Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics

Supporting Information

Diversification of EPR signatures in Site directed spin labeling using a β-phosphorylated nitroxide

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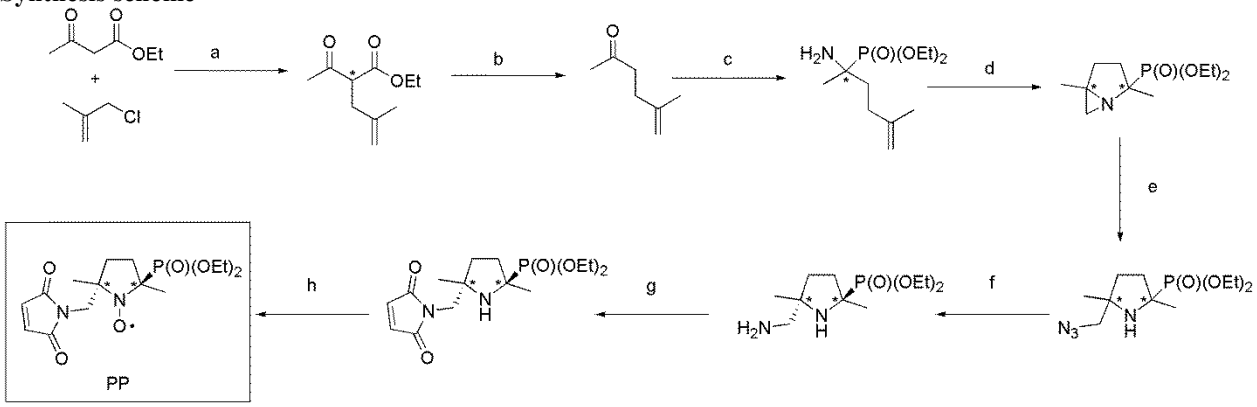
Synthesis and characterization of spin label PP

Materials. All reactions were carried out in oven–dried glassware. All chemicals were purchased from Sigma-Aldrich and used as received. Flash column chromatography was performed using 63–200 µm silica gel (Merck). Thin–layer chromatography (TLC) was performed using 60 F–254 silica gel plates (Merck). Visualization of TLC plates was accomplished with UV light and iodine or ethanolic phosphomolybdic solution.

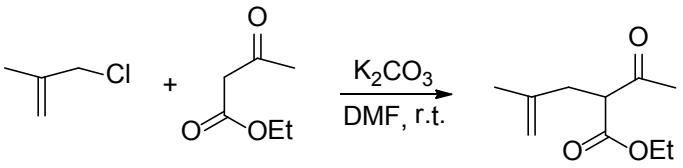
Physical measurements. ^{1}H, ^{13}C, ^{31}P NMR were measured on Bruker Avance-300 and Bruker Avance-400 NMR spectrometers. ^{1}H NMR and ^{13}C NMR spectra were referenced to the residual non-deuterated solvent peak. ^{31}P NMR chemical shift were given to an external reference (85% H_3PO_4). Chemical shifts are reported in ppm and coupling constants in Hz. High-resolution mass spectra were obtained on 3200 QTRAP or QStar Elite (Applied Biosystems SCIEX).

Synthesis scheme



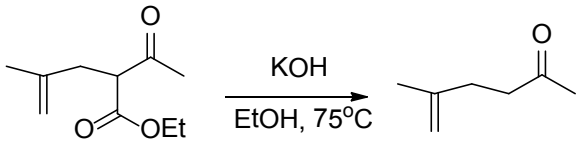
Synthesis of spin-label PP. (a) K_2CO_3, DMF, rt; (b) KOH, EtOH, 75 °C; (c) Diethylphosphite, NH_3, rt; (d) I_2, $NaHCO_3$, CH_2Cl_2, rt; (e) NaN_3, NH_4Cl, MeCN, 80 °C; (f) H_2, Pd/C, EtOH, rt; (g) rt *(1)* Maleic anhydride, *(2)* $(COCl)_2$ (h) *m*-CPBA, CH_2Cl_2, rt.

- **Ethyl 2-acetyl-4-methylpent-4-enoate**



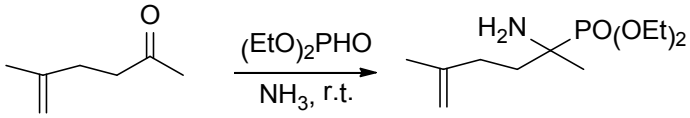
The title compound was prepared according to the procedure.[1] To a solution of ethyl 3-oxobutanoate (0.33 mol, 43 g) and 3-chloro-2-methylprop-1-ene (0.33 mol, 30 g) in 500 ml of DMF, anhydrous K_2CO_3 (1 mol, 138 g) was added. The reaction mixture was stirred at rt for 48 hours, then diluted with water and extracted with mixture of Et_2O/hexane (2:1). The organic solution was washed with water, brine, dried over $MgSO_4$ and evaporated. The crude product was purified by distillation at reduced pressure to give a colorless oil, 35 g, yield 58%. ^1H (400 MHz, $CDCl_3$) : 1.26 (t, J = 7.2 Hz, 3H), 1.73 (s, 3H), 2.24 (s, 3H), 2.55-2.58 (m, 2H), 3.66 (t, J = 7.6 Hz, 1H), 4.19 (q, J = 7.1 Hz, 2H), 4.69 (s, 1H), 4.78 (s, 1H).

