Protein Spin Labeling with a Photocaged Nitroxide Using Diels-Alder Chemistry

Anandi Kugele, Bjarne Silkenath, Jakob Langer, Valentin Wittmann, Malte Drescher

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EPR spectroscopy of diamagnetic bio-macromolecules is based on site-directed spin labeling (SDSL). Here, we present a novel labeling strategy for proteins. We developed and synthesized a nitroxide-based spin label that can be ligated to proteins by an inverse-electron-demand Diels-Alder (DAinv) cycloaddition to genetically encoded non-canonical amino acids (ncAA). The nitroxide moiety is shielded by a photoremovable protecting group (PPG) with an attached tetraethylene glycol unit to achieve water solubility. We demonstrate SDSL of two model proteins with the PaNDA (Photoactivatable Nitroxide for DAinv reaction) label. Our strategy features high reaction rates combined with high selectivity, as well as high stability of the nitroxide in E. coli lysate.

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Electron paramagnetic resonance (EPR) spectroscopy together with site-directed spin labeling (SDSL) is a valuable and established tool to elucidate structure, function and dynamics of proteins and protein complexes.[1] Nitroxide-based spin labels are the best established and convenient ones,[2] as they are small, non-disturbing and exhibit excellent spectroscopic properties.[3] In particular, they are perfect to display rotational dynamics via line shape analysis[3,4] and can be used for distance determination.[5] The most commonly used spin label for EPR spectroscopy is the methanethiosulfonate spin label (MTSSL).[6] It reacts specifically with accessible sulfhydryl moieties in proteins, i.e. cysteines. MTSSL application requires elimination of native cysteines by genetic engineering and introduction of strategically positioned ones. However, the choice of native amino acids as tag for spin labels limits bioorthogonality. When selecting genetically encoded non-canonical amino acids (ncAA) as targets for SDSL instead, selectivity can be achieved even in cells and removing of functionally-relevant cysteines is not required.[6] Utilizing an orthogonal aminoacyl-tRNA-synthetase (aaRS)-tRNA pair capable of selectively charging a nonsense suppressor tRNA (e.g. an amber codon) with an ncAA is an established method.[7] The expansion of the genetic code by incorporation of ncAA has brought a plethora of potential conjugation techniques, as a broad range of ncAA with specific functionalities is available.[6]

However, in combination with spin labeling only few ncAA and corresponding chemical reaction schemes were employed to date.[9] An ideal spin labeling procedure should exhibit high reaction rates, but still be selective. The independence from any potentially cytotoxic catalyst further simplifies the reaction, while water-solubility is a prerequisite for in vivo use. Gadolinium(III)- and trityl-based spin labels are stable in cells and very efficient for distance measurements, however, they cannot provide dynamic information as nitroxides do.[9b, 10] In contrast, traditional nitroxide-based labels feature limited redox stability in the cellular environment and their EPR signal vanishes within minutes.[11] Thus, for routine in vivo use, it is crucial to increase the nitroxide stability. Recently, a tetraethyl-modified maleimido-proxyl-based spin label with enhanced stability was introduced.[12] We here present a novel approach addressing both ncAA-mediated spin labeling and nitroxide stability (Figure 1).

Introduction of the novel label is achieved by inverse-demand Diels-Alder (DAinv) reaction[13] of an 1,2,4,5-tetrazenine with a strained alkyne (cyclooctyne)[14] or alkene [trans-cyclooctene, TCO].[15] This reaction forms the corresponding pyridazine and dihydropyridazine, respectively. Due to its excellent water-compatibility, the DAinv reaction has proven suitable for a broad range of biochemical applications both in vitro and in vivo.[13, 16] Strain-promoted DAinv cycloadditions have not been used for site-directed spin labeling of proteins so far (notably, recently an in vitro transcribed RNA segment was site-specifically labeled with a tetrazine-nitroxyl moiety[17]). Moreover, we aimed for addressing nitroxide stability by using a protection strategy. So far, alkylation,[18] silylation,[19] acylation,[20] as well as photoremovable protecting groups (PPGs)[21] have been demonstrated to protect nitroxides and to release them when needed. Especially photoirradiation for deprotection is interesting, as it enables spatial and temporal control over the release of functional groups. In particular, o-nitrobenzyl derivatives for diverse functionalities, even for native amino acid side chains,[22] were pioneered since 1966 and are since the best established PPGs.[23] Their application for the protection of nitroxide spin labels during oligonucleotide synthesis has been introduced by Seven et al. in 2014 and pursued by Weinrich et al.[21, 24] Figure 1 A depicts the structure of the new spin label, named PaNDA, referring to “Photoreactivatable Nitroxide for DAinv reaction”. It is composed of a 3,6-

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**Abstract:** EPR spectroscopy of diamagnetic bio-macromolecules is based on site-directed spin labeling (SDSL). Here, we present a novel labeling strategy for proteins. We developed and synthesized a nitroxide-based spin label that can be ligated to proteins by an inverse-electron-demand Diels-Alder (DAinv) cycloaddition to genetically encoded non-canonical amino acids (ncAA). The nitroxide moiety is shielded by a photoremoveable protecting group (PPG) with an attached tetraethylene glycol unit to achieve water solubility. We demonstrate SDSL of two model proteins with the PaNDA (Photoactivatable Nitroxide for DAinv reaction) label. Our strategy features high reaction rates combined with high selectivity, as well as high stability of the nitroxide in *E. coli* lysate.
diarylsubstituted 1,2,4,5-tetrazine as diene component of a DArin reaction attached to an α-nitrobenzyl-protected TEMPO derivative that delivers an EPR-active nitroxyl radical upon photoradiation and subsequent air oxidation. Oligoethylene glycol chains have been used before to enhance water solubility of tetrazine derivatives.\(^{[25]}\) Accordingly, we attached a tetraethylene glycol monomethyl ether to ensure water solubility of the PaNDA label. This approach is elegant insofar, as the rather bulky PPG along with the tetraethylene glycol chain is cleaved off by irradiation.

