Design and synthesis of paramagnetic probes for structural biololgy

Liu, W.

Chapter V

The application of spin labels

Abstract

Nitroxide radicals are commonly used as a paramagnetic center for electron paramagnetic resonance spectroscopy and nuclear magnetic resonance spectroscopy. Compared with other paramagnetic centers, such as lanthanoid chelating probes, the small size of nitroxide radicals is advantageous. Among all of the nitroxide radicals, *S*-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate (MTSL) is the most widely used probe. However, the flexible linker complicates the data analysis. To overcome this drawback, a new doubled-armed nitroxide probe and a pyridyl-MTSL analog were synthesized. The results showed that the doubled-armed nitroxide was unstable after attaching to protein. Also, nitroxide radicals were introduced on an inhibitor of P450cam and the cocrystal structures were obtained. These results suggest that substrate/inhibitor functionalized nitroxide radicals might be a potential approach to study protein-ligand complexes.

Introduction

In recent years, nitroxide radicals have not only been used for determining protein topography, local and global structure and dynamics by electron paramagnetic resonance (EPR) spectroscopy,[189] but also for characterizing the dynamic complexes and low populated states of proteins by nuclear magnetic resonance (NMR) spectroscopy.[190] Among all of the nitroxide radical probes, MTSL is the most widely used (Chart 1). It can be site-specifically linked to a protein *via* a disulfide bridge to a Cys residue that is introduced by site-directed mutagenesis. The small size of MTSL is advantageous in comparison with other paramagnetic centers, such as lanthanoid chelating probes. The relatively small probe is unlikely to disturb the structure and the exposed hydrophobic surface of the labeled proteins. However, the mobility of the nitroxide group due to the rotatable bonds complicates the data analysis. It is for the reason that an ensemble of nitroxide positions has to be used to properly represent the paramagnetic center. To reduce the mobility of MTSL, two approaches have been developed. One of those is generating a second link between the nitroxide probe and a protein. Several bifunctional probes have been reported, as shown in Chart 1, and used for EPR studies.[191-193] The other approach is to introduce a bulky group next to the attachment site. Due to steric effects, the internal motion of the nitroxide probe is reduced and one of those derivatives, HO-3606, is shown in Chart 1.[194,195] In this chapter the synthesis of a new double-armed spin label (DASL), HO-3606 and several inhibitor-based nitroxide probes is described. These probes offer new approach for paramagnetic NMR studies on protein-protein and protein-ligand complexes.

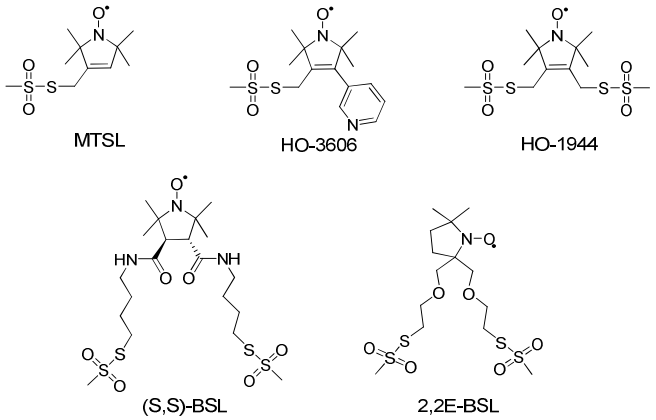
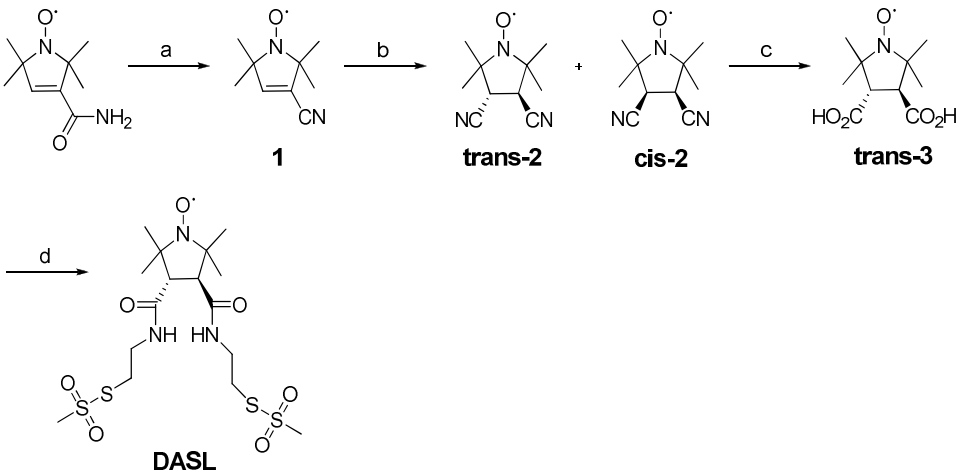


Chart 1. Structures of published spin label probes. Single-armed probes: MTSL[196], HO-3606[195]; double-armed probes: HO-1944[192], (S,S)-BSL[191] and 2,2E-BSL[193].

