological processes and their localization within a biological structure[34] is often crucial for the understanding of for example, a function of protein. Previous studies indicated, that for distance measurements involving the CYP101 low-spin iron(III), RIDME is better suited than PELDOR.[35] Therefore, trityl labels **1** and **3** were tested for such RIDME measurements. Labels **1** and **3** were chosen because **1** is the analog of the nitroxide standard MTSSL and **3** enables in-cell measurements.

In Figure 3a, the in vitro Q-band RIDME time trace of CYP101 labeled with **1** (C58T1) and the corresponding distance distribution are shown. The time trace is well-modulated and has a high SNR of 20.84 S/N min$^{-1/2}$. For the sake of comparison, also the RIDME data for the MTSSL-labeled analog is shown (C58R1). Here, the SNR was calculated as 13.08 S/N min$^{-1/2}$. Thus, the trityl sample provides a 60% better SNR than the MTSSL sample, which matches expectations, as only about 40% of the nitroxide spectrum but all of the trityl spectrum is excited. The observed modulation depth for C58T1 is 15% lower than the 35% obtained for C58R1. However, this can be attributed to a lower iron content in CYP101 C58T1 (see the Supporting Information), as the double integral of the iron signal for equally concentrated samples is 171 a.u. and 263 a.u. for C58T1 and C58R1, respectively, yielding a ratio of 0.65, which matches the ratio of the modulation depths. These differences in iron contents in CYP101 preparations are a known phenomenon.[31,35,36]

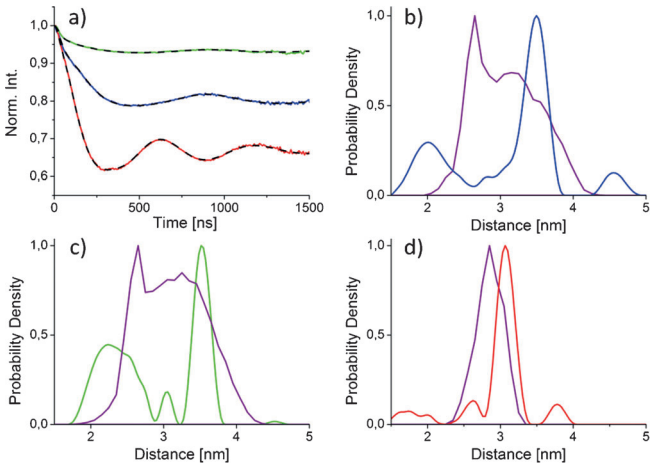

Figure 3. a) Q-band RIDME time traces of CYP101 mutant C58 labeled with **1** (blue solid line), **3** (green solid line) and MTSSL (red solid line) overlayed with the corresponding DeerAnalysis fits (black dashed lines). The corresponding distance distributions are shown in b) for C58T1, c) C58T3 and d) C58R1. The purple lines are the mtsslWizard predictions.

The distance distribution for C58T1 (Figure 3b) reveals two maxima at 2.0 nm and 3.5 nm with a standard deviation of 0.2 nm and 0.15 nm, respectively. To translate this spin–spin distances into biologically relevant C_{Alpha} distances, it is necessary to match them with in silico predictions, for example, using mtsslWizard.[37] At this point, however, the mtsslWizard prediction differs from the experimental result in terms of mean distance and width of distribution, although it encompasses the experimental distribution and also predicts the experimentally obtained bimodality, which is probably due to label–protein interactions. In order to exclude that the bimodality is an artifact of the background correction, the two peaks were demonstrated to prevail for various backgrounds (see the Supporting Information). Compared to C58R1, which revealed a nitroxide–Fe distance of 3.06 nm with a standard deviation of 0.11 nm (Figure 3d), the T1 side-chain shifts the distribution to 0.4 nm longer distances because of the three bonds longer linker and causes a broadening of the distribution.

Also, in the case of CYP101 mutant C58 labeled with **3** (C58T3), a modulated time trace could be obtained with a SNR calculated as 10.85 S/N min$^{-1/2}$. The decrease in SNR relative to CYP101 mutant C58T1 is due to the lower labeling efficiency of **3**. The modulation depth of this sample is only 7% and thus a factor of 3 lower than found for C58T1, but matches again with an iron content reduced by the same factor (see the Supporting Information). However, even with this rather low labeling efficiency and low iron content a good quality RIDME time trace could be obtained. The distance distribution for C58T3 reveals a trityl–Fe^{III} distance with maxima at 2.4 nm and 3.5 nm and standard deviations of 0.25 nm and 0.12 nm, respectively. Within error, the mtsslWizard prediction matches the experimental inasmuch as it predicts a bimodal distribution, but again differs in terms of mean distance and width of distribution. The distance distributions obtained for labels **1** and **3** are of a smaller width than those found in the literature for other trityl labels where standard deviations of 0.19 nm and 0.37 nm were reported.[15,17] This is partially due to a smaller label volume and a smaller number of rotatable bonds (see the Supporting Information), but it should be considered that the width of the distribution is also dependent on the labeling site and therefore not immediately comparable between different proteins.

In order to demonstrate that in-cell measurements are feasible, xenopus laevis oocytes were injected with CYP101 C58T3 (final in-cell concentration 250 μm). The injected cells were placed in a quartz tube, shock frozen and subjected to trityl-Fe^{III} Q-band RIDME measurements (Figure 4).