For the synthesis of PaNDA label 1, carboxy TEMPO 5 was quantitatively transformed into hydroxylammonium salt 6 making use of the acid-dependent redox triad of nitroxy radical, oxoammonium cation, and hydroxylamine (Scheme 1 A).\(^{[26]}\) Conveniently, the carboxylic acid is simultaneously protected under these conditions. Nitrobenzaldehyde 7 was reduced to the benzyl alcohol and the phenol group selectively alkylated with tetraethylene derivative 8 to yield nitrobenzyl alcohol 9 (Scheme 1 B). Appel reaction gave benzyl bromide 10 that was used to alkylate 6 followed by ester hydrolysis yielding carboxylic acid 11. Finally, amide bond formation with tetrazine derivative 12\(^{[27]}\) delivered PaNDA label 1 in an overall yield of 42 % starting from nitrobenzaldehyde 7.

To initially test and optimize the deprotection we evaluated the UV/Vis absorption spectrum of the PaNDA spin label (Figure 2 A). It exhibits a prominent feature for the photocage group at approx. 300 nm. To this end, we tested the time-dependent deprotection efficiency at 302 nm. LC-MS of the PaNDA spin label proved almost complete conversion to the desired nitroxide after irradiation for 10 min (Figure 2 B). Accordingly, an EPR signal appeared only after irradiation (Figure 2 C).

To test and establish our labeling and deprotection method with proteins, we chose the green fluorescent protein (GFP) and the E. coli oxidoreductase thioredoxin (TRX). TRX was the first protein to be modified by a DArin reaction in 2008,\(^{[15a]}\) and there are previous EPR studies available for data comparison.\(^{[16a, 16c, 16e]}\) Both model proteins exhibit native cysteines, thus providing the possibility to test for orthogonal labeling without affecting cysteines. The first ncAA that can undergo a DArin reaction, a lysine-derived cyclooctyne, was introduced for copper-free click reaction in 2011,\(^{[29]}\) before also a tetrazine,\(^{[30]}\) norbornenes,\(^{[14a, 31]}\) trans-cyclooctenones,\(^{[14a]}\) and a spherohexene\(^{[32]}\) were genetically encoded. The rationally designed Methanosarcina mazei mutant tRNA\(^{[P]}\)/PyrrS\(^{SF}\) (Y306A, Y384F) possesses an enlarged binding pocket suitable to incorporate these bulky ncAA in response to the amber stop codon.\(^{[14a, 29, 33]}\) Therefore, we co-expressed amber stop codon mutants of C-terminally His6-tagged model proteins together with the previously reported tRNA\(^{[P]}\)/PyrrS\(^{SF}\) synthetase in E. coli. We tested two different ncAA, i.e. \(N^\delta-(\text{cyclooct-2-yn-1-yloxy})\text{carbonyl})L\)-lysine (SCO-L-lysine 2a, Figure 1 B) and \(N^\delta-(\text{(E)-cyclooct-2-en-1-yloxy})\text{carbonyl})L\)-lysine (TCO-L-lysine 3a), which are well known for high integration rates and which exhibit excellent reaction kinetics.\(^{[14a, 34]}\) The expression in presence of 1 mM ncAA yielded between 4 and 7 mg/L of purified full-length GFP-Y39→2a, GFP-Y39→3a, TRX-R74→2a or TRX-R74→3a, respectively (Figure S11-S12 and S16-S17). We even succeeded in the incorporation of SCO in response to two amber stop codons in a protein, which was, to our knowledge, not reported before (TRX-G34/R74→2a; Figure S13).

Next, we wanted to assess the spin labeling potential of the ncAA-containing proteins with the PaNDA spin label \(\text{in vitro}\) (for the general procedure see Figure 1 C). For this purpose, we mixed the purified proteins with the PaNDA spin label and established mild and universally applicable reaction conditions (30 min, 20 °C). We separated the labeled proteins from excess label \(\text{via Ni-NTA resin, and measured EPR spectra before and after irradiation (Figure 3). The data recorded before irradiation indicate that the PPG is stable enough to survive the labeling and washing procedure. EPR spectra after irradiation show, that both model proteins comprising 2a or 3a were successfully labeled and deprotection works. Spectra of the wildtype (wt) proteins incubated with the PaNDA spin label exhibit no EPR signal, indicating a high chemoselectivity of this reaction (Figure 3, upper line). By circular dichroism spectroscopy we further proved that the labeled proteins kept their secondary structure throughout the process (Figure S20). By analyzing the double integral of the EPR spectra, we found that the deprotection of the PaNDA spin label was maximal already after 2 min of irradiation at 302 nm (Figure S21). To substantiate our data, full-length ESI-MS spectra were recorded (Figure S18-S19). Peaks can be assigned to successfully labeled and deprotected protein, but no mass peaks were found referring to still protected protein-label-complex (occurrence of peaks with \(\Delta = -150\) Da for 2a or 2b, or \(\Delta = -152\) Da for 3a or 3b indicated partial degradation of the ncAA to lysine). Thus, the design of our spin label allows for fast and selective labeling as well as convenient and quantitative deprotection.

Next, we assessed the performance of our protection strategy in biological environments. We reasoned, that reducing conditions could potentially intervene with the spontaneous oxidation step converting the irradiation-derived hydroxylamine into a nitroxide.\(^{[21]}\) To this end, we chose E. coli lysate, because it contains relevant cell components and provides reducing conditions. We first labeled TRX-R74→2a with the PaNDA spin label and deprotected the obtained TRX-R74→2b as described above. Then we incubated the lysate with this protein to confirm nitroxide degradation. After 80 min, the EPR signal was < 1 μM (Figure 4). Then, we incubated labeled and still protected TRX-R74→2b with the same amount of fresh E. coli lysate for 80 min. No EPR signal was detected in this period of time, meaning that the protecting group remained stable on the protein. After irradiation, the EPR signal increased, resulting in approx. 50 % spin concentration compared to deprotection in buffered solution. This demonstrates that a spontaneous oxidation step forming the nitroxide is actually possible in reducing environment, and that, in principle, the deprotection can be performed in E. coli lysate to recover the EPR signal. However, points that need to be addressed before the system can be used for
in-cell EPR measurements involve the delivery of the PaNDA spin label into cells and removal of unbound label. Moreover, labeling and deprotection efficiencies need to be confirmed in cells. In conclusion, we have developed an innovative SDSL approach based on genetically encoded ncAAs amenable to DAInv chemistry and on a newly designed spin label. The PaNDA spin label provides an appealing combination of a tetrazine moiety and a PPG. The use of DaInv chemistry for spin labeling illustrates the assets and versatility of this reaction. For in-cell labeling or for binding studies in cellular environments the PPG potentially enables prolonged incubation times before data acquisition. Thus, our method opens up new vistas for the study of proteins by EPR spectroscopy.

