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# CycloPeptidic Photosensitizer Prodrugs as Proteolytically Triggered Drug Delivery Systems of Pheophorbide a With Defined Structures for Selective PhotoDiagnosis and PhotoDynamic Therapy: Part I - Self-quenched Prodrugs – Electronic Supplementary Information

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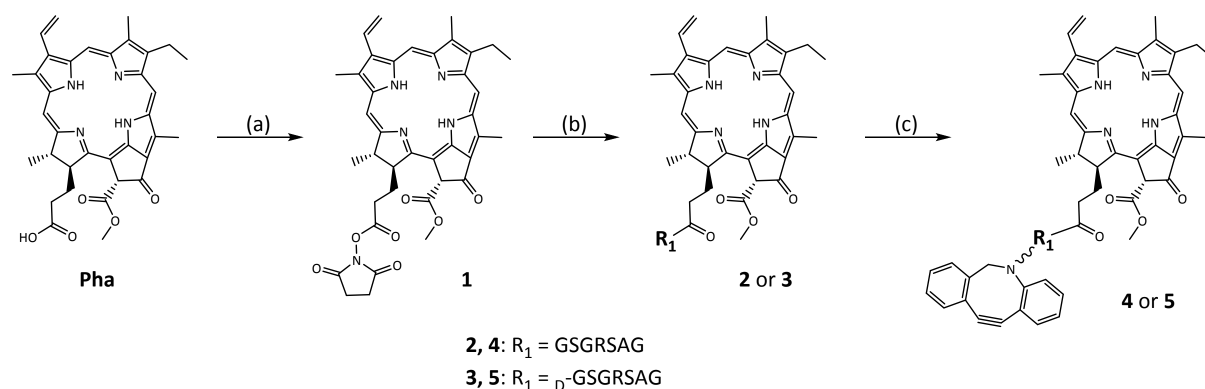
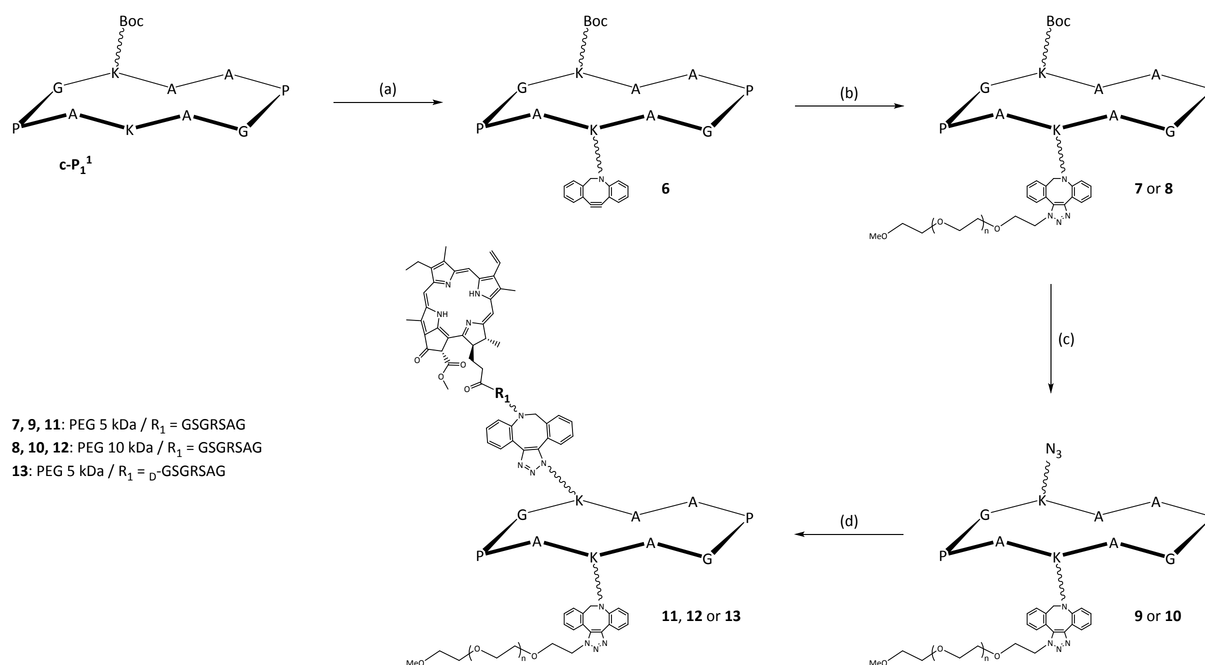
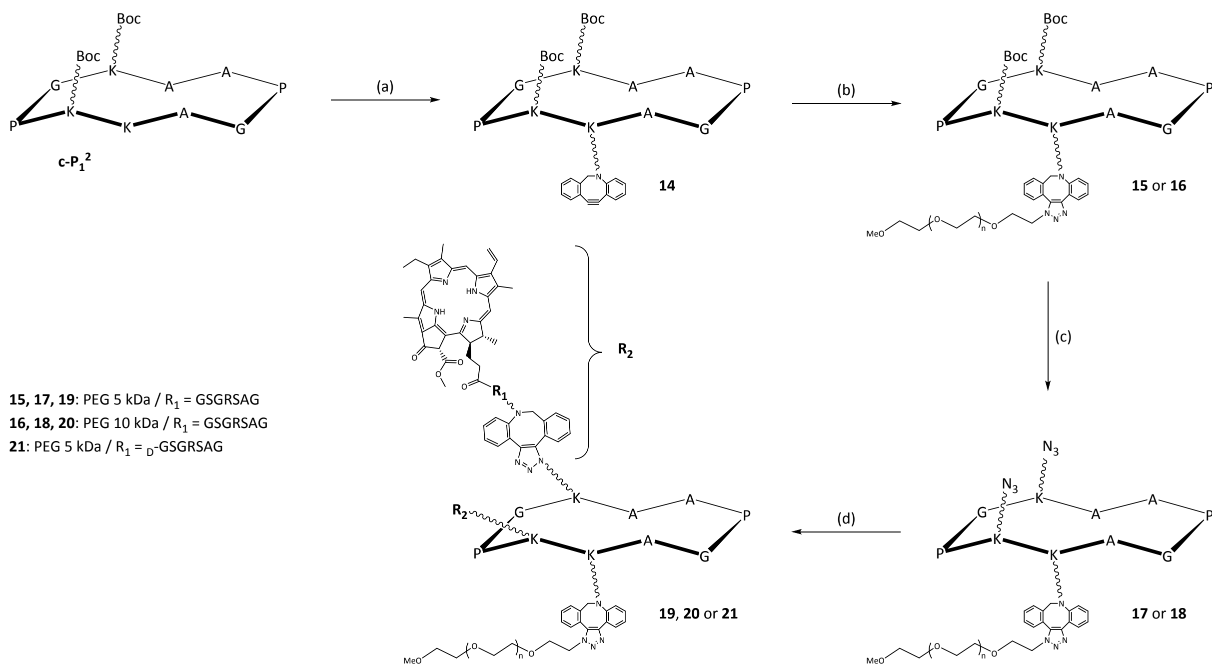