Results and Discussion

Synthesis of double armed spin label (DASL)

According to the literature, several bifunctional nitroxide probes have been developed (Chart 1). However, those probes have some drawbacks. For example, **2,2E-BSL** is a water-soluble bifunctional probe, but two diastereoisomers are generated when it is attached to a protein.[193] In contrast to **2,2E-BSL**, the other two bifunctional probes, **HO-1944** and **(S,S)-BSL**, have a *C2*-symmetry. The *C2*-symmetric **HO-1944** circumvents the problem of diastereoisomers, but the structure of the attachment site is slightly altered when this rigid probe is linked to a protein.[192] For **(S,S)-BSL**[191], the inefficient labeling results in single- or double-armed attachment. The single-armed labeled protein has to be removed by an additional thiol reactive column, which complicates the purification and reduces the yield. Moreover, the long linker might be a potential drawback of bifunctional nitroxide probes due to their flexibility. To enhance the rigidity and labeling efficiency, a modified double-armed spin label (**DASL**) was designed and the synthesis of **DASL** is depicted in Scheme 1. A commercially available radical compound 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl was treated with TsCl in pyridine to yield the cyanide **1**. The treatment of **1** with potassium cyanide gave di-functionalized **2**. After hydrolyzing the cyano groups, the di-carboxylic acid **3** was obtained. Finally, the carboxylic acid was coupled with aminoethyl-MTS yielding **DASL**. EPR was used to determine the amount of radical in the **DASL** samples.



Scheme 1. Synthesis of DASL. (a) TsCl, pyridine, RT, 36 h; (b) KCN; (c) i) NaOH; ii) HCl; (d) aminoethyl-MTS, NHS, EDC, DMF, RT, 30 h.

To demonstrate the rigidity of double-armed labeling, **DASL** was attached to a model protein, Paz E51C/E54C. Paz was reduced with DTT in an ice bath for 1 h, washed, reacted with 10 equiv. excess **DASL** for 1 h, and purified by Superdex 75 column (GE Healthcare). For comparison MTSL was attached to Paz E51C. Both proteins were kindly provided by Dr. Monika Timmer (Leiden University, Inst. Chemistry). HSQC and EPR spectra of the tagged proteins were immediately recorded after labeling. In Figure 1, the [^{15}N, ^{1}H]-HSQC spectra of MTSL and **DASL** labeled Paz as well as the ratio of paramagnetic and diamagnetic peaks are presented. MTSL shows a stronger PRE effect than **DASL**. This unexpected result might arise from the low labeling efficiency or the instability of the probe itself. In order to quantify the degree of labeling, the protein sample was checked by EPR (Figure 2) and LC/MS. The EPR spectra were kindly measured by Mr. Martin von Son. The EPR experiments showed that only 30% of the sample was paramagnetic, whereas the LC/MS showed that the labeling was more than 95% (data now shown). The MS data rule out the possibility of inefficient labeling and show that **DASL** was attached *via* both arms. Further tests demonstrated that, the nitroxide radical of free **DASL** slowly decomposed when it was prepared as a stock solution in DMSO and stored in a fridge. Also, the excess DTT coming from the activation step might reduce the radical. To avoid those possibilities, **DASL** solution was prepared freshly and the excess DTT was removed carefully (see protein labeling experiment). However, the EPR results had no significant improvement. The scaffold of **DASL** is a saturated five-membered ring, pyrrolidine, and it presents significantly higher mobility compared with an unsaturated five-membered ring, pyrroline.[197] Several pyrrolidine ring based spin label probes have been studied and tested with proteins, suggesting that such probes are still stable, although information about the stability was not reported explicitly. The degradation of **DASL** attached to Paz could be a consequence of decreased stability of the mobile five-membered ring. In Paz E51C/E54C, the attachment site is located in a loop region and the distance between the two cysteines is around 10Å, which could cause strain in **DASL** attachment because the maximum distance that can be spanned by the arms is around 10 Å. Although **DASL** was successfully tagged on the test protein *via* two arms, the flexible saturated pyrrolidine may be further twisted by the attachment, which could result in a more reactive nitroxide. Hence, another test protein with two cysteines closer together could be tested to check whether that yields a more stable probe.