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Figure 1. Protein labeling with the PaNDA label. A) Structure of the PaNDA spin label 1 with its particular functional features colored in orange (tetrazine moiety), red (photocaged TEMPO), green (PPG with tetraethylene glycol chain in blue. B) Structures of the ncAA SCO-L-lysine 2a and TCO-L-lysine 3a used in this study. The respective products upon reaction with 1 are shown as 2b and 3b for the ncAA 2a and 3a, respectively. C) Incorporation of ncAA into proteins (exemplarily shown for TRX-R74→2a (pdb entry 2TRX[33]), and in vitro labeling and deprotection conditions.
Scheme 1. Synthesis of PaNDA label 1. NMM = N-methylmorpholine, PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate.

Figure 2. Deprotection study of the PaNDA spin label 1. A) UV absorption spectrum of 75 µM of 1 in methanol. B) LC-MS traces showing the absorption at 254 nm of 1 before (purple) and after irradiation (black) for 10 min at 302 nm. After irradiation, 1 is virtually completely consumed and nitroxide 13 the main product. Small amounts of intermediate 14 and side product 15 are visible. C) EPR spectrum of 100 µM of 1 in water, before (purple) and after irradiation (black).
Figure 3. Site-directed spin labeling of proteins by DAinv cycloaddition and subsequent deprotection of the PPG. EPR spectra of GFP and TRX bearing amino acids as indicated (wildtype shown in the first row) and reacted with the PaNDA spin label after purification. Spectra are shown before (purple) and after (black) irradiation. The signal intensities are drawn on the same scale for all spectra in one column. Approx. 1 µM spin concentration for TRX-R74 (wt) + 1 indicates minor remaining impurities of unreacted PaNDA after purification.

Figure 4. Recovery of nitroxide radicals is possible in E. coli lysate. E. coli cells were lysed and mixed with preliminarily irradiated TRX-R74→2b (black squares) and the spin concentration was quantified at different time points until the signal had vanished (< 1 µM) after 80 min. In comparison, a non-irradiated sample of TRX-R74→2b (blue circles) was incubated with lysate for 80 min, showing no EPR signal. After irradiation for 2 min at 302 nm, a spin concentration of approx. 15 µM was found (see Figure S22 for EPR spectra at each time point).
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Experimental Procedures

Synthesis of the PaNDA spin label (1)

General methods

Technical solvents were distilled prior to use. Dry solvents were either purchased from Sigma Aldrich, Acros or were dried and distilled. For degassing of dry solvents, the freeze-pump-thaw method was applied. Deuterated solvents for NMR spectroscopy were purchased from Deutero.

All reactions were monitored by Thin Layer Chromatography (TLC) with silica gel 60 F254 coated on aluminum sheets from Merck. UV active compounds were detected at 254 nm. Additionally, different staining solutions followed by gentle heating were used for the visualization of the reactants (the composition of the solvents is stated as a ratio of volumes (v/v)):

- Anisaldehyde solution: EtOH (150 mL), acetic acid (15 mL), conc. H2SO4 (5 mL), p-methoxybenzaldehyde (3.7 mL)
- Ninhydrin solution: EtOH (200 mL), acetic acid (3 mL), ninhydrin (0.2 g)
- Potassium permanganate solution: 0.1 % KMnO4 in 1 M NaOH

For the preparative Flash Chromatography (FC) silica gel 60 (Geduran Si 60, 0.040-0.063 mm particle size) from Merck was used. Solvent mixtures are specified as volume ratio (v/v) and all solvents were distilled prior to usage. Additionally, FC was performed on a MPLC-Reveleris X2 system from Grace.

High Pressure Liquid Chromatography with Mass Detection (LC-MS) analysis was performed with LCMS2020 by Shimadzu (pumps: LC-20 AD, auto sampler: SIL-20A HT, UV-Vis detector: SPD-20A, oven: CTO-20AC, communications bus module: CBB-20A, ESI detector, software LCMS-Solution) with an EC 125/4 C18, 3 μM 40 column (Machery Nagel) and a binary gradient of acetonitrile and water supplemented with 0.1 % formic acid. The flow rate was 0.4 mL min⁻¹.

Nuclear magnetic resonance (NMR) spectra were recorded on spectrometers Avance III 600 MHz and on Avance III 400 MHz from Bruker at room temperature. The resonance signals of different deuterated solvents were used as internal standards: CDCl3 (δH = 7.26 ppm, δC = 77.16 ppm), DMSO-d6 (δH = 2.50 ppm, δC = 39.5 ppm). In addition to first-order analysis, 1H, 1H homo- and 1H, 13C heteronuclear two-dimensional correlation spectra like HSQC, COSY, TOCSY and HMBC were recorded for the assignment of signals. JH-HC couplings were determined from non-decoupled HSQC and HMBC spectra. The multiplicities of the resonances are abbreviated as followed: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublets of doublets), t (triplet), dt (doublet of triplets), td (triplet of doublets), m (multiplet). The recorded NMR spectra were analyzed by using the software MestReNova v12.0.20080 by Mestrelab Research. NMR spectra can be found under the “Results and Discussion” section in Figure S1-S10 of this Supporting Information.

High resolution masses were measured on a micrOTOF II instrument from Bruker in positive mode. Electrospray was used as ionization method (ESI) and the time of flight (TOF) method was used for detection. The recorded mass spectra were analyzed by using the software Xcalibur v3.0 by Thermo Fischer Scientific.

Synthesis of 4-(Ethoxycarbonyl)-1-hydroxy-2,2,6,6-tetramethylpiperidin-1-ium-chlorid (6)

Carboxy-TEMPO 5 (50 mg, 250 μmol, 1.0 equiv.) was dissolved in EtOH (3 mL) and 37 % HClaq (1 mL). The reaction mixture was stirred at 50 °C for 4 h. The solvent was then evaporated under reduced pressure. The solvent was then
coevaporated with 1 M HCl (2 x 2 mL). The product 6 was obtained without further purification as a white foam (65 mg, 245 μmol, 98 % yield).