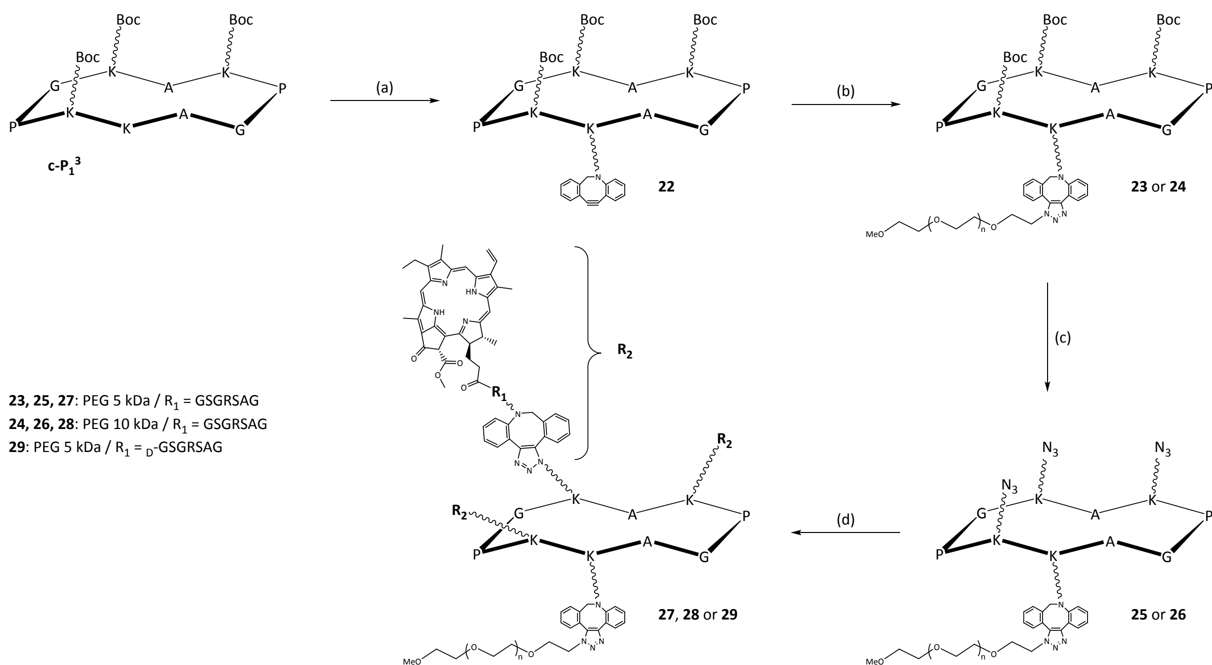

Scheme S1 - Generic synthesis pathway for the Pha-peptide-DBCO conjugates. The text on the bottom depicts the different conjugates obtained depending on the nature of the amino acids of the linker. *Reagents and conditions*: (a) NHS, EDC, DMAP, argon (ar.), dark, room temperature (r. t.), overnight (o/n); (b) DIPEA, GSGRSAG (TFA salt) or $_D$-GSGRSAG (TFA salt), ar., dark, r. t., o/n; (c) DBCO-$NH_2$, HATU, DIPEA, ar., dark, r. t., o/n.