- **5-methylhex-5-en-2-one**



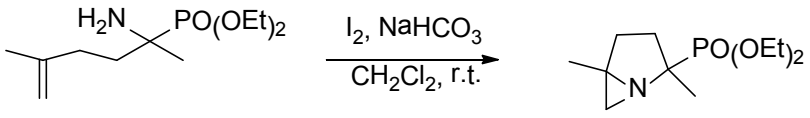
The 5-methylhex-5-en-2-one was prepared according to the procedure.[2] The ester (33 mmol, 6 g) was added to a solution of KOH (75 mmol, 4.2 g.) in 100 ml EtOH. The solution was stirred at 75 °C for 1 hour, neutralized with 10 % HCl and extracted with EtOAc. The combined organic phases were washed with brine, dried over $MgSO_4$ and concentrated to give a colorless oil, 3.1 g, yield 84 %. The obtained product was used in the next step without further purification. ^1H ($CDCl_3$, 400 MHz): 1.73 (s, 3H) ; 2.17 (s, 3H) ; 2.27-2.33 (m, 2H) ; 2.56-2.60 (m, 2H) ; 4.66 (s, 1H) ; 4.73 (s, 1H).

- **Diethyl (1-amino-1,4-dimethylpent-4-en-1-yl)phosphonate**



Diethyl (1-amino-1,4-dimethylpent-4-en-1-yl)phosphonate was prepared according to the procedure.[3] A stream of ammonia was bubbled through a mixture of ketone (20 mmol, 2.3 g.) and $(EtO)_2PHO$ (47 mmol, 6.5 g) at rt overnight. The reaction mixture was stirred with aqueous ammonia for 0.5 hour, diluted with chloroform and acidified with 10 % HCl. The aqueous solution was basified with $NaHCO_3$ and extracted with $CHCl_3$. The organic layers were dried over $MgSO_4$ and evaporated. Colorless oil, 3.5 g, yield 67%. It was used in the next step without further purification ^1H ($CDCl_3$, 400 MHz) δ: 1.20 (d, 3H, J = 16.1 Hz) ; 1.27 (t, 6H, J = 7.0 Hz) ; 1.42 (br. s, 2H, NH_2) ; 1.67 (s, 3H); 1.64-1.72 (m, 2H), 2.00-2.19 (m, 2H); 4.04-4.12 (quart, 4H, J = 7.2 Hz) ; 4.63 (s, 2H). ^{31}P: ($CDCl_3$, 120MHz) : δ = 31.5.

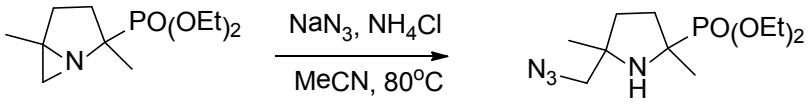
- **Diethyl (2,5-dimethyl-1-azabicyclo[3.1.0]hex-2-yl)phosphonate**



The aminophosphonate (5.68 g, 22.8 mmol) was dissolved in 50 mL of CH_2Cl_2, then 150 ml of 6 % solution of $NaHCO_3$ was added. A solution of I_2 (6.44 g, 25.4 mmol) in 150 mL of CH_2Cl_2 was added dropwise. The mixture was stirred at room temperature for 3 hours. Solid $Na_2S_2O_3$ was added to the reaction mixture and stirred for 30 minutes. The organic phase was decanted and the aqueous phase was extracted with CH_2Cl_2. The organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The product was obtained as a mixture of two

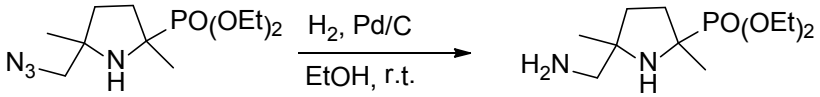
diastereomers, colorless oil, 4.8 g, yield 85%. The product was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): 1.22-1.34 (m, 12H), 1.41-1.55 (m, 3H), 1.95-2.19 (m, 3H), 4.05-4.21 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) : 16.4, 16.5, 16.5, 16.6, 19.8, 19.8, 21.0, 21.8, 23.8, 29.4, 29.5, 30.0, 30.1, 30.6, 30.8, 31.2, 32.7, 33.7, 33.7, 46.0, 46.2, 47.9, 61.6, 62.0, 62.1, 62.4, 62.5, 62.8, 64.5, 64.9, 66.7, 67.3. ^{31}P NMR ($CDCl_3$, 120 MHz): δ (ppm) = 29.9 (major) and 29.5 (minor). HRMS: calculated for $C_{11}H_{23}NO_3P$ $[M+H]^+$ 248.1410, found: 248.1404.