$^1$H NMR (400 MHz, CDCl$_3$, 300 K) δ = 11.52 (s, 1H, N-H), 10.97–10.48 (br. s, 1H, OH), 4.12 (q, 2H, $J$ = 7.1 Hz, CH$_2$-CH$_3$), 2.79 (tt, 1H, $J$ = 13.6 Hz, 3.5 Hz, CH), 2.44 (dt, 2H, $J$ = 13.6 Hz, 3.5 Hz, CH$_3$), 2.05 (dt, 2H, $J$ = 13.6 Hz, 3.5 Hz, CH$_3$), 1.66 (s, 6H, CH$_3$), 1.38 (s, 6H, CH$_3$), 1.24 (t, 3H, $J$ = 7.1 Hz, CH$_2$-CH$_3$).

$^{13}$C NMR (101 MHz, CDCl$_3$, 300 K) δ = 172.6 (C-carbonyl), 68.0 (C-quart.), 61.2 (CH$_2$-CH$_3$), 38.4 (CH$_3$), 33.5 (CH), 27.9 (CH$_3$), 20.4 (CH$_3$), 14.1 (CH$_3$-CH$_2$) ppm.

HRMS (ESI-TOF) calcd. for C$_{12}$H$_{22}$NO$_3$ [M + H]$^+$ m/z = 230.1751, found: 230.1743.

Synthesis of 2,5,8,11-Tetraoxatridecan-13-yl-4-methylbenzenesulfonate (8)

![Chemical structure of 2,5,8,11-Tetraoxatridecan-13-yl-4-methylbenzenesulfonate (8)](image)

C$_{16}$H$_{22}$O$_7$S
362.44 g/mol

The reaction procedure was adopted from Wang et al.[1]

Synthesis of 3-(Hydroxymethyl)-4-nitrophenol (16)

![Chemical structure of 3-(Hydroxymethyl)-4-nitrophenol (16)](image)

C$_7$H$_7$NO$_4$
169.14 g/mol

Benzaldehyde 7 was reduced to the corresponding alcohol 16 in a yield of 97 %.

The reaction procedure was adopted from Hu et al. [2]

Synthesis of (5-((2,5,8,11-Tetraoxatridecan-13-yl)oxy)-2-nitrophenyl)methanol (9)

![Chemical structure of (5-((2,5,8,11-Tetraoxatridecan-13-yl)oxy)-2-nitrophenyl)methanol (9)](image)

C$_{16}$H$_{24}$NO$_8$
359.38 g/mol

Oligoethylene glycol 8 (3.1 g, 8.45 mmol, 1.3 equiv.) and nitrophenol 16 (1.1 g, 6.5 mmol, 1.0 equiv.) were dissolved in dry MeCN (50 mL) and K$_2$CO$_3$ (2.7 g, 19.6 mmol, 3 equiv.) was added. The reaction was heated to reflux overnight and then filtered. The solvent was evaporated under reduced pressure. The combined organic layers were dried over MgSO$_4$ and the crude product was purified by FC (petroleum ether/ethyl acetate 1:1 to pure ethyl acetate). The product 9 was obtained as a yellow oil (1.46 g, 4.63 mmol, 71 % yield).

$R_f$ = 0.41 (DCM/acetone 95:5).
Compounds were dissolved in dry THF (5 mL) and stirred for 1 h. The solution was filtered. The solvent was removed under reduced pressure. The crude product was purified by FC (DCM/acetone 95:5). The product 10 was obtained as pale yellow oil (200 mg, 474 μmol, 85 % yield).

Rf = 0.49 (DCM/acetone 95:5).

1H NMR (400 MHz, CDCl3, 300 K) δ = 8.12 (d, 1H, J = 9.2 Hz, H-3), 7.06 (d, 1H, J = 2.8 Hz, H-6), 6.93 (dd, 1H, J = 9.2 Hz, 2.8 Hz, H-4), 4.48 (s, 2H, benzyl-CH2), 4.22 (t, 2H, J = 4.2 Hz, aryl-CH2-PEG), 3.88 (t, 2H, J = 4.2 Hz, aryl-CH2-CH2-PEG), 3.78–3.61 (m, 10H, CH2-PEG).

13C NMR (101 MHz, CDCl3, 300 K) δ = 162.8 (C-5), 140.8 (C-2), 135.7 (C-1), 128.5 (C-3), 118.4 (C-6), 114.70 (C-4), 76.8 (CH2-O-CH3), 72.0 (CH3), 71.0 (CH3), 69.4 (CH2-CH2-O-benzyl), 68.4 (CH2-O-benzyl), 59.1 (CH3), 29.9 (benzyl) ppm.

HRMS (ESI-TOF) calcd. for C38H32BrNO7 [M + Na]+ m/z = 444.0634, found: 444.0615.

Synthesis of 1-(5-(2,5,8,11-Tetraoxatridecan-13-yl)oxy)-2,2,6,6-tetramethylpiperidine-4-carboxylic acid (11)

The TEMPO-H-salt 6 (75 mg, 282 μmol, 1.0 equiv.) was dissolved in dry, degassed THF (3 mL). 60 % NaH dispersion in mineral oil (68 mg, 1.7 mmol, 6.0 equiv.) was added. The reaction mixture was stirred for 5 min and the bromide 10 (298 mg, 705 μmol, 2.5 equiv.) was added in THF (3 mL). The reaction mixture was stirred for 1 h at room temperature. 60 % NaH dispersion in mineral oil (68 mg, 1.7 mmol, 6.0 equiv) was added and the reaction mixture was then heated to reflux for 2.5 h. The reaction was stopped by the addition of a solution of 5 % H2O in MeOH (2 mL) and the solution was stirred for 30 min. The solvents were then evaporated under reduced pressure. The crude product was purified by FC (ethyl acetate:acetic acid 99:1) to obtain the product 11 as a colorless oil (145 mg, 267 μmol, 95 % yield).