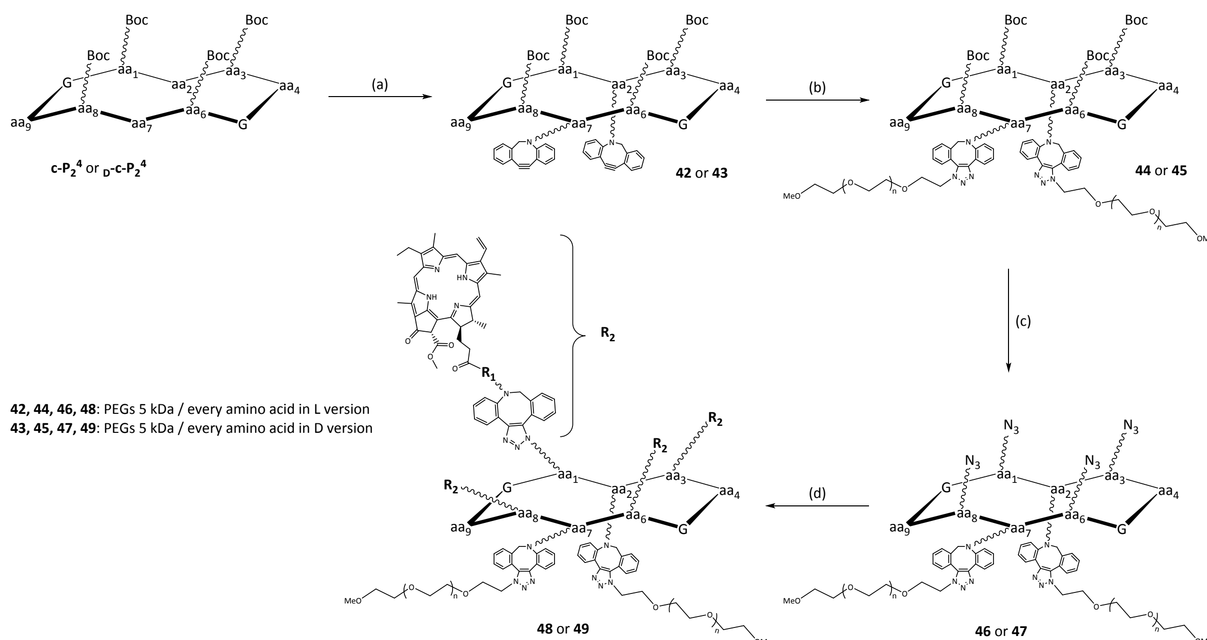

Scheme S2 - Generic synthesis pathway for monosubstituted-monoPEGylated conjugates. The text on the left side depicts the different conjugates obtained depending on the nature of the amino acids of the linker and the molecular weight of the PEG. *Reagents and conditions*: (a) DBCO-NHS, argon (ar.), room temperature (r. t.), overnight (o/n); (b) α-azido-ω-methoxy-PEG (5 or 10 kDa), ar., r. t., o/n; (c) TFA/DCM (50:50), 30 min, r. t.; APA, DIPEA, HATU, ar., r. t., o/n; (d) **(4)** or **(5)**, ar., dark, r. t., o/n.



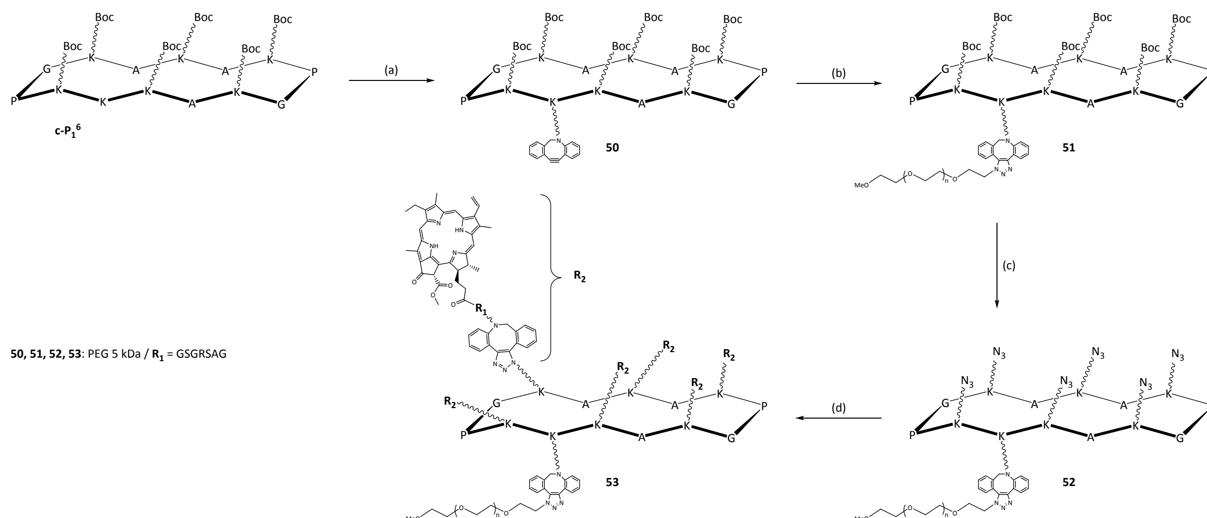

Scheme S3 - Generic synthesis pathway for disubstituted-monoPEGylated conjugates. The text on the left side depicts the different conjugates obtained depending on the nature of the amino acids of the linker and the molecular weight of the PEG. *Reagents and conditions*: (a) DBCO-NHS, argon (ar.), room temperature (r. t.), overnight (o/n); (b) α-azido-ω-methoxy-PEG (5 or 10 kDa), ar., r. t., o/n; (c) TFA/DCM (50:50), 30 min, r. t.; APA, DIPEA, HATU, ar., r. t., o/n; (d) **(4)** or **(5)**, ar., dark, r. t., o/n.