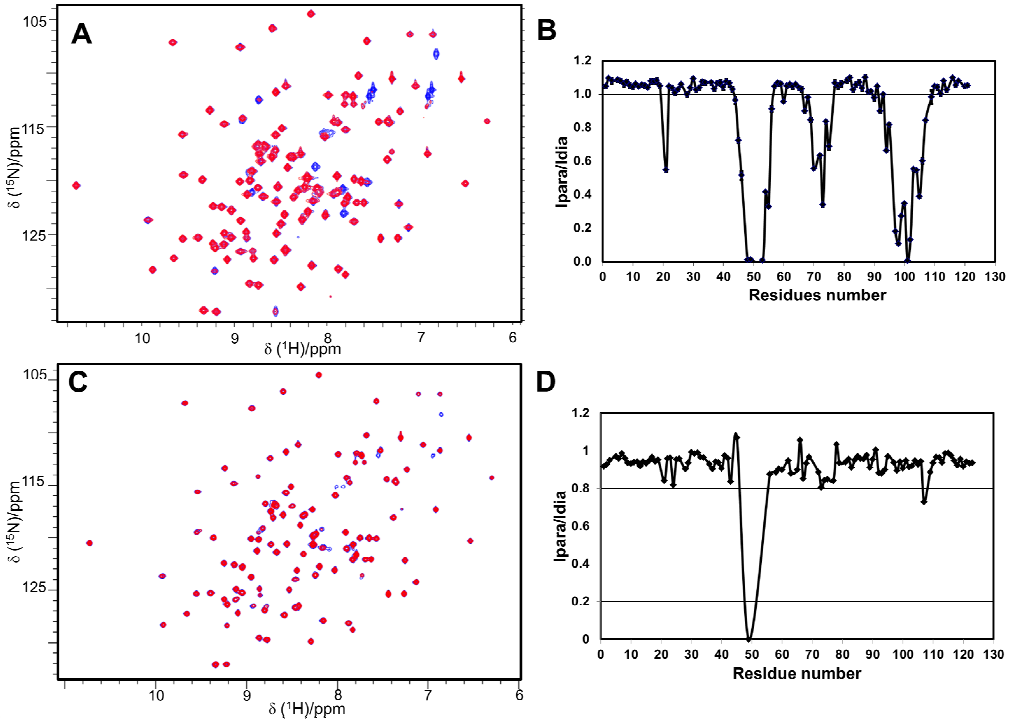


Figure 1. The HSQC spectra and PREs effect of MTSL (A, B) and DASL labeled Paz (C, D). (A, C) Overlay of [^{15}N,^{1}H]-HSQC spectra of MTSL (Red) and 1-Acetyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl methanethiosulfonate (Blue) tagged Paz E51C (A) and DASL (Red) and reduced DASL (Blue) tagged Paz E51C/E54C (C). (B, D) I_{para}/I_{dia} plots of the spectra in panel A (B) and C (D). MTS and DAS are the diamagnetic controls of MTSL and DASL, respectively.

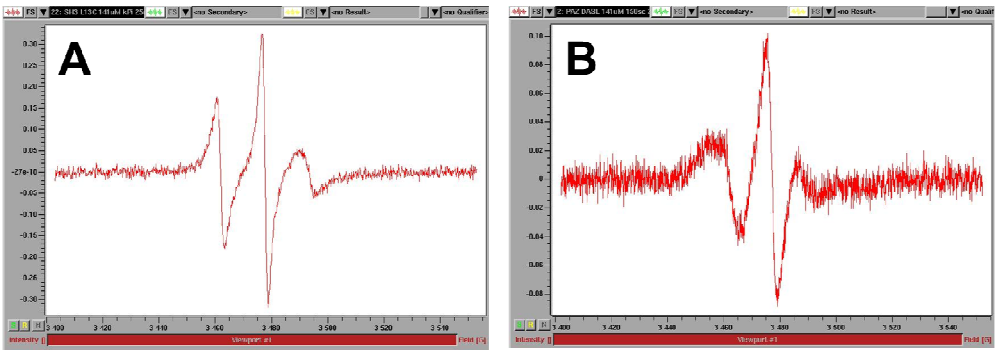
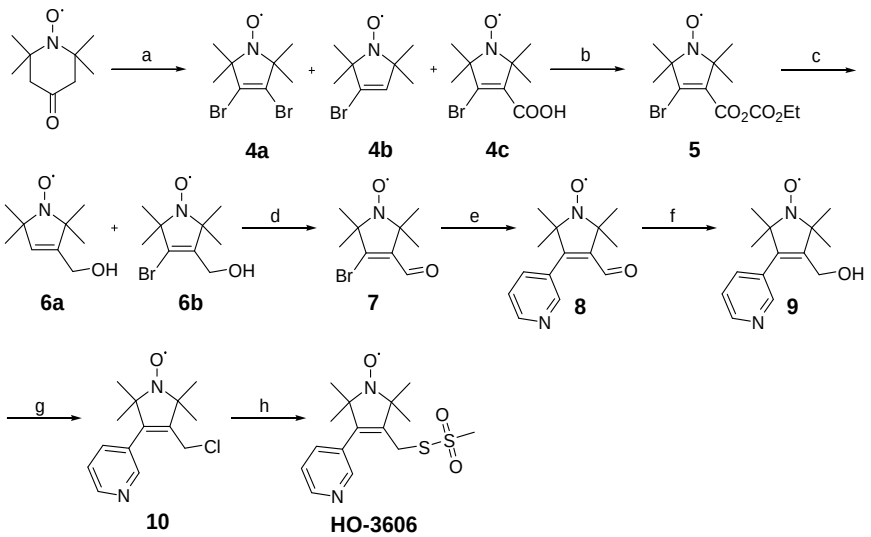


Figure 2. The EPR spectra of MTSL labeled Paz E51C (A) and DASL E51C/E54C (B). The EPR spectra were recorded at ambient temperature.