- **Diethyl [5-(azidomethyl)-2,5-dimethylpyrrolidin-2-yl]phosphonate**



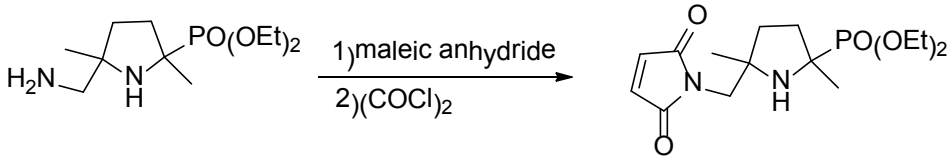
A solution of the aziridine (15 mmol, 3.7 g), NaN_3 (18.2 mmol, 1.18 g, 1.2 eq) and NH_4Cl (16.6 mmol, 0.89 g, 1.1 eq) in 95 ml MeCN was heated for 5 hours at 80°C. The reaction mixture was diluted with MeCN, filtered through Celite® and evaporated. The crude product was purified by column chromatography (eluent CH_2Cl_2/EtOH 95/5). The product was obtained as a mixture of diastereoisomers with the ratio 3:1, colorless oil, 2.4 g, yield 55%. Major diastereomer: ^{1}H NMR ($CDCl_3$, 400 MHz) 1.21 (s, 3H), 1.33 (t, J = 7.02, 6H), 1.37 (d, J = 15.29 Hz, 3H), 1.65-1.83 (m, 3H), 2.30-2.40 (m, 1H), 3.12-3.30 (m, 2H), 4.11-4.21 (m, 4H). ^{31}P NMR : ($CDCl_3$, 162 MHz) : δ (ppm) = 30.35. Minor diastereomer: ^{1}H NMR ($CDCl_3$, 400 MHz) 1.21 (s, 3H), 1.32 (t, J = 7.02 Hz, 6H), 1.39 (d, J = 15.29 Hz, 3H), 1.65-1.83 (m, 3H), 2.30-2.40 (m, 1H), 3.12-3.30 (m, 2H), 4.11-4.21 (m, 4H). ^{31}P NMR : ($CDCl_3$, 162 MHz) : δ (ppm) = 29.14. ^{13}C NMR ($CDCl_3$, 75 MHz): 16.4, 16.5, 16.6, 25.5, 25.6, 25.6, 26.2, 26.9, 33.9, 33.9, 34.4, 34.9, 35.0, 35.1, 59.7, 59.9, 61.3, 61.5, 61.5, 61.6, 62.0, 62.1, 62.6, 62.6, 63.4, 63.5. HRMS: calculated for $C_{11}H_{24}N_4O_3P$ $[M+H]^+$ 291.1581, found 291.1580.

- **Diethyl [5-(aminomethyl)-2,5-dimethylpyrrolidin-2-yl]phosphonate**



To a solution of the amine (2.6 mmol, 0.7 g) in 150 mL of EtOH 210 mg of Pd/C was added. The mixture was stirred for 6 hours under hydrogen atmosphere at rt. The reaction mixture was filtered through a Celite and evaporated. Only the major diastereomer was obtained as a yellowish oil (450mg, 71%). The obtained diamine was used in the next step without further purification. ^{1}H NMR : ($CDCl_3$, 400 MHz) : 1.14 (s, 3H), 1.33 (t, J = 7.24 Hz, 6H), 1.34 (d, J = 15.44 Hz, 3H), 1.66-1.78 (m, 3H), 2.14 (br. s, 3H), 2.30-2.39 (m, 1H), 2.49-2.61 (m, 2H), 4.11-4.22 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz): 16.2 (triplet like, J = 5.2 Hz, 2C), 25.4 (d, J = 8.1 Hz), 25.6, 34.1, 34.5 (d, J = 5.5 Hz), 51.5, 59.9 (d, J = 160.7 Hz), 61.7 (d, J = 7.7 Hz), 62.3 (d, J =7.7 Hz), 63.5 (d, J = 7.2 Hz). ^{31}P NMR: ($CDCl_3$, 162 MHz) : δ (ppm) = 31.0. HRMS: calculated for $C_{11}H_{26}N_2O_3P$ $[M+H]^+$ 265.1676, found 265.1679.

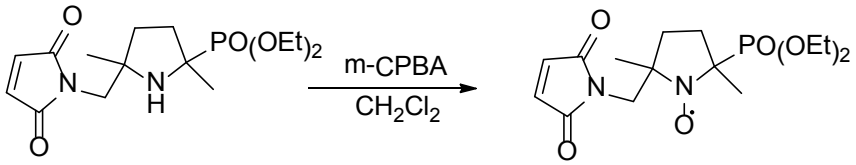
- **Diethyl {5-[(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl]-2,5-dimethylpyrrolidin-2-yl}phosphonate**