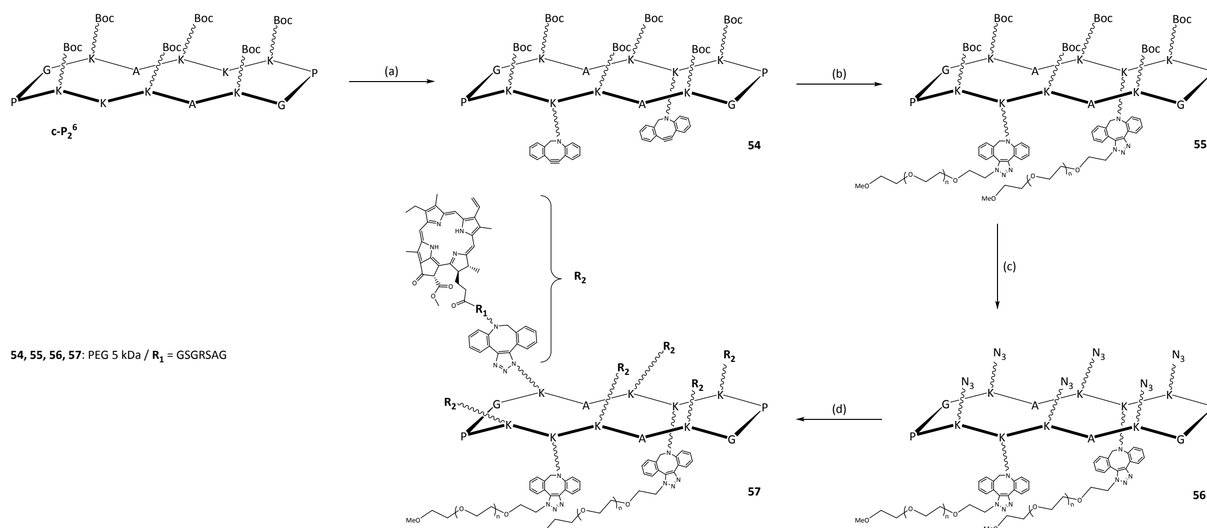

Scheme S4 - Generic synthesis pathway for trisubstituted-monoPEGylated conjugates. The text on the left side depicts the different conjugates obtained depending on the nature of the amino acids of the linker and the molecular weight of the PEG. *Reagents and conditions*: (a) DBCO-NHS, argon (ar.), room temperature (r. t.), overnight (o/n); (b) α-azido-ω-methoxy-PEG (5 or 10 kDa), ar., r. t., o/n; (c) TFA/DCM (50:50), 30 min, r. t.; APA, DIPEA, HATU, ar., r. t., o/n; (d) **(4)** or **(5)**, ar., dark, r. t., o/n.




Scheme S5 - Generic synthesis pathway for tetrasubstituted-diPEGylated conjugates. The text on the left side depicts the different conjugates obtained depending on the nature of the amino acids of the RAFT and the linker and the molecular weight of the PEG. *Reagents and conditions*: (a) DBCO-NHS, argon (ar.), room temperature (r. t.), overnight (o/n); (b) α-azido-ω-methoxy-PEG (5 kDa), ar., r. t., o/n; (c) TFA/DCM (50:50), 30 min, r. t.; APA, DIPEA, HATU, ar., r. t., o/n; (d) **(4)** or **(5)**, ar., dark, r. t., o/n.




Scheme S6 - Generic synthesis pathway for hexasubstituted-monoPEGylated conjugates. The text on the left side depicts the different conjugates obtained depending on the nature of the amino acids of the linker and the molecular weight of the PEG. *Reagents and conditions*: (a) DBCO-NHS, argon (ar.), room temperature (r. t.), overnight (o/n); (b) α-azido-ω-methoxy-PEG (5 kDa), ar., r. t., o/n; (c) TFA/DCM (50:50), 30 min, r. t.; APA, DIPEA, HATU, ar., r. t., o/n; (d) **(4)** or **(5)**, ar., dark, r. t., o/n.




Scheme S7 - Generic synthesis pathway for hexasubstituted-diPEGylated conjugates. The text on the left side depicts the different conjugates obtained depending on the nature of the amino acids of the linker and the molecular weight of the PEG. *Reagents and conditions*: (a) DBCO-NHS, argon (ar.), room temperature (r. t.), overnight (o/n); (b) α-azido-ω-methoxy-PEG (5 kDa), ar., r. t., o/n; (c) TFA/DCM (50:50), 30 min, r. t.; APA, DIPEA, HATU, ar., r. t., o/n; (d) **(4)** or **(5)**, ar., dark, r. t., o/n.

Table S1 – Fluorescence emission values ($\lambda_{ex}$ = 410 ± 9 nm and $\lambda_{em}$ = 670 ± 9 nm) and detection of $^1O_2$ ($\lambda_{ex}$ = 405 nm and $\lambda_{em}$ = 1270 nm) of the synthesized conjugates; a dash means that the corresponding experiment was not performed. [a] Conjugates at 3 μM of equivalent of Pha; [b] conjugates at 30 μM of equivalent of Pha.