Synthesis of functionalized spin label

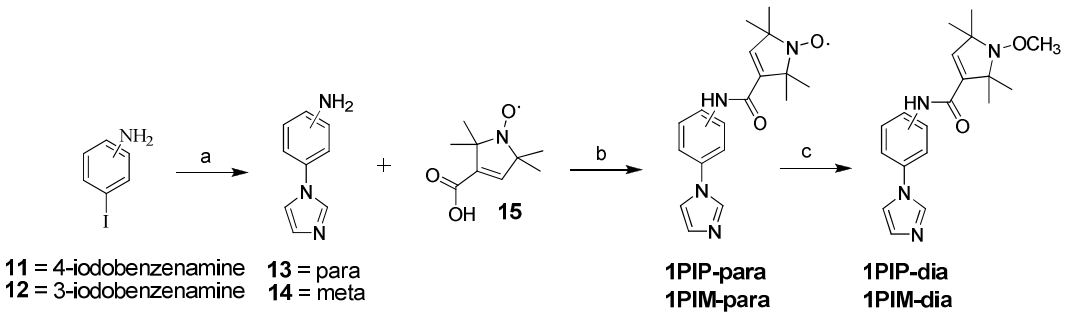
In 2011, Prof. Hubbell and co-workers published a rigid spin label, **HO-3606**, which is a 4-pyridyl analog of MTSL.[195] This substituted probe displays highly restricted motion and the EPR spectra were similar to the 4-phenyl analog of MTSL,[194] for which a single rotamer was found in the crystal structure of tagged protein (PDB code 1ZUR). In contrast, two rotamers were found in the crystal structure of MTSL tagged protein.[198] The observed PRE effects agreed with the theoretically predicted result using a single nitroxide position instead of an ensemble. Consequently, the data analysis can be simplified by using **HO-3606**. The synthesis of **HO-3606** is depicted in Scheme 2. Following the literature protocol, commercially available 4-Oxo-TEMPO was treated with NaOBr yielding the Favorskii rearranged product **4c**. The carboxylic acid group of **4c** was activated with ethyl chloridocarbonate and reacted with $NaBH_4$ to obtain **6b**. Oxidation of **6b** with CrO_3 in pyridine gave aldehyde **7**. Compound **7** was submitted to Suzuki condition to yield **8**. Treatment of **8** with $NaBH_4$ in ethanol and conversion of the hydroxyl group in **9** into a chloride yielded **10**. Finally, the chloride **10** was transformed with $NaSSO_2CH_3$ in a solution mixture of acetone and water, yielding **HO-3606**.



Scheme 2. Synthesis of HO-3606. (a) NaOBr, water/1,4-dioxane; (b) $ClCO_2Et$, Et_3N, Et_2O, 0 °C to RT, 3 h; (c) $NaBH_4$, EtOH, 0 °C, 4 h; (d) CrO_3, pyridine, 0 °C, 30 mins; (e) $Pd(PPh_3)_4$, dioxane, 3-pyridineboronic acid; (f) $NaBH_4$, EtOH, 0 °C, 4 h; (g) i) MsCl, CH_2Cl_2, 0 °C; ii) LiCl, acetone; (h) $NaSSO_2CH_3$, acetone/water, 40 °C.

Synthesis of 1-phenylimidazole analogs

A new approach to use paramagnetic centers to study protein complexes is to label enzyme ligands or inhibitors with a paramagnetic center that highlights the active site and interaction surface of the enzyme upon formation of the protein-ligand complex. 1-phenylimidazole (1PI) is a known inhibitor of P450cam and the co-crystal structure was solved by Poulos and Howard.[199] The crystal structure (PDB code 1PHD) shows that the imidazole ring of 1PI sits above the haem and the phenyl group occupies the camphor binding site (Figure 3A). Based on the co-crystal structure, several 1PI analogs were synthesized and used for studying the protein-ligand interaction. The synthesis of 1PI analogs is shown in Scheme 3. The compounds **13**[200,201] and **14**[200,201] were activated with HATU and coupled with spin labeled **15** yielding target molecules **1PIP-para** and **1PIM-para**. In order to have diamagnetic controls, the nitroxide radical was blocked with a methyl group by using $FeSO_4 \cdot 7H_2O$, H_2O_2 and DMSO.[202,203]



Scheme 3. Synthesis of spin labeled 1-phenylimidazole analogs. (a) imidazole, CuI, K_3PO_4, DMF, 40 ^oC 40 h; (b) HATU, TEA, DMF, RT, 12 h; (c) $FeSO_4 \cdot 7H_2O$, H_2O_2, DMSO.