A toluene solution of the amine (2 mmol, 528 mg) and maleic anhydride (3 mmol, 300 mg) was stirred at rt overnight. The red oil deposited on the bottom of the flask was decanted, dissolved in dichloromethane and treated with oxalyl chloride (2 mmol, 255 mg). After stirring 24 h at rt the solution was washed with water, the aqueous layer was neutralized with $NaHCO_3$ and extracted with $CHCl_3$. The solvent was evaporated and the crude product was purified by column chromatography (eluent EtOH/Et_2O 1:4), 48 mg oil, yield 7 %. ^{1}H NMR ($CDCl_3$, 400 MHz) : 1.15 (s, 3H), 1.28 (d, J = 15.35 Hz, 3H), 1.31 (t, J = 7.09 Hz, 6H), 1.64-1.78 (m, 3H), 2.27-2.42 (1H, m), 3.52 (dd, $J_{A,B}$ = 14.05 Hz, 2H), 4.11-4.17 (dq, J_1 = 6.94 Hz, J_2 = 2.5 Hz, 4H), 6.74 (s, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz): 16.5 (triplet like, J = 5.2 Hz, 2C), 25.1 (d, J = 8.8 Hz), 27.1, 34.0 (d, J = 1.1 Hz), 35.1 (d, J = 5.5 Hz), 47.8,

60.6 (d, J = 166.7 Hz), 62.2 (d, J = 7.7 Hz), 62.7 (d, J = 7.2 Hz), 64.1 (d, J = 8.3 Hz), 134.2 (2C), 171.4 (2C). ^{31}P NMR ($CDCl_3$, 162 MHz) : 30.0 ppm. HRMS: calculated for $C_{15}H_{26}N_2O_5P$ $[M+H]^+$ 345.1574, found 345.1572.

- **{2-(diethoxyphosphoryl)-5-[(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl]-2,5-dimethylpyrrolidin-1-yl}oxidanyl - PP**



A solution of maleimide (0.029 mmol, 10 mg) and m-CPBA (0.058 mmol, 10 mg) in CH_2Cl_2 was stirred for 3 h at rt. The reaction mixture was washed with $Na_2S_2O_3$, $NaHCO_3$ then dried with $MgSO_4$. The solvent was evaporated and the crude product was purified by flash chromatography (eluent EtOAc/Et_2O 1/1). The nitroxyde was obtained as a yellowish oil (4.7 mg, yield 45 %) HRMS : calculated for $C_{15}H_{25}N_2O_6P$ $[M+H]^+$ 360.1445, found 360.1442.

Figures

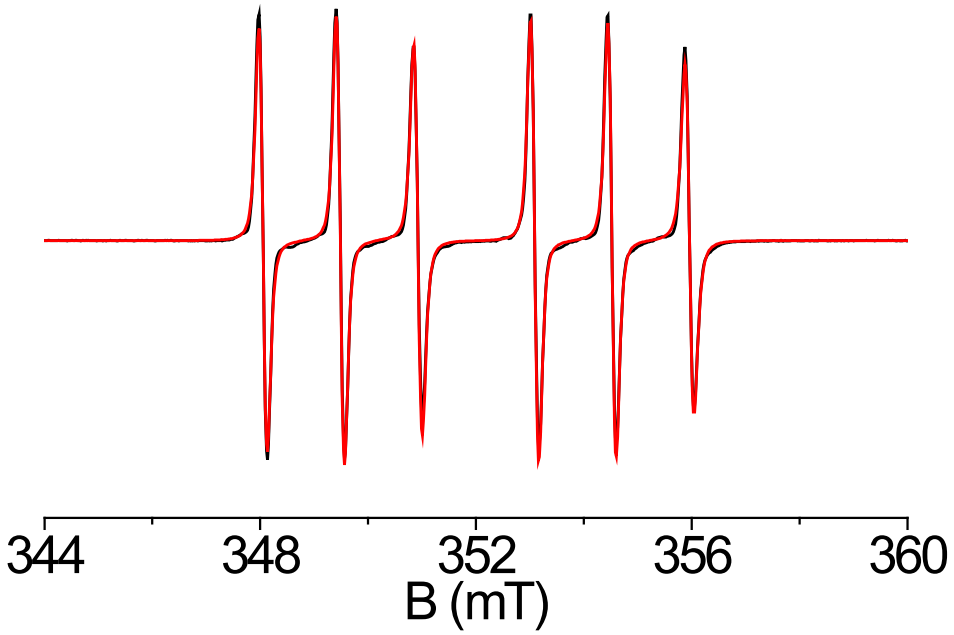


Figure S1. Experimental (black line) and simulated (red line) X-band EPR spectra of PP recorded at room temperature using a 200 µM solution in 10 mM sodium phosphate buffer at pH 7. The microwave power was 10 mW and the magnetic field modulation amplitude was 0.1 mT. Simulation was performed using ROKI software [4] with the following parameters: $g_{\perp} = 2.0069$, $g_{//} = 2.0021$, $A_{P\perp} = 4.7$ mT, $A_{P//} = 5.6$ mT, $A_{N\perp} = 0.7$ mT, $A_{N//} = 2.7$ mT $\tau = 0.17$ ns.