| *Conjugate* | *F (a.u.)* [a] | *$^1O_2$ detected (counts per second)* [b] |
|---|---|---|
| $uPA$-$cPPP_{1/5}$ | 14494 ± 773 | 185.5 ± 0.6 |
| $uPA$-$cPPP_{2/5}$ | 484 ± 22 | 52.2 ± 0.5 |
| $uPA$-$cPPP_{3/5}$ | 254 ± 23 | 35.9 ± 0.7 |
| $uPA$-$cPPP_{4/5}$ | 179 ± 5 | 22.0 ± 0.7 |
| $uPA$-$cPPP_{4/5}^2$ | 191 ± 18 | - |
| $uPA$-$cPPP_{6/5}$ | 94 ± 12 | 21.2 ± 0.7 |
| $uPA$-$cPPP_{6/5}^2$ | 142 ± 16 | - |
| $uPA$-$cPPP_{1/10}$ | 17151 ± 630 | - |
| $uPA$-$cPPP_{2/10}$ | 1130 ± 15 | - |
| $uPA$-$cPPP_{3/10}$ | 366 ± 29 | - |
| $uPA$-$cPPP_{4/10}$ | 158 ± 24 | - |
| $uPA$-$cPPP_{D1/5}$ | 24941 ± 1134 | - |
| $uPA$-$cPPP_{D2/5}$ | 1697 ± 51 | - |
| $uPA$-$cPPP_{D3/5}$ | 197 ± 16 | - |
| $uPA$-$cPPP_{D4/5}$ | 146 ± 16 | - |
| $uPA$-$_D$-$cPPP_{D4/5}$ | 238 ± 9 | - |
| $uPA$-$_D$-$cPPP_{D4/5}^2$ | 219 ± 15 | - |

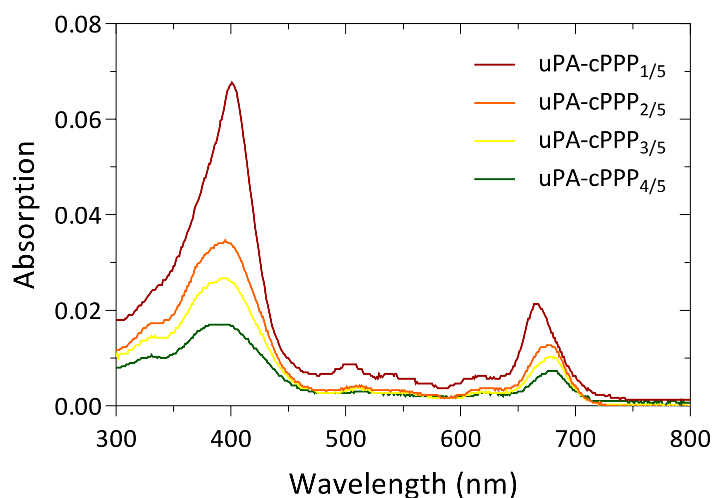



Figure S1 - Absorption spectra of uPA-cPPP$_{1/5}$ (red line), uPA-cPPP$_{2/5}$ (orange line), uPA-cPPP$_{3/5}$ (yellow line) and uPA-cPPP$_{4/5}$ (green line) at 3 µM of Pha equivalents. With an increased loading in Pha onto the cyclopeptidic template, one can observe a hypsochromic shift at the maximum of absorbance in the 400 nm area and a bathochromic shift at the maximum of absorbance in the 670 nm area.

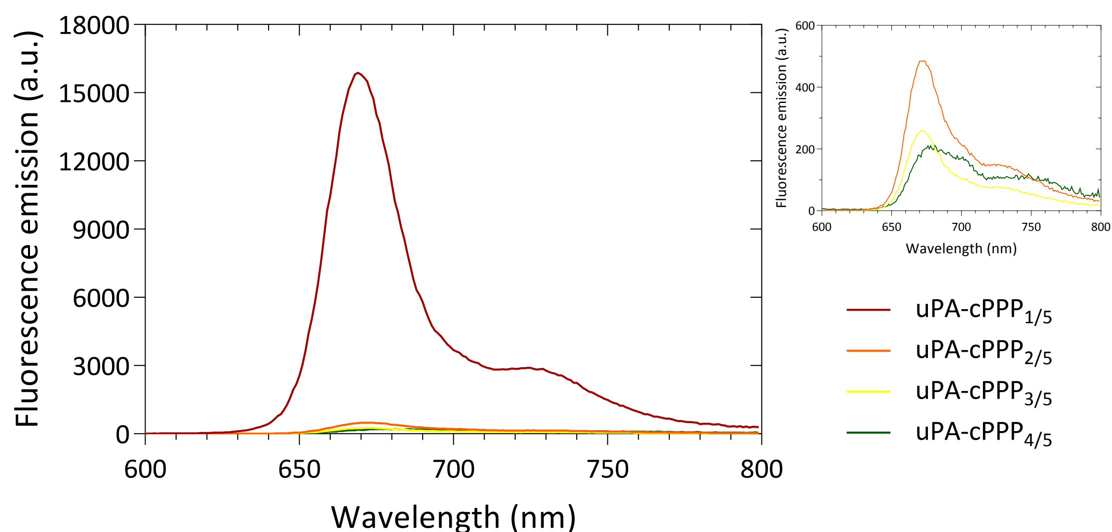



Figure S2 - Fluorescence emission of uPA-cPPP$_{1/5}$ (red line), uPA-cPPP$_{2/5}$ (orange line), uPA-cPPP$_{3/5}$ (yellow line) and uPA-cPPP$_{4/5}$ (green line) at 3 µM of Pha equivalents, $\lambda_{ex}$ = 410 ± 9 nm.