Rf = 0.48 (ethyl acetate:acetic acid 99:1).
$^1$H NMR (400 MHz, CDCl$_3$, 300 K) $\delta$ = 8.15 (d, 1H, $J$ = 9.1 Hz, H-3), 7.39 (d, 1H, $J$ = 2.8 Hz, H-6), 6.88 (dd, 1H, $J$ = 9.1 Hz, 2.8 Hz, H-4), 5.23 (s, 2H, benzyl-CH$_2$), 4.24 (t, 2H, $J$ = 4.7 Hz, aryl-CH$_2$-PEG), 3.91 (t, 2H, $J$ = 4.7 Hz, aryl-CH$_2$-CH$_2$-PEG), 3.74 (m, 8H, O-CH$_3$), 3.67 (m, 8H, O-CH$_3$), 3.54 (m, 2H, O-CH$_3$), 3.37 (s, 3H, O-CH$_3$), 2.66 (tt, 1H, $J$ = 13.2 Hz, 3.5 Hz, CH$_3$-TEMPO), 1.77 (dt, 2H, $J$ = 13.6 Hz, 3.5 Hz, CH$_2$-TEMPO), 1.68 (dt, 2H, $J$ = 13.6 Hz, 3.5 Hz, CH$_2$(TEMPO)), 1.23 (s, 6H, CH$_3$), 1.22 (s, 6H, CH$_3$) ppm.

$^{13}$C NMR (101 MHz, CDCl$_3$, 300 K) $\delta$ = 163.2 (C-5), 139.7 (C-2), 138.6 (C-1), 127.6 (C-3), 113.4 (C-6), 112.8 (C-4), 75.6 (benzyl), 72.0 (CH$_3$-O-CH$_3$), 70.7 (CH$_2$(PEG)), 69.5 (CH$_3$-benzyl), 59.8 (C$_{quat}$), 59.1 (O-CH$_3$), 41.9 (2x CH$_2$(TEMPO)), 32.8 (CH$_3$), 20.9 (CH$_3$) ppm.

HRMS (ESI-TOF) calcd. for C$_{26}$H$_{42}$N$_2$O$_{10}$ [M + H]$^+$ m/z = 543.2912 , found: 543.2902.

**Synthesis of Boc-4-(6-(pyrimidinyl)-1,2,3,4-tetrazin-3-yl)benzylamine (17)**

![Diagram of Boc-4-(6-(pyrimidinyl)-1,2,3,4-tetrazin-3-yl)benzylamine](image)

C$_{18}$H$_{19}$N$_2$O$_2$

365.40 g/mol

The reaction procedure was adopted from Willems et al.[3]

**Synthesis of 11-(5-((2,5,8,11-Tetraoxatridecan-13-yl)oxy)-2-nitrobenzyl)oxy)-2,2,6,6-tetramethyl-N-(4- (6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzyl)piperidine-4-carboxamide (1)**

![Diagram of 11-(5-((2,5,8,11-Tetraoxatridecan-13-yl)oxy)-2-nitrobenzyl)oxy)-2,2,6,6-tetramethyl-N-(4- (6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzyl)piperidine-4-carboxamide](image)

C$_{36}$H$_{57}$N$_5$O$_9$

798.89 g/mol

The Boc-protected tetrazine 17 (10 mg, 27 μmol, 1.0 equiv.) was dissolved in a mixture of DCM:TFA (1:1, 2 mL) and stirred for 30 min. The solvents were removed under reduced pressure to yield the free amine 12 which was used without further purification. The photo-protected spin label 11 (7 mg, 27 μmmol, 1.0 equiv.) was dissolved in dry DCM (1 mL) and PyBOP (15 mg, 30 μmol, 1.1 equiv.) was added. To this solution N-methyImorpholine (6 μL, 57 μmol, 2.1 equiv.) was added. The solution was stirred for 5 min. Then the solution containing the spin label was transferred to the tetrazine and peptide grade, dry DMF (1 mL) was added. The solution was stirred for 3 h. The reaction mixture was diluted with DCM (4 mL)
and washed with saturated NaHCO₃-solution (3 x 2 mL) and H₂O (2 x 2 mL). The combined organic layers were dried over MgSO₄. The solvent was removed under reduced pressure. The crude product was purified by FC (ethyl acetate /acetone 95:5 to 93:7). The product 1 was obtained as a pink oil (16 mg, 20 μmol, 75 % yield).

Rf = 0.43 (ethyl acetate:acetone 97:3).

1H NMR (400 MHz, CDCl₃, 300K) δ = 9.13 (d, 2H, J = 4.9 Hz, H-6'', H-4''), 8.69 (d, 2H, J = 8.1 Hz, H-6', H-2'), 8.14 (d, 1H J = 9.1 Hz, H-3), 7.59 (t, 1H, J = 4.9 Hz, H-5'''), 7.52 (d, 2H, J = 8.1 Hz, H-3', H-5'), 7.37 (d, 1H J = 2.8 Hz, H-6), 6.87 (dd, 1 H J = 9.1, 2.8 Hz, H-4), 6.09 (s, 1H, NH), 5.25 (s, 2H, benzyl-CH₂(N₉-CH₂)), 4.58 (d, 2H J = 5.9 Hz, benzyl-CH₂(Tetrazine)), 4.23 (t, 2H J = 4.7 Hz, Aryl-O-CH₂-PEG), 3.90 (t, 2H J = 4.7 Hz, Aryl-O-CH₂-CH₂-PEG), 3.80 – 3.59 (m, 10H, CH₂), 3.35 (s, 3H, O-CH₃), 2.60 (tt, 1H J = 12.6, 3.5 Hz, CH(TEMPO)), 1.92 – 1.69 (m, 4H, CH₂(TEMPO)), 1.24 (s, 12H, CH₃) ppm.

13C NMR (101 MHz, CDCl₃, 300K) δ = 174.8 (C-carbonyl), 164.4 (C₂₅₋₅), 163.2(C-5), 163.2 (C₅₋₃), 159.72 (C₃₋₁), 158.5 (C-6'', C-4'''), 144.4 (C-5''), 139.8 (C-2), 130.6 (C-2''), 129.4 (C-6', C-2'), 128.7 (C-3', C-5', C-4'), 127.6 (C-3), 122.6 (C-5'''), 113.6 (C-6), 112.9 (C-4), 75.6 (C-benzyl(N₉-CH₂)), 72.1 (CH₂-O-CH₂), 71.03 (CH₂PEG), 70.7 (CH₂PEG), 70.6 (CH₂PEG), 69.5 (Aryl-O-CH₂-CH₂-PEG), 68.2 (Aryl-O-CH₂-PEG), 60.1 (CH₂(TEMPO)) 59.2 (O-CH₃), 43.3 (benzyl-CH₂(TETRAMER)), 42.5 (2 x CH₂(TEMPO)), 36.7 (CH(TEMPO)), 32.6 (CH₃), 21.1 (CH₃) ppm.