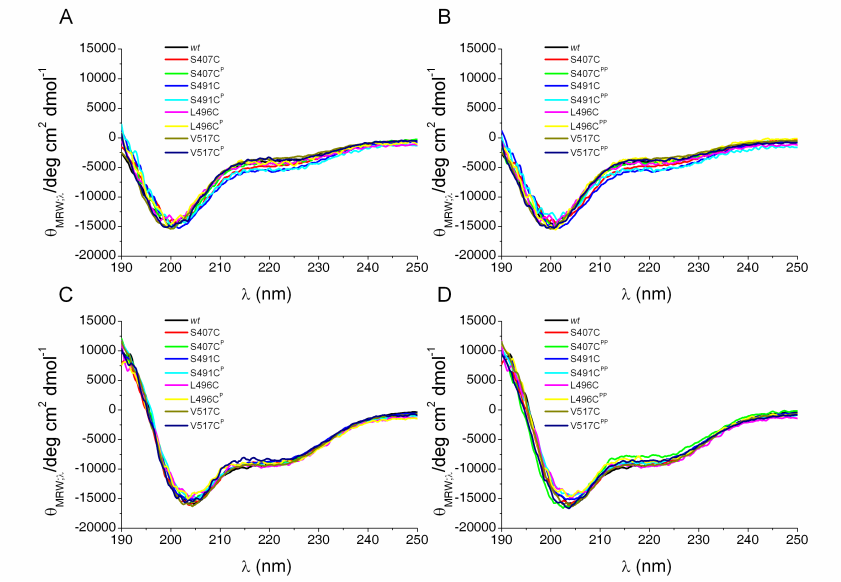


Figure S2. Far-UV CD spectra of wt, unlabeled variants and labeled $N_{TAIL}{}^{P}$ (left) and $N_{TAIL}{}^{PP}$ (right) variants at 0% **(A, B)** and 20% **(C, D)** TFE (v/v).

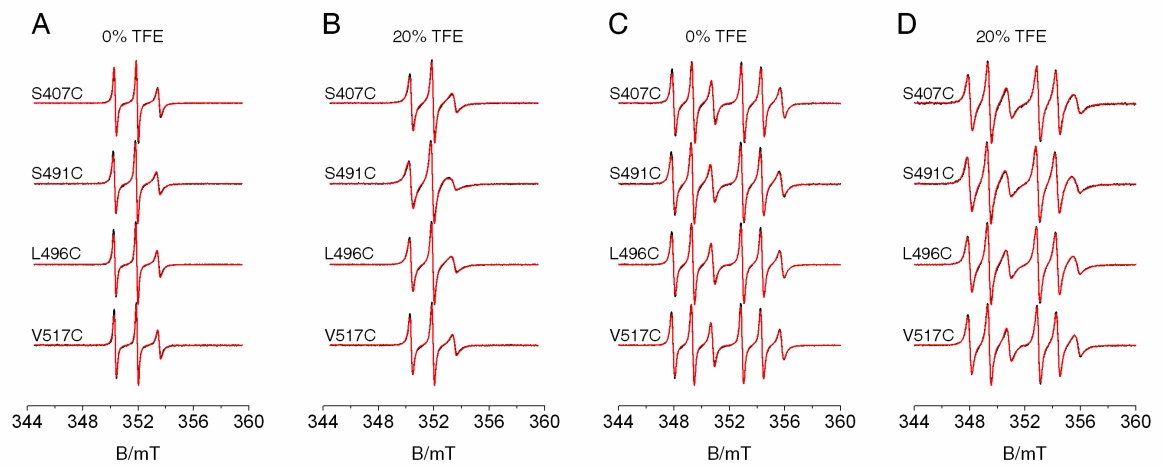


Figure S3. Amplitude normalized rt EPR spectra (black) of P **(A, B)** and PP **(C, D)** spin labeled N_{TAIL} variants in the presence of 0% ***(A, C)*** and 20 % ***(B, D)*** TFE (v/v). Simulated spectra (red) using ROKI software[4]are superimposed on the experimental spectra.