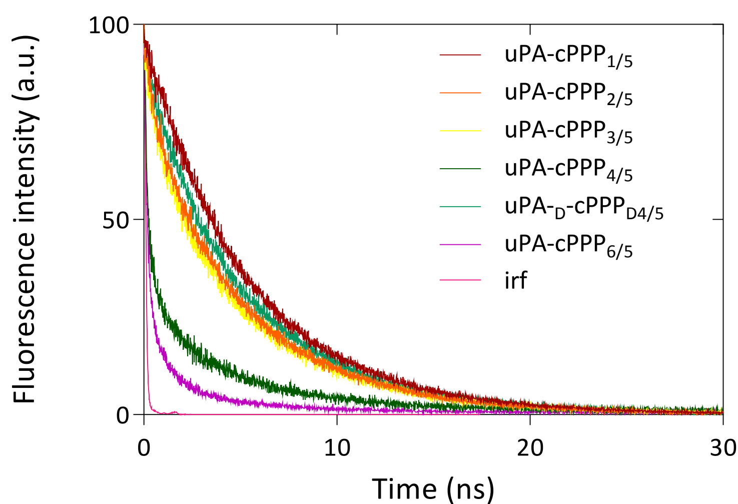



Figure S3 – Normalized fluorescence decays of the considered compounds in water at 3 µM of Pha equivalents, $\lambda_{ex}$ = 395 nm and $\lambda_{em}$ = 670 nm.

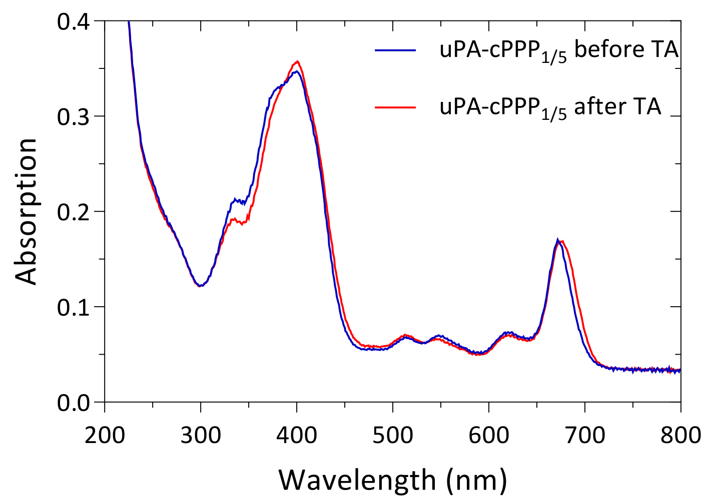



Figure S4 - Steady-state absorption spectra of uPA-cPPP$_{1/5}$ in water before (blue line) and after (red line) the ns-µs TA measurement.

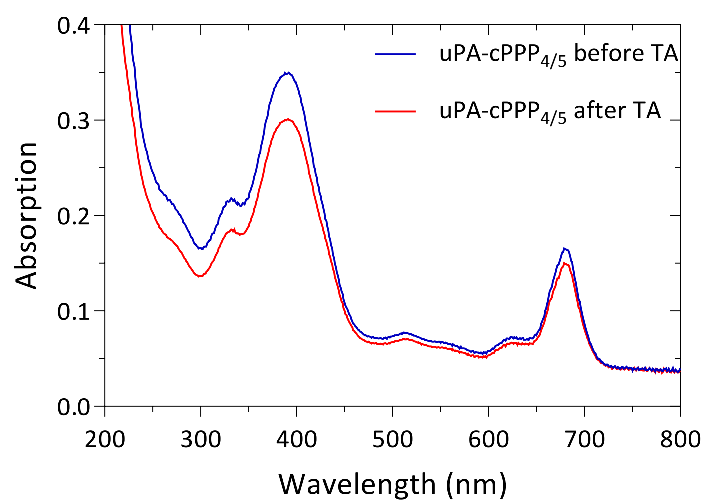



Figure S5 - Steady-state absorption spectra of uPA-cPPP$_{4/5}$ in water before (blue line) and after (red line) the ns-µs TA measurement.

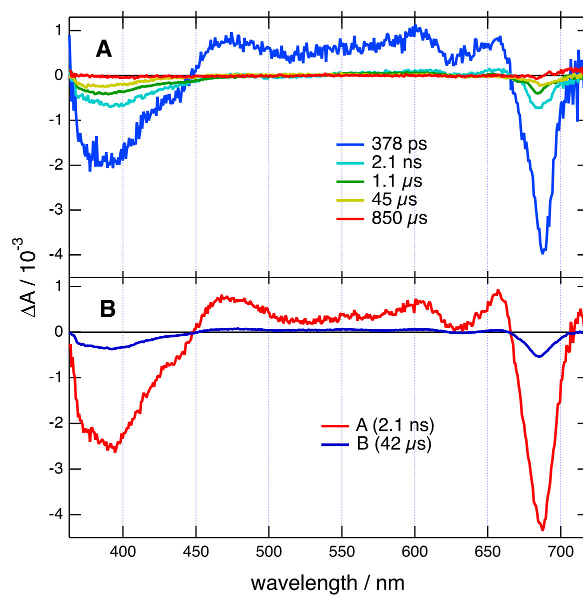



Figure S6 - A) TA spectra measured at different time delays after 355 nm excitation of uPA-cPPP$_{4/5}$ in water B) Species-associated difference spectra obtained from a global analysis assuming a A → B → C schem.