HRMS (ESI-TOF) calcd. for C₆H₁₁NO₄S [M + H]+ m/z = 790.3888, found: 790.3871.
Plasms and transformation of E. coli

The plasmids pEVOl_PylRS_AF and pBAD_TRX_His6_R74TAG or pBAD_TRX_His6_G34TAG_R74TAG, respectively, were used for the expression of E. coli thioredoxin (TRX-R74→ncAA or TRX-G34/R74→ncAA). For expression of TRX wildtype (TRX wt) only the pBAD_TRX_His6 plasmid was transformed into E. coli. The plasmids pEVOL_PylRS_AF and pBAD-Flag-GFP-Y39TAG-6His were used for the expression of the GFP mutant, which contains the ncAA at amino acid residue 38 instead of tyrosine (GFP-Y39→ncAA). For expression of the GFP wildtype (GFP wt) only the pBAD_Flag-GFPwt-6His plasmid was transformed into E. coli.

The plasmids were co-transformed into chemically competent BL21-gold (DE3) E. coli as follows. The reaction tube containing E. coli and an appropriate amount of the particular plasmid(s) was mixed by flicking and incubated 30 min on ice. The cells were then heat shocked at 42 °C for 30 sec and incubated for another 2 min on ice, before being added to 1 mL pre-warmed (37 °C) Super Optimal Broth with catabolite repression (SOC-medium). The cells were then incubated for 1 h at 37 °C and 1400 rpm, before grown on an LB-Agar plate (Lennox; ROTH) containing 34 μg/mL chloramphenicol (ROTH) and 50 μg/mL carbenicillin (ROTH) overnight.

Expression and purification of TRX and GFP and incorporation of genetically encoded artificial amino acids

For all steps performed in medium, transformed E. coli strains were grown in LB-medium (Lennox; ROTH), containing 34 μg/mL chloramphenicol and 50 μg/mL carbenicillin (or only carbenicillin for expression of wildtype proteins). Cells were shaken at 37 °C and 180 rpm.

TRX-R74→ncAA, TRX-G34/R74→ncAA, TRX wildtype, GFP-Y39→ncAA or GFP wildtype were expressed in E. coli co-transformed with the respective plasmid(s) (see above) as follows. For overnight cultures, 10 mL LB-medium were inoculated with one colony of the particular E. coli from the agar plate. The next day, the overnight culture was diluted 1/100 (typically to a final volume of 1 L), and incubated until an OD<sub>600</sub> of 0.2 – 0.3 was reached. At this point, 1 mM SCOM-L-lysine (2α) or TCO-L-lysine (3α, both ncAA were bought from SICHEM) were added from freshly prepared stock solutions (therefore, the ncAA were dissolved in 0.1 M NaOH in 60 mM or 80 mM for 2α or 3α, respectively). Cells were further grown until an OD<sub>600</sub> of 0.4 – 0.6 was reached. Protein expression was induced for 4 – 6 h with 0.2 % L-arabinose (ROTH) from a 20 % w/v stock solution. For wildtype proteins, only L-arabinose but no ncAA was used for the expression. Expression was stopped and cells were harvested by centrifugation (4 °C, 4000 rpm, 10 min). The supernatant was discarded, and the pellets were stored at -20 °C, until proteins were isolated and purified.

Proteins were purified using HisPur Ni-NTA resin (Thermo Fisher Scientific) as described elsewhere. Samples were dialyzed in Slide-A-Lyzer MINI Dialysis Devices (3.5 K MWCO, Thermo Fisher Scientific) against PBS buffer, pH 7.4 (MERCK), at 4 °C. Resulting protein concentration was determined photometrically with the use of an Eppendorf BioPhotometer D30 via absorption at 280 nm (with a Factor F<sub>p</sub> = 1/A<sub>0.1%</sub> = 0.651 g/L for TRX or 1.35475 g/L for GFP; A<sub>0.1%</sub> = ε<sub>0.1%</sub>/MM<sub>0</sub> is the absorbance of the protein at 0.1 %, ε<sub>0.1%</sub> is the molar extinction coefficient of the protein, and MM<sub>0</sub> is the relative molar mass of the protein).

Integration of the ncAA and purity were confirmed by SDS-PAGE. Briefly, samples for SDS-PAGE analysis and 5 μL of a BIO-RAD Precision Plus Protein™ Dual Color Standard were applied to a 15 % SDS-gel and run at 90 V in a BIO-RAD Mini-PROTEAN Tetra System. After Coomassie Blue staining (Brillant Blau R 250, ROTH), gels were imaged using a BIO-RAD ChemiDocTM Imaging System (Figure S11-S13).

For additional proof for expression of the correct product and for assessing fidelity of the aminoacyl-tRNA-synthetase, full-length ESI-MS spectra were recorded (Figure S16-S17).

Site-directed spin labeling of TRX and GFP with the PaNDA spin label

The labeling protocol was based on a reaction mixture of 200 μL of 40 μM protein (in PBS, pH 7.4) containing a 10-fold molar excess of PaNDA spin label (from a 10 mM stock solution in DMSO, which was stored at -20 °C). The mixture was incubated for 30 min at 20 °C and 0 rpm in an Eppendorf ThermoMixer C. The excess reactants were then removed by using HisPur Ni-NTA resin (Thermo Fisher Scientific). 120 μL of bead slurry were washed three times with MQ-H<sub>2</sub>O in a centrifuge tube, before it was incubated together with the labeling mixture for 30 min at 4 °C under constant rotation. In a centrifuge column (Pierce Centrifuge Columns, 0.8 mL, Thermo Fisher Scientific) the beads with adherent proteins were washed approx. ten times with washing buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8). For elution of the spin-labeled proteins, the beads were incubated for 20 min with 60 μL of washing buffer containing 500 mM imidazole, before proteins were collected by centrifugation. All wash steps and elution were performed in a benchtop centrifuge.