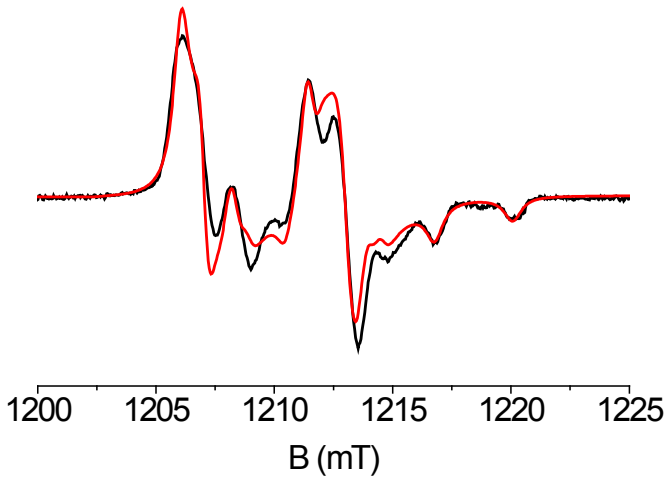


Figure S4. Experimental (black line) and simulated (red line) Q-band EPR spectra of S407C N_{TAIL}^{PP} variant, recorded at 100K using a 100 µM solution in the presence of 30% (v/v) glycerol in 10 mM sodium phosphate buffer at pH 7. The microwave power was 1 µW and the magnetic field modulation amplitude was 0.25 mT. Simulation was performed using ROKI software [4] with the parameters : g_{xx} = 2.0088, g_{yy} = 2.0059, g_{zz} = 2.0020, A_{Pxx} = 5.2 mT, A_{Pyy} = 5.7 mT, A_{Pzz} = 6.3 mT, A_{Nxx} = 0.4 mT, A_{Nyy} = 0.6 mT, A_{Nzz} = 3.3 mT.