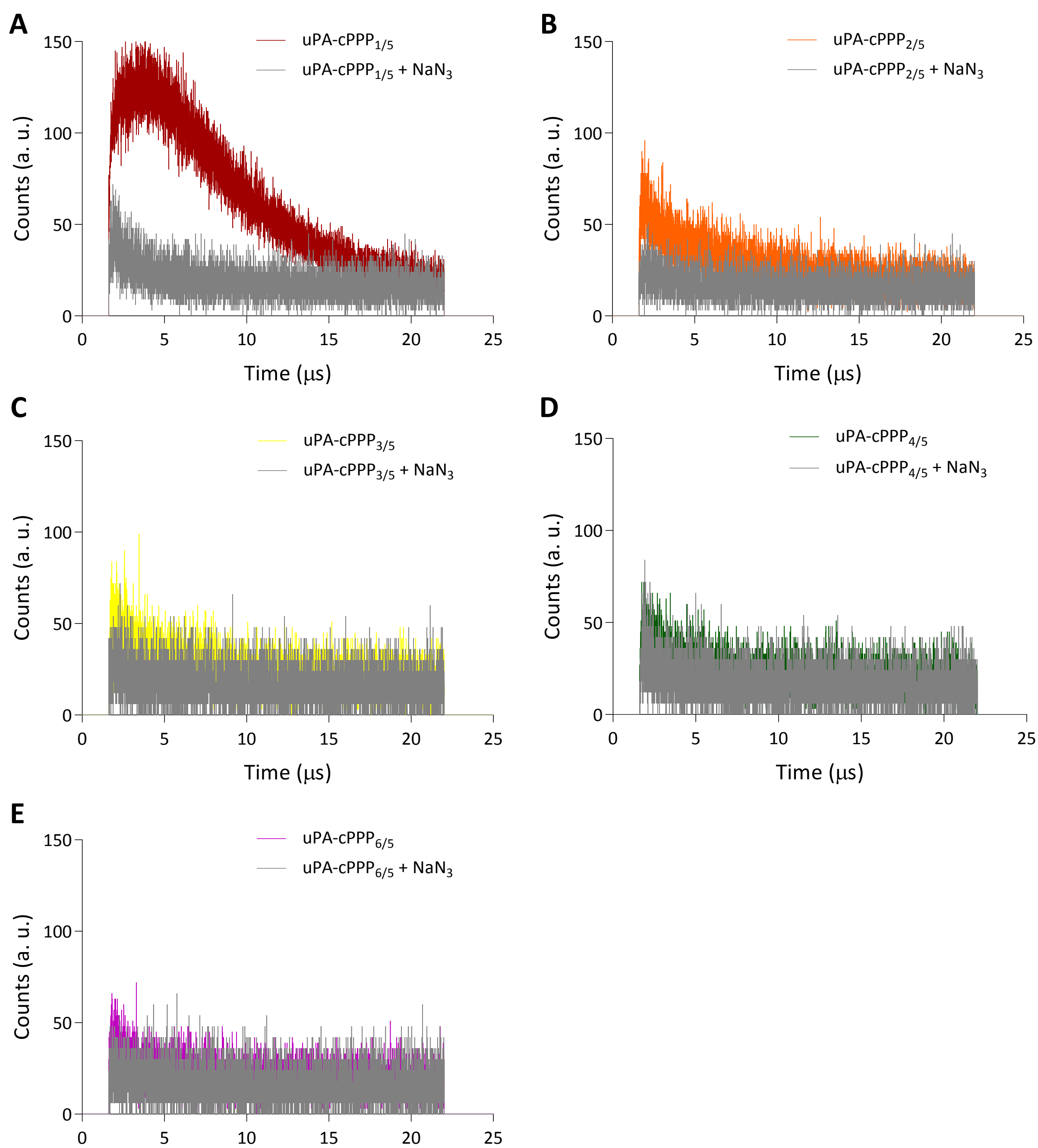



Figure S7 – Singlet oxygen luminescence decay of uPA-cPPP$_{1/5}$ (A), uPA-cPPP$_{2/5}$ (B), uPA-cPPP$_{3/5}$ (C), uPA-cPPP$_{4/5}$ (D) and uPA-cPPP$_{6/5}$ (E) at 30 μM of Pha equivalents (coloured curves), and acquired backgrounds after addition of a 2 M solution of $NaN_3$ (grey curves). Excitation occurred at 405 nm (40 kHz, 5 mW average power) over 10 minutes.