P450cam crystallization

For protein-substrate/inhibitor recognition, conformational changes play an important role. Generally, X-ray crystallography provides a straightforward manner to visualize the recognition. Recently, Prof. Goodin and co-workers reported that the substrate recognition of P450cam occurs by a stepwise mechanism, in which the closed, intermediate, and open conformations are involved.[204] Here, the 1PI analogs were also co-crystallized with P450cam by Mr. Yoshitaka Hiruma (Leiden University, Inst. Chemistry) and P450cam was kindly provided by Dr. Monika Timmer (Leiden University, Inst. Chemistry). The co-crystal structures of 1PI analogs with P450cam were solved by Mr. Erik van Orlé and Dr. Navraj Pannu

(Leiden University, Inst. Chemistry). They exhibit an open conformation. Interestingly, the para-substituted 1PIP-dia presents a reversed conformation in comparison with 1PI (Figure 4B). The nitroxide points to the haem iron and the imidazole ring sits in the channel. In contrast, the imidazole ring of meta-substituted 1PIM sits above the haem and the nitroxide is oriented forward the channel. These results could be explained by the structure of active site. The meta-position of 1PI points to the active channel and, conversely, the para-position faces to P450cam side-chains. Thus, linkage of groups to the para-position results in a steric clash with the protein, which explains the observed reverse orientation of the ligand (Figure 4E). The EPR spectra showed that **1PIM-para** is immobilized by P450cam (data not shown). Further studies of the protein-ligand interaction are being carried out by Mr. Martin von Son and Dr. Martina Huber (Leiden University, Inst. Physics).

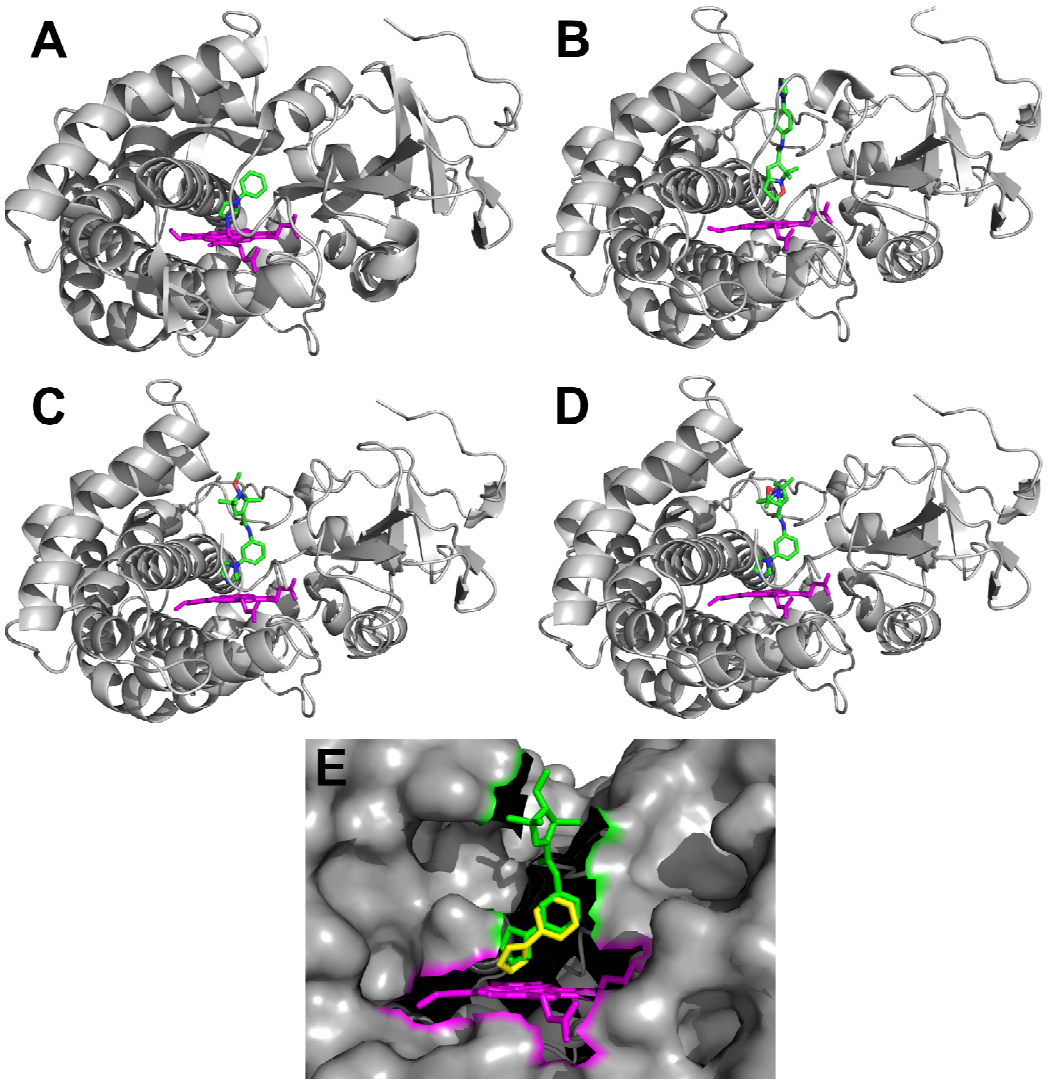


Figure 4. Crystal structures of 1PI and 1PI analogs. (A) The crystal structure of 1PI with P450cam (closed state) (PDB code 1PHD).[199] (B-D) The crystal structures of 1PIP-dia (B), 1PIM-dia (C), and 1PIM-para (D) with P450cam C334A present in the open state. The proteins are shown in grey, the haem in purple, the carbons of 1PI and its analogs in green, nitrogens in blue and oxygens in red. (E) Detail of the crystal structure of 1PIM-dia. The P450cam is shown in grey surface, haem in magenta, 1PIM-dia in green, and 1PI in yellow. Note that the para-position of 1PI faces the protein, whereas the meta-position faces the substrate channel in the open state.