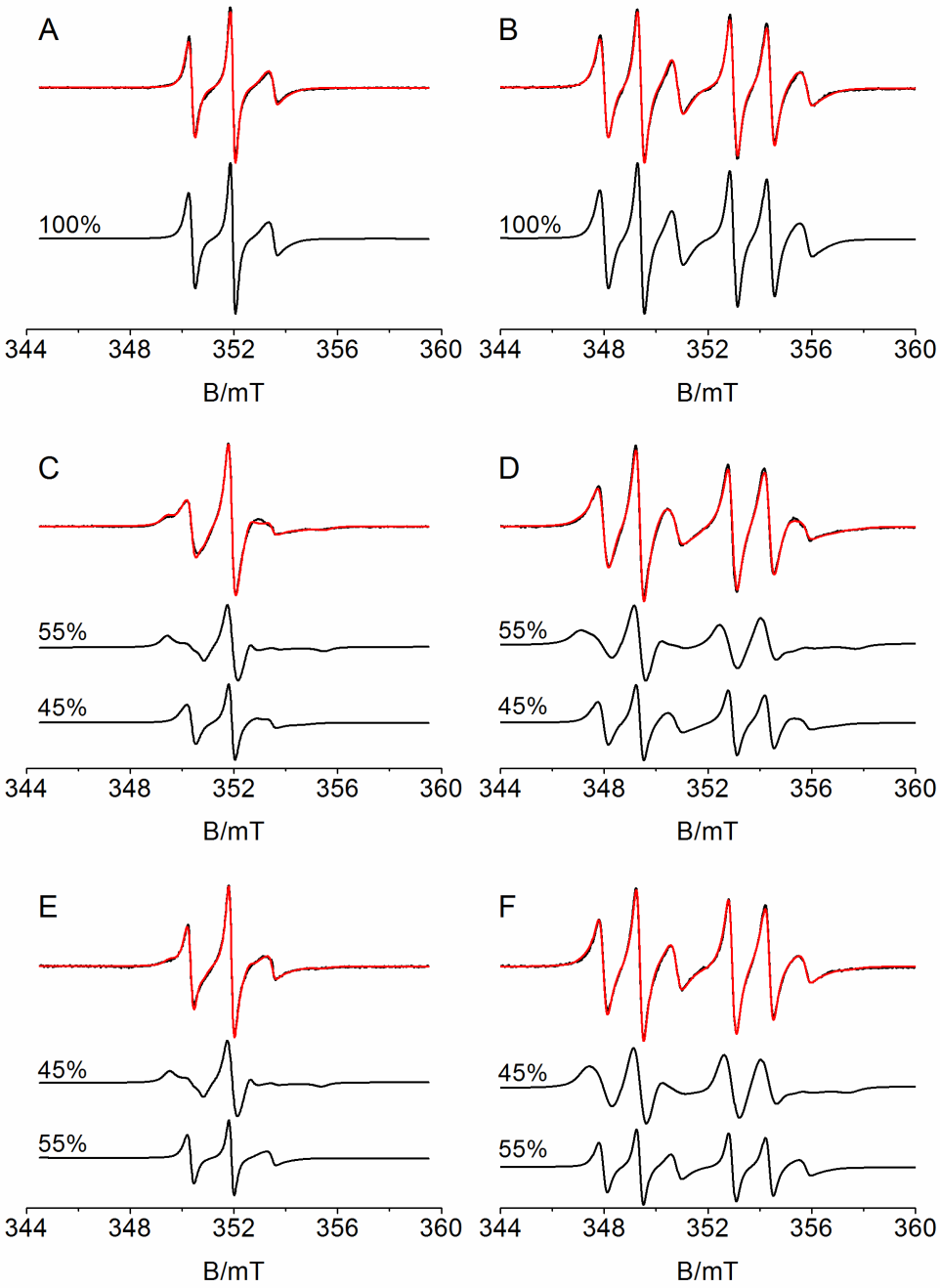


Figure S5. Amplitude normalized room temperature EPR spectra (black lines) of ***(A, B)*** S407C N_{TAIL} variant labeled with ***(A)*** P and ***(B)*** PP, ***(C, D)*** L496C N_{TAIL} variant labeled with ***(C)*** P and ***(D)*** PP and ***(E, F)*** V517C N_{TAIL} variant labeled with ***(E)*** P and ***(F)*** PP in 30% sucrose (w/v). The simulated spectra (red lines) obtained using the ROKI software[4] are superimposed with the experimental one. Amplitude normalized individual components corresponding to bound and unbound forms are displayed. The the following parameters have been used for the simulations: for P : $g_{\perp} = 2.0068$ (± 0.0005), $g_{//} = 2.0018$ (± 0.0005), $A_{N\perp} = 0.70$ (± 0.06) mT, $A_{N//} = 2.7$ (± 0.1) mT ; for PP : $g_{\perp} = 2.0070$ (± 0.0005), $g_{//} = 2.0022$ (± 0.0005), $A_{P\perp} = 4.7$(± 0.2) mT, $A_{P//} = 5.5$ (± 0.4) mT, $A_{N\perp} = 0.7$ (± 0.1) mT, $A_{N//} = 3.3$ (± 0.2) mT.

Molecular dynamics

- ***Starting coordinates***

The starting structure was taken from the X-ray structure of the XD/N_{TAIL} chimera (pdb entry 1T6O).[5] Mutants S491C and L496C were built *in silico* using Swiss-PdbViewer (aka DeepView) software,[6] by changing serine 491 or leucine 496 into cysteine.
The protonation state of each residue was assigned with respect to physiological conditions by using the H^{++} server.[7] Histidine 498 was protonated at the ε nitrogen atom. Partial charges for probes P and PP were obtained from R.E.D server.[8] C-term and N-term atoms from N_{TAIL} were capped with N-methylamine and acetyl, respectively.
For each probe, four systems were built:
-N_{TAIL} alone modified at position 491
-N_{TAIL} alone modified at position 496
-N_{TAIL} modified (with P or PP) at position 491, in the vicinity of XD. In this case, the modified N_{TAIL} (with P or PP) were manually translated ~5 Å away from XD in order to avoid steric clashes between XD and the probe and to prepare unrestrained MDs dedicated to sample the binding event between the two sub-systems.
-N_{TAIL} modified at position 496 in bound to XD built from the X-Ray structure of the chimera.
The systems were then neutralized by adding one chloride ion in the case of N_{TAIL} alone or five ions when it was in interaction with XD. Finally, the water phase was modeled as a box extended to a distance of 10 Å from any solute atom.

- ***Molecular Dynamics Simulations***

Molecular dynamic simulations were carried out with either sander or pmemd within the AMBER12 suite of programs. [*AMBER 12*, University of California, San Francisco][9] ff03 parameters were used for all amino-acids and gaff parameters were obtained with Antechamber for P and PP. P and PP charges were computed with the RED server. Periodic boundaries conditions were applied and all bonds involving hydrogen atoms were constrained by using the SHAKE algorithm. A time step of 2 fs was used. An 8 Å cut-off was applied to non-bonded van der Waals interactions and the non-bonded pair list was updated every 15 steps. Particle Mesh Ewald parameters were chosen to obtain a grid spacing close to 1 Å and a 9 Å direct space cut-off.
A series of simulations described further were performed to relax water molecules and remove close contacts. 10000 steps of minimisation followed by 20 ps of Molecular Dynamics (MD) simulation with constraints (20 kcal/(mol.Å²)) applied on the solute atoms. These calculations were repeated 3 times, reducing the restraints by 5 kcal/(mol.Å²) at each round. Then, 10000 steps of minimisation without any restraint were carried out. Further, the system was slowly heated from 0 to 300 K over a period of 14 ps, using a Langevin thermostat (with a damping constant of 2 ps^{-1}), respectively. The system was then equilibrated in NVT conditions during 2 ns. An additional 10 ns equilibration simulation was carried out in NPT conditions. Finally 100 ns production with NPT conditions was performed for each structure.
To simulate the association of $N_{TAIL\_491\_PP}$ with XD, a different protocol was used. After having energy minimised the system, several MDs (10 for PP and 3 for P) simulations with different initial velocities were run for 10 ns. In 3 out of the 10 systems for PP, and 3 out of the 3 systems for P, a binding event was observed within the allowed time, with final structures showing N_{TAIL} bound to XD in a position equivalent to that of the Chimera. One typical structure of each system was selected for a further 100 ns MD run.

- ***Analysis***

Atomic fluctuations:
The quantification of the probe mobility was estimated by the atomic fluctuations of the radical oxygen atom. The 100 ns trajectory was imaged and centered on the initial structure. Rotations and translations of the system were removed by fitting the coordinate to the first structure of the trajectory. Then the atomic fluctuations were calculated for all the atoms of the probe.

Binding event:
The RMSd between the crystal structure and the structure observed during the unconstrained simulation (heating, equilibration and production phase) was calculated on the trajectory centered on the P_{XD} X-Ray structure, using VMD RMSd trajectory tools. The results are presented figures S6 and S7. The RMS deviation rapidly falls into the 2-3 Å range, emphasizing structures closely related to that of the X-Ray of the chimera. Figure S7 shows a top view of the comparison between the complexes and the chimera X-ray structure.