Wildtype proteins were also incubated with the PaNDA spin label as described above to exclude unspecific labeling (Figure S18-S19). Full-length ESI-MS of spin labeled proteins (after irradiation) proves the conversion of the ncAA-containing proteins into labeled ones (Figure S18-S19).
Deprotection of the PaNDA spin label by irradiation

A typical sample of 30 μL was filled into a glass capillary (HIRSCHMANN® ringcaps®; inner diameter 1.02 mm), and sealed with tube sealing compound (Kimble Cha-Seal) on one end. The capillary was placed on a table, and covered with a 302 nm handheld UV lamp (UVLM-28 EL Series UV Lamp. 8 Watt, 302/365 nm, analytikJena). Samples were irradiated for 2 min (or longer, if indicated; Figure S21). The sample in the capillary was directly used for subsequent EPR spectrometric measurements.

EPR measurements

EPR spectra were recorded at a BRUKER EMXnano X-band continuous wave EPR spectrometer at room temperature (approx. 22 °C). A typical sample volume of 30 μL was filled into a glass capillary (HIRSCHMANN® ringcaps®; inner diameter 1.02 mm). Spectra were recorded at a modulation amplitude of 1 G, microwave attenuation 15 dB, and a sweep width of 150 G. Typically, 20 scans of 60.06 sec scan time each were accumulated to improve the signal-to-noise ratio. Quantitative spin concentrations of samples were obtained with the use of the built-in EMXnano reference-free spin counting module (Xenon software, Bruker). Spectra were plotted with MATLAB R2018a (The MatWorks, Inc. 3 Apple Hill Drive, Natick, MA 01760-2098, USA).

Full-length mass spectrometry of proteins

Before being subjected to mass spectrometry, the buffer of the protein samples was replaced by MQ-water in 3K spin filters (Amicon Ultra-0.5 mL Centrifugal Filters, MERCK). Protein masses were recorded by an amaZon speed ETD mass spectrometer (Bruker) with a flow rate of 4 μl/min at the Proteomics Facility of the University of Konstanz. Mass spectrometric data were evaluated using the Data Analysis Version 4.4 (Bruker) software (Figure S16-S19).

Measurement of circular dichroism (CD) spectra

Before CD measurements, the buffer of the protein samples was replaced by MQ-water using 3K spin filters. CD spectra were recorded in a JASCO J-715 Spectropolarimeter. Spectra were recorded at room temperature, using a 0.5 mm cuvette. Ten scans each were accumulated to improve signal-to-noise levels. 1000 data points were received between 280 and 180 nm. The data were baseline-corrected, and subsequently background-corrected with a sample containing only MQ-water. Noisy data at low and high wavelengths were cut off for the final presentation of the data. For the calculation of the molar residue ellipticity out of the given spectrometer unit (CD-signal in mdeg), the formula \( MRE = \frac{MRW^*CD-signal/1000}{10*d*c} \) with \( M \) in g/mol, \( N_{aa} \) (number of amino acid residues in the protein), \( MRW = \frac{M}{N_{aa}} \), c in g/mL, and \( d = 0.05 \) cm was used (Figure S20).

Experiments with E. coli lysate

This section describes the experiment shown in Figure 4 (main text); EPR spectral raw data of every time point are given in Figure S22. For the preparation of E. coli lysate 5 mL of E. coli overnight culture (carrying pBAD_TRX_His6 plasmids, but without expression) were prepared. After 17 hours cells were harvested by centrifugation for 10 min at 4 °C and 4000 rpm, before the supernatant was discarded. Cells were lysed with 200 μL of B-PER reagent (Thermo Fisher Scientific) containing 1 mM PMSF, and incubated for 10 min on ice with regular vortexing. The cell lysate was cleared by centrifugation for 2 min at 4 °C and 14000 rcf, and the resulting supernatant was stored on ice and further used as reducing environment for experiments. In the first part of the experiment a TRX-R74→2b sample was irradiated and a spin concentration of 89 μM was determined. Then 15 μL of this protein were mixed with 15 μL of the E. coli lysate, and EPR spectra were measured every ten min. For these experiments three scans at each time point were accumulated. The resulting plot of the spin concentration against the time showed the expected degradation kinetics of nitroxides in reducing environments. In the second part of this experiment, 15 μL of the protein, which was not irradiated in advance, were mixed with 15 μL of E. coli lysate, and EPR spectra were measured every twenty min. After 80 min the sample was irradiated and EPR spectra were measured every ten min to see first an increase followed by degradation of the signal (Figure 4).
Supporting Figures

NMR spectra (Figures S1-S10)

Figure S1. $^1$H NMR spectrum (400 MHz, CDCl$_3$, 300 K) of compound 9.

Figure S2. $^{13}$C NMR spectrum (101 MHz, CDCl$_3$, 300 K) of compound 9.
Figure S3. $^1$H NMR spectrum (400 MHz, CDCl$_3$, 300 K) of compound 10.

Figure S4. $^{13}$C NMR spectrum (101 MHz, CDCl$_3$, 300 K) of compound 10.
Figure S5. $^1$H NMR spectrum (400 MHz, CDCl$_3$, 300 K) of compound 6.

Figure S6. $^{13}$C NMR spectrum (101 MHz, CDCl$_3$, 300 K) of compound 6.
Figure S7. $^1$H NMR spectrum (600 MHz, CDCl$_3$, 300 K) of compound 11.

Figure S8. $^{13}$C NMR spectrum (150 MHz, CDCl$_3$, 300 K) of compound 11.
Figure S9. $^1$H NMR spectrum (600 MHz, CDCl$_3$, 300 K) of compound 1.

Figure S10. $^{13}$C NMR spectrum (150 MHz, CDCl$_3$, 300 K) of compound 1.
SDS-PAGE images (Figures S11-S13)

**Figure S11.** 15 % SDS-PAGE gel analysis of GFP wildtype and GFP-Y39→ncAA before and after purification via His6-tag. “-ncAA” indicates the absence of ncAA during expression.

**Figure S12.** 15 % SDS-PAGE gel analysis of TRX wildtype and TRX-R74→ncAA before and after purification via His6-tag. “-ncAA” indicates the absence of ncAA during expression.