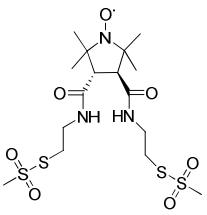
Conclusion

Rigid spin label probes were successfully synthesized and tagged on a test protein. The EPR spectra clearly showed that the mobility of probe was reduced due to two-point attachment. However, the nitroxide of DASL is unstable after attaching to this model protein. A possible explanation is the combination of strain caused by the attachment site and the mobility of the saturated five-membered ring. Hence, another test protein with closer cysteines should be tried to establish the power of DASL.

Substrate/inhibitor analogues can be considered the next generation of probes, which can interact with a designated target protein. In this context, 1-phenylimidizole derivatives, inhibitors of P450cam, were synthesized and the ligands were co-crystalized with P450cam. The imidazole ring of 1PIM-dia and 1PIM-para sits above the haem iron and the nitroxide sits in the channel. By contrast, the nitroxide group faces the haem iron in the case of 1PIP-dia. Both 1PI analogs are locked in the substrate channel, forcing the protein to be in an open state. These initial results suggest this approach is promising for the study of protein-ligand complexes. The spin labels in the P450cam analog may help to study the dynamics of the opening and closing in solution using PRE NMR spectroscopy.

Methods and Materials

Compound **1**[205,206], **2**[207], **3**[207], **8**[195], **9**[195], **10**[195], **HO-3606**[195], **13**[200,201] and **14**[200,201] were prepared according to the literature methods. All other chemicals were used as purchased without further purification. TLC-analysis was conducted on DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm). Flash chromatography was performed on Screening Devices silica gel 60 (0.04-0.06 mm). A Biocad Vision HPLC (PerSeptive Biosystems, inc.) and an Akta Basic FPLC (GE Healthcare Inc.) were used for purifications. Analytical, semipreparative, and preparative reversed phase C18 columns were obtained from Phenomenex (Torrance, CA). Superdex 75 column was obtained from GE Healthcare. NMR spectra were recorded on a Bruker AV-400 (400/100 MHz) and Bruker Avance-III 600 (600/150 MHz) spectrometer. A LCQ LCMS system and a Finnigan LTQ Orbitrap system were used for HRMS and protein conjugation analysis. FTIR was performed on a Perkin-Elmer (Shelton, CT) Paragon 1000 FTIR spectrometer. Melting points were obtained using a SMP3 scientific melting apparatus (Stuart, Bibby Sterlin Ltd.)

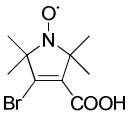


DASL

To a solution of **3** (300 mg, 1.3 mmol) in DMF (11 mL) was added NHS (320 mg, 2.7 mmol), DMAP (340 mg, 2.7 mmol), and aminoethyl-MTS (654 mg, 2.7 mmol) and the solution mixture was cooled to 0 ^{o}C. DCC (557 mg, 2.7 mmol) dissolved in 1 mL DMF was then added to the solution mixture and the reaction was allowed to move to RT and stirred continuously for 16 h. The DMF was removed under vacuo and the residue was diluted with chloroform (20 mL). The organic solution was washed with $NaHCO_{3(aq.)}$, dried by $MgSO_4$, concentrated under vacuo and purified by silica chromatography. The title compound was obtained in 60% yield. R_f = 0.25 (5% MeOH in CH_2Cl_2). HR-MS m/z: 505.1038 $[M+H]^+$, calcd $[C_9H_{15}N_2O]^+$: 505.1039.

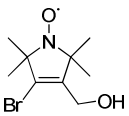
Protein labeling experiment and NMR spectroscopy

To attach DASL to the Paz, protein sample (1 mL, 150 ~ 300 μM) was treated with DTT (final concentration 5 mM) at 0 °C for 1 h to remove possible dimers. The reaction mixture was loaded on a PD-10 column (GE Healthcare) pre-equilibrated with labeling buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.0) to remove DTT. Instead of regular 3.5 mL eluent collection, 3 mL eluent was collected. Although the protein sample might partly lost, the excess DTT can be removed efficiently. To avoid any reoxidation by air, the buffer was degassed and the PD-10 column kept under an argon atmosphere. To the eluted protein 10 equivalents of DASL or MTSL and their control were added. Those compounds were dissolved in DMSO and prepared freshly. The solution was stirred 1 h at 4 °C. The probe attached Paz sample was concentrated to 500 μL and purified over a Superdex 75 gel filtration column. The purified DASL tagged protein was separated to two portions, one was for paramagnetic sample and the other for diamagnetic control. For diamagnetic control, five equiv. sodium ascorbate were added to reduce the radical. The NMR and EPR spectra were immediately recorded after sample preparation. For EPR and NMR sample, Paz spin-label (100 ~ 150 μM) were prepared in 20 mM sodium phosphate, 150 mM NaCl buffer and 6% (v/v) D_2O at pH = 7. All [^{15}N, ^{1}H]-HSQC were recorded at 298 K on a Bruker Avance III 600 MHz spectrometer. Data were processed with NMRPipe and analyzed with CCPNMR Analysis version 2.1. Assignments of the resonances were based on previous work.