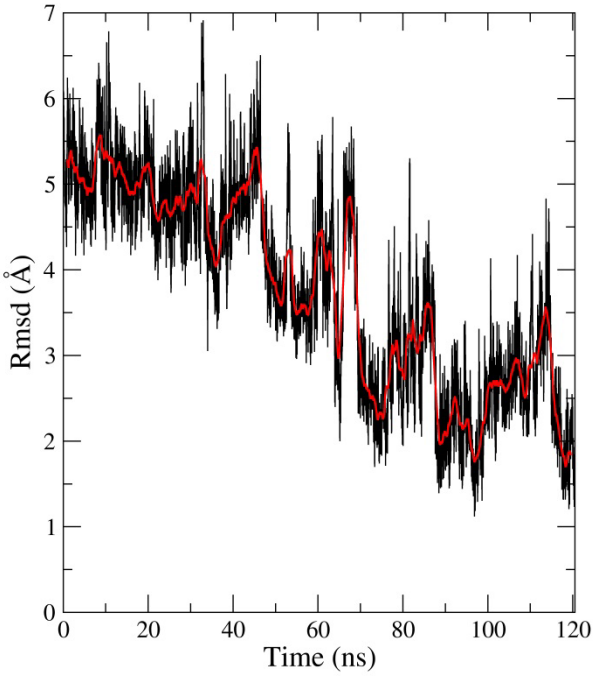


Figure S6. Root Mean Square deviation with respect to the X-Ray structure (on all atoms except hydrogen atoms) of N_{TAIL} modified with the probe P at position 491, during the whole simulation (heating phase, equilibration and production) with XD. The red curve is a running average over 100 points. The binding process is initiated by a contact between the hydrophobic residues of the $N_{TAIL\_491\_P}$ C-term and XD. The first contact is observed in the early phase of the equilibration process, after 7 ns.

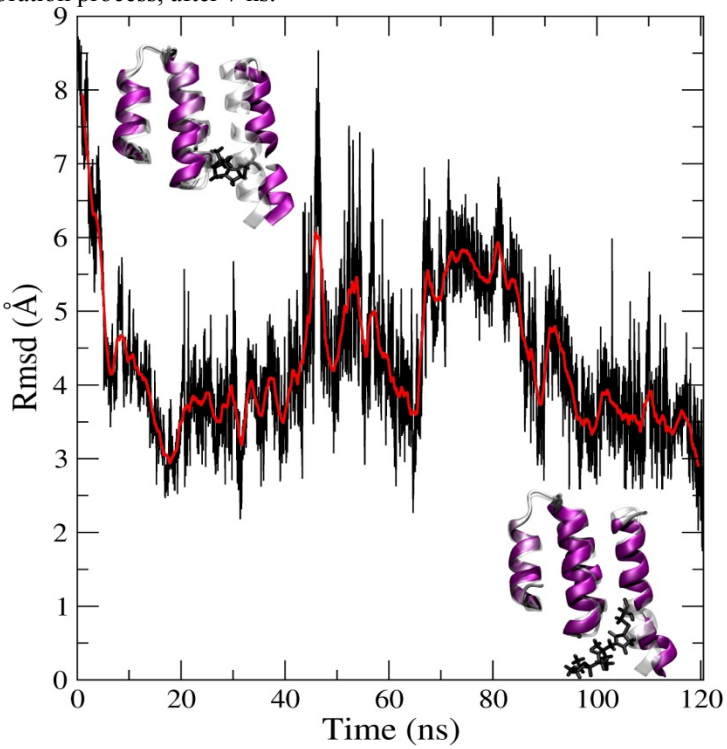


Figure S7. Root Mean Square deviation with respect to the X-Ray structure (on all atoms except hydrogen atoms) of NTAIL modified with the probe PP at position 491, during the whole simulation (heating phase, equilibration and production) with XD. The red curve is a running average over 100 points. The binding process between $N_{TAIL\_491\_PP}$

and XD occurs in the early beginning phase of the equilibration process (after 5 ns). A comparison with the X-Ray structure (in white) is provided. PP is shown in black.

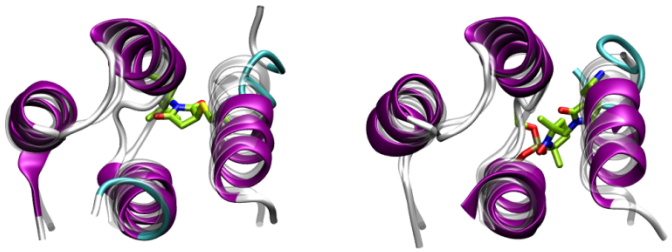


Figure S8. Top views of a typical structure of P_{XD}/N_{TAIL} S491C^{P} (left) and S491C^{PP} (right) sampled during the 100 ns MD. The structures are superimposed with the X-Ray structure (in white). PP and P are shown in licorice. The colors are as follows: O: red; C: green; N: blue, S: yellow, P: tan; α-helix: magenta; coil: blue

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