The obtained RIDME time trace (see the Supporting Information) was background corrected using a polynomial of the same order as for the in vitro time trace revealing a modulation depth of 4% and a SNR of 1.97 S/N min$^{-1/2}$. The

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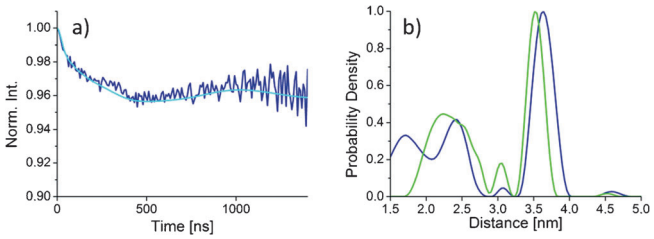

Figure 4. a) The time trace of in-cell RIDME on **3** (dark blue) and its DEER Analysis fit (cyan). b) The corresponding distance distribution (blue line) overlayed with the in vitro distribution (green line).

modulation depth is of a similar order of magnitude as compared to Q-band PELDOR results published for Gd^{III} spin labels,[6] although it should be stressed that the low modulation depth here is due to the difficulty in controlling the iron content of the CYP101 protein. Also, it should be noted that oocytes contain 40 μM Mn^{II} which may reduce the modulation depth because of relaxation enhancement of the Fe center compared to the in vitro measurement of the identically labeled protein. The in-cell SNR is less than for the in vitro measurement by a factor of 10, because of the factor 10 smaller protein amount injected into the cells. While no exact numbers are given for the aforementioned Q-Band Gd^{III} PELDOR measurements, the average SNR of the Gd^{III} experiments is visibly better than what was achieved for the measurement presented here. The most likely reason for this is the 10 fold higher label concentration used for the protein sample in the Gd^{III} case. Very good SNR was also achieved for a Gd^{III} model compound in cell where the injected amount of spins exceeds the amount of trityl used here by one order of magnitude higher taking spin labeling efficiencies into account. Other works on Gd^{III}–Gd^{III} distance measurements in E. coli cells show a moderately increased SNR but exhibit only a weak modulation depth of 0.6% or less, at in-cell concentrations that are again one order of magnitude higher than what is shown here.[38] Such high concentrations could not be achieved for the CYP101 protein used here and are beyond being biologically relevant in-cell concentrations. However, recent works on Gd^{III} at W-band could achieve timetraces with better SNR than the timetraces presented here at concentrations as low as 20 μM. While these results are very encouraging, the shown timetraces so far did not show any modulations beyond the initial decay and yielded very broad distance distributions, although this was to be expected because of the high flexibility of the investigated protein.[39,40] The SNR achieved in the trityl–Fe^{III} measurements presented here and its comparison to the results achieved with other spin labels shows that at this point, distances of about 3.5 nm constitute an upper limit for what can be measured within cells. This is mainly due to the drastic SNR decrease after 1000 ns but maybe balanced in cases where the percentage of protein bound metal ion is higher than obtained here, because it would increase the modulation depth.

The corresponding distance distribution is very similar to the in vitro experiment supporting a correct background treatment and can be reproduced from different sample preparations. It shows mean distances of 3.6 nm and 2.45 nm and standard deviations of 0.16 nm and 0.2 nm, respectively. The smaller peaks at 1.8 and 3.1 nm are attributed to the noise. The in-cell distance distribution is thus slightly broader than the distribution obtained in vitro, and shows a shift of the mean distances by roughly 1 Å. This can either be due to the effect of noise on the DEER analysis fit, or may signify a small change of conformation of the protein under in-cell conditions. The current data does not allow a distinction between the two possibilities, but future studies of different labeling positions may allow estimating the extent of a possible structural difference in vitro and in cell.

In summary, the synthesis of four new trityl spin labels, the corresponding labeling procedures and a first application in trityl–Fe^{III} distance measurements using RIDME in vitro and within cells was presented. To the best of our knowledge this is the first in-cell distance measurement with trityl spin labels and also the first measurement between a spin label and a native metal cofactor in cells. The presented trityl labels work very well for the RIDME experiment, where they have a significantly better SNR than nitroxide labels and allow an unambiguous distance determination as opposed to Gd^{III} labels, where higher harmonics of the dipolar coupling complicate the analysis of the distance distributions, although it was demonstrated on model compounds that this problem can be compensated.[41,42] In addition, Gd^{III} labels cannot be used for room-temperature distance measurements because of their fast relaxation times,[43] so that trityl spin labels represent an important addition to the spin label repertoire. The presented study does thus hold promise for the future investigation of metal ion binding sites under truly biological conditions.[20]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: distance measurements · EPR spectroscopy · in-cell spectroscopy · protein structures · spin labeling

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Communications

EPR Spectroscopy

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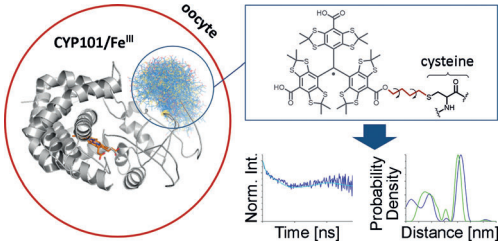
Versatile Trityl Spin Labels for Nanometer Distance Measurements on Biomolecules In Vitro and within Cells



Distance measurements: Four new triarylmethyl (trityl) labels for the site-directed spin labeling of proteins and oligonucleotides have been synthesized. Their application in EPR distance measurements is demonstrated on CYP101 protein under in vivo conditions

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