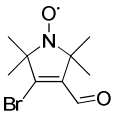
4-bromo-3-(carboxylic acid)-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-1-yloxyl radical (4c)

A solution mixture of H_2O (100 mL) and 1,4-dioxane (25 mL) containing 4-Oxo-TEMPO (5 g, 29.4 mmol) and NaOH (5 g, 125 mmol) was added over 10 min into a 150 mL NaOBr solution which was prepared by Br_2 (5.2 mL) and NaOH (20 g) at -2 to -5 ^oC. The reaction mixture was stirred at the same temperature for another 20 min, followed by adding $Na_2S_2O_3$ (2 g) in 6 mL H_2O and the reaction was heated until 35 ^oC for 30 min. After cooling to 0 ~ 5 ^oC, the precipitate was filtered off and the filtrate was acidified by using concentrated HCl to yielding pure compound **6** (40 %). Spectroscopic data were in agreement with those reported in the literature.[208] HR-MS m/z: 264.0230 $[M+H]^+$, calcd $[C_9H_{14}BrNO_3]^+$: 264.0229.



4-bromo-3-(hydroxymethyl)-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-1-yloxyl radical (6b)

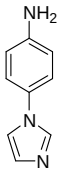
To a dry solution of **4c** (1 g, 3.8 mmol) and TEA (0.56 mL, 4.6 mmol) in Et_2O (40 mL) was added ethyl chloridocarbonate (500 mg, 4.6 mmol) under 0 ^oC, and then the reaction mixture was stirred and allowed to warm up to RT over 3 h. The reaction was extracted with water, dried by $MgSO_4$ and concentrated without further purification. The concentrated residue was dissolved in 4 mL EtOH under -30 ~ -40 ^oC and $NaBH_4$ (56 mg) was added into the solution mixture, stirring was continued for an additional 2 h at 0 ^oC. When the reaction was finished, the solvent was removed by vacuo and the residue was diluted with CH_2Cl_2, washed with brine, and recrystallized by using toluene. The title compound was obtained in 50% yield. Spectroscopic data were in agreement with those reports in the literature.[208] R_f = 0.26 (PET/ETOAc, 3/1). HR-MS m/z: 249.0359 $[M+H]^+$, calcd $[C_9H_{16}BrNO_2]^+$: 249.0359.



4-bromo-3-formyl-2,2,5,5-tetramethyl-2,5-dihydro-*1H*-pyrrol-1-yloxyl radical (7)

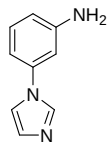
A solution mixture of CrO_3 (1.33 g) and pyridine (2.16 mL) in 6.7 mL dry CH_2Cl_2 was stirred for 15 mins in an ice bath, after which **6b** (562 mg, 2.3 mmol) dissolved in 3.3 mL dry CH_2Cl_2 was added. After an additional 20min stirring, Et_2O was added into the mixture and the precipitate was washed with Et_2O. The Et_2O was collected, washed with 5% $HCl_{(aq)}$, water, sat. $NaHCO_3$, brine, dried by $MgSO_4$ and

purified by silica column chromatography. Spectroscopic data were in agreement with those reports in the literature.[208] R_f = 0.77 (PET/EtOAc, 3/1).



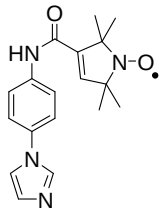
4-(1H-imidazol-1-yl)benzenamine (13)

The title compound was prepared according to literature procedures.[200,201] ^{1}H NMR (400 MHz, d^4-MeOD): δ = 7.91 (t, 1H, *J* = 1.2 Hz), 7.37 (t, 1H, *J* = 1.2 Hz), 7.22 (dt, 2H, *J* = 8.8 Hz, *J* = 3.2Hz), 7.08 (t, 1H, *J* = 1.2 Hz), 6.79 (td, 2H, *J* = 8.8 Hz, *J* = 3.2 Hz). ^{13}C NMR (100 MHz, d^4-MeOD): δ = 149.3, 136.9, 129.4, 129.0, 123.9, 120.3, 116.7. FTIR: 3340.7, 3184.5, 3113.1, 1608.6, 1517.9, 1253.7, 827.5 cm^{-1}. HR-MS m/z: 160.1076 $[M+H]^+$, calcd $[C_9H_{10}N_3]^+$: 160.0869.