**Figure S13.** 15 % SDS-PAGE gel analysis of TRX-G34/R74→2a (2a = SCO-L-lysine, short: SCO) before and after purification via His6-tag. “FT” (flow-through) indicates the proteins not bound to Ni-NTA resin, while “1./2./3. El.” indicates three subsequent elution steps of the desired protein from the Ni-NTA resin.
Primary sequences of model proteins (Figures S14-S15)

MDYKDDDKVSKGEELFTGVVIPVLEDGDVNGHKFSVSVEGEGDATYKTLKFLCTTGGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKQHDFKSAEMEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNLRELK
GIDFKEKGNILGHKLEYNYNHVNYIMADKQKGIKANFKIRHNIEGDGQPLADHYQQNTPIDGPGVLL
PDNHYLYSTQSALSBDPNEKRDHMVLLEFVTAAGITLGMDELYKHHHHHH

Figure S14. Amino acid sequence of GFP wildtype.

MGDKIHLTDSDFTDVIKADGAILVDFWAECGPCKMIAPILDEADYEYQKLTVAKLNIDQNGTPAP
KYGIRGIPTLLLKNGEVAATKVGSNGQMLKEFLDANLAGSGSGERQHMDSPLGTDDDDKHHHHHHH

Figure S15. Amino acid sequence of TRX wildtype.
Figure S16. Full-length ESI-MS spectra of GFP samples after expression in E. coli and purification:

Left: 28711 Da: GFP wildtype
Middle: 28828 Da: GFP-Y39→3a (calcd. 28711 Da + 117 Da = 28828 Da)
Right: 28826 Da: GFP-Y39→2a (calcd. 28711 Da + 115 Da = 28826 Da)
Figure S17. Full-length ESI-MS spectra of TRX samples after expression in *E. coli* and purification:

**Left:**
- 14767 Da: TRX wildtype (this exact mass was found before, compare Schmidt et al.[5])

**Middle:**
- 14891 Da: TRX-R74→3a (calcd. 14767 Da + 124 Da = 14891 Da)
- 14739 Da: TRX-R74→Lys (calcd. 14891 Da – 152 Da = 14739 Da)

**Right:**
- 14889 Da: TRX-R74→2a (calcd. 14767 Da + 122 Da = 14889 Da)
- 14739 Da: TRX-R74→Lys (calcd. 14889 Da – 150 Da = 14739 Da)

Peaks at 14739 Da indicate degradation of TCO-L-lysine 3a or SCO-L-lysine 2a to lysine.
Figure S18. Full-length ESI-MS spectra of GFP samples incubated with the PaNDA spin label, then purified and irradiated:

Left: 28709 Da: GFP wildtype (compare Figure S16)

Middle: 28676 Da: GFP-Y39→Lys (calcd. 28828 Da – 152 Da = 28676 Da)

28828 Da: unlabeled GFP-Y39→3a (compare Figure S16)

28826 Da: unlabeled GFP-Y39→2a (compare Figure S16)

29230 Da: labeled GFP-Y39→2b* after irradiation (calcd. 28826 Da + 419 Da – 16 Da + 1 Da = 29230 Da)

The asterisk (*) indicates the elimination of the oxygen from the nitroxide, leading to the corresponding amine, due to mass spectrometry-induced fragmentation. This effect was also seen for the unbound spin label in solution (see Figure 2 B).

In the spectrum in the middle (GFP-Y39→3a spin labeled with the PanDA spin label) no peak can be assigned to successfully spin labeled protein (GFP-Y39→3b). However, in the EPR spectrum (Figure 3) successful labeling was evidenced.
Figure S19. Full-length ESI-MS spectra of TRX samples incubated with the PaNDA spin label, then purified and irradiated:

Left: 14767 Da: TRX wildtype (compare Figure S17)

Middle:
- 14739 Da: TRX-R74→Lys (compare Figure S17)
- 14893 Da: unlabeled TRX-R74→3a (compare Figure S17)
- 15296 Da: labeled TRX-R74→3b* after irradiation (calcd. 14893 Da + 419 Da – 16 Da + 1 Da = 15297 Da)

Right:
- 14739 Da: TRX-R74→Lys (compare Figure S17)
- 15294 Da: labeled TRX-R74→2b* after irradiation (calcd. 14889 Da + 419 Da - 16 Da + 1 Da = 15293 Da)
- 15310 Da: labeled TRX-R74→2b after irradiation (calcd. 15294 Da - 1 Da + 16 Da = 15309 Da)

The asterisk (*) indicates the elimination of the oxygen from the nitroxide, leading to the corresponding amine, due to mass spectrometry-induced fragmentation.
Further supplementary figures (Figures S20-S22)

**Figure S20.** CD analysis of GFP (left) and TRX (right). Wildtype proteins (blue line) were measured after expression (without labeling), while the red and black lines refer to labeled proteins. All samples were desalted before measurement, and spectra were baseline- and background-corrected.

**Figure S21.** Evaluation of the deprotection kinetics of the photocage group of the PaNDA spin label attached to a protein. Each time point stems from an individual TRX-R74→2a sample, that was labeled with the PaNDA spin label as described above (TRX-R74→2b), and irradiated at 302 nm for the indicated durations. The maximum indicates, that irradiation for 2 min yielded the highest deprotection.
**Figure S22.** EPR spectra of the experiment shown in Figure 4 (main text). All spectra are drawn on the same y-axis range. The spectrum “10 min after irradiation” shown in the 2nd column on the right was measured with 25 dB microwave attenuation instead of 15 dB, leading to a higher signal-to-noise ratio (SNR).

| cleared *E. coli* lysate + irradiated TRX-R74→2b | cleared *E. coli* lysate + TRX-R74→2b |
|-------------------------------------------------|---------------------------------------|
| 0 min                                           | 0 min                                 |
| ![Heartbeat](image)                             | ![Heartbeat](image)                  |
| 20 min                                          | 40 min                                |
| ![Heartbeat](image)                             | ![Heartbeat](image)                  |
| 60 min                                          | 80 min                                |
| ![Heartbeat](image)                             | ![Heartbeat](image)                  |
| 10 min after irradiation                        | 10 min after irradiation              |
| ![Heartbeat](image)                             | ![Heartbeat](image)                  |
| 20 min after irradiation                        | 30 min after irradiation              |
| ![Heartbeat](image)                             | ![Heartbeat](image)                  |

![Graph](image)
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Author Contributions

M. D., V. W. and A. K. designed the concept. M. D. and V. W. acquired funding. Organic Syntheses were performed by B. S. EPR spectroscopy and biochemistry was performed by A. K., supported by J. L. A. K., M. D., V. W. and B. S. wrote the paper. All authors discussed the results, commented on the manuscript and approved the submitted version.