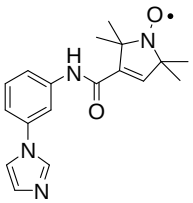
3-(1H-imidazol-1-yl)benzenamine (14)

The title compound was prepared according to literature procedures.[200,201] ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.81 (s, 1H), 7.22-7.15 (m, 2H), 6.75-6.72 (m, 1H), 6.66-6.64 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 147.9, 138.2, 135.4, 130.5, 129.9, 118.1, 113.7, 110.9, 107.5. FTIR: 3415.9, 3340.7, 3199.9, 3111.2, 1606.7, 1458.2, 1234.4, 1055.0, 740.7 cm^{-1}. HR-MS m/z: 160.1076 $[M+H]^+$, calcd $[C_9H_{10}N_3]^+$: 160.0869.



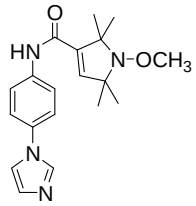
1PIP-para

To a solution of **13** (283 mg, 1.8 mmol) in 20 mL DMF was added HATU (640 mg, 2 mmol). After stirring for 30 second, spin labeled **5** (334 mg, 1.8 mmol) was added into the mixture. The reaction was allowed to stir at RT for 16 h. The residue was diluted in water and extracted with CH_2Cl_2. The organic layer was dried by $MgSO_4$, filtered and concentrated. The concentrated crude compound was purified by HPLC (0.1% NH_4OAc and a 10-50% acetonitrile gradient on C18 preparative column). FTIR: 3493.1, 3304.1, 3228.8, 3134.3, 2978.1, 2935.6, 1668.4, 1521.8, 1319.3, 1068.6 cm^{-1}. HR-MS m/z: 326.1737 $[M+H]^+$, calcd $[C_{18}H_{22}N_4O_4]^+$: 326.1737.



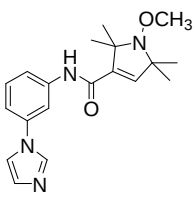
1PIM-para

Following the same protocol with **1PIP-para**, **1PIM-para** was synthesized and purified by HPLC (0.1% NH_4OAc and a 10-50% acetonitrile gradient on C18 preparative column). FTIR: 3448.7, 3253.9, 3205.7, 3082.3, 2978.1, 2931.8, 1664.6, 1600.9, 1550.8, 1440.8, 1217.1, 796.6 cm^{-1}. HR-MS m/z: 326.1738 $[M+H]^+$, calcd $[C_{18}H_{22}N_4O_2]$: 326.1737.



1PIP-dia

A mixture of **1PIP-para** (160 mg, 0.49 mmol) and $FeSO_4 \cdot 7H_2O$ (178 mg, 0.98 mmol) was dissolved in 10 mL DMSO, and then H_2O_2 (140 μL, 1.43 mmol) was added into the reaction mixture. After stirring for 1 h at RT, the residue was diluted with water and extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, concentrated under reduce pressure and purified by HPLC (0.2% TFA and a 25-45% acetonitrile gradient on C18 preparative column) yielding colorless oil **1PIP-dia** (30 mg, 18%). R_f = 0.75 (10% MeOH in CH_2Cl_2). ^{1}H NMR (600 MHz, d^4-MeOD): δ = 9.42 (s, 1H), 8.04 (t, 1H, *J* = 3 Hz), 7.91 (t, 2H, *J* = 13.2 Hz), 7.75 (t, 1H, *J* = 2.4 Hz), 7.67 (t, 2H, *J* = 13.2 Hz), 6.44 (s, 1H), 3.75 (s, 3H), 1.48-1.38 (br, 12H). ^{13}C NMR (150 MHz, d^4-MeOD): δ =165.6, 141.9, 141.3, 141.0, 135.6, 131.9, 124.1, 122.9, 122.8, 121.9, 72.7, 69.8, 65.7, 27.5, 21.4. FTIR: 3111.2, 2974.2, 2935.6, 1666.5, 1521.8, 1197.8, 1128.4, 829.4, 719.5 cm^{-1}. HR-MS m/z: 341.1974 $[M+H]^+$, calcd $[C_{19}H_{25}N_4O_2]^+$: 341.1972.



1PIM-dia

Following the same protocol with **1PIP-dia**, **1PIM-dia** was synthesized and purified by HPLC (0.2% TFA and a 25-45% acetonitrile gradient on C18 preparative column). ^{1}H NMR (600 MHz, d^4-MeOD): δ = 9.46 (s, 1H), 8.24 (s, 1H), 8.06 (s, 1H), 7.77 (s, 1H), 7.65 (d, 1H, *J* = 8.4 Hz), 7.58 (t, 1H, *J* = 8.4 Hz), 7.44 (d, 1H, *J* = 7.8 Hz), 6.45 (s, 1H), 3.78 (s, 3H), 1.47-1.37 (br, 12H). ^{13}C NMR (150 MHz, d^4-MeOD): δ = 165.7, 142.0, 141.5, 140.9, 136.8, 135.9, 131.9, 122.9, 122.8, 122.1, 118.9, 115.6, 72.8, 69.9, 65.7, 29.4, 23.2. FTIR: 3410.1, 3128.5, 2991.6, 1666.5, 1606.7, 1170.8,

1130.3, 796.6, 719.5 cm^{-1}. HR-MS m/z: 341.2152 $[M+H]^+$, calcd $[C_{19}H_{25}N_4O_2]^+$: 341.1972.