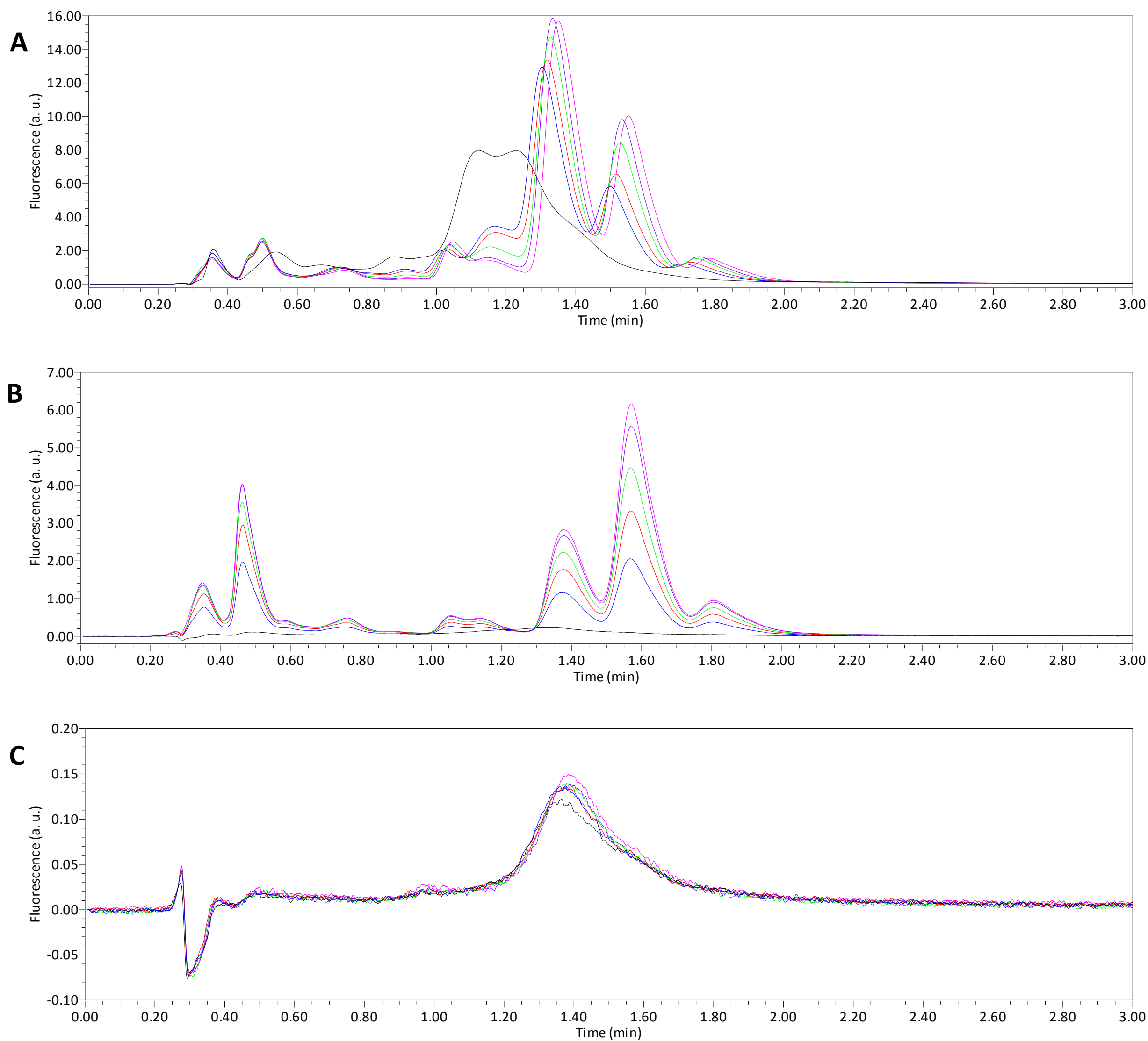



Figure S8 – Digestion by trypsin (42 µL of trypsin-EDTA solution 10x) of uPA-cPPP$_{1/5}$ (A), uPA-cPPP$_{4/5}$ (B) and uPA-$_{D}$-cPPP$_{D4/5}$ (C) at 30 µM of Pha equivalents followed by UPLC (isocratic gradient over 3 minutes, 47.5% solvent B with $H_2O$ + 0.1% FA as solvent A and ACN + 0.1% FA as solvent B, fluorescence detection with $\lambda_{ex}$ = 410 nm and $\lambda_{em}$ = 670 nm, gain of 10) at 0 (black curve), 5 (blue curve), 15 (red curve), 30 (green curve), 60 (purple curve) and 120 minutes (pink curve).

Figure S9 – Digestion by uPA (1000 U) of uPA-cPPP$_{1/5}$ (A), uPA-cPPP$_{4/5}$ (B) and uPA-$_{D}$-cPPP$_{D4/5}$ (C) at 30 µM of Pha equivalents followed by UPLC (isocratic gradient over 3 minutes, 47.5% solvent B with $H_2O$ + 0.1% FA as solvent A and ACN + 0.1% FA as solvent B, fluorescence detection with $\lambda_{ex}$ = 410 nm and $\lambda_{em}$ = 670 nm, gain of 10) at 0 (black curve), 5 (blue curve), 15 (red curve), 30 (green curve), 60 (purple curve) and 120 minutes (pink curve).

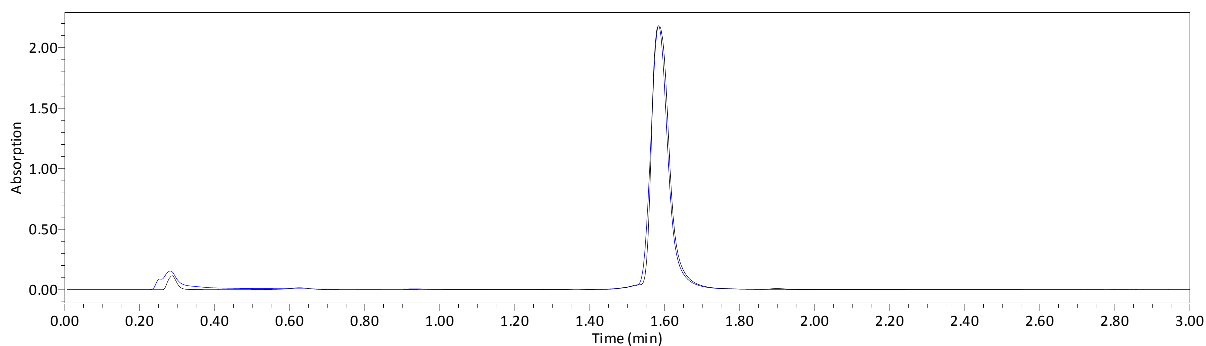



Figure S10 – Follow-up by UPLC (linear gradient of 30-100% solvent B with $H_2O$ + 0.1% FA as solvent A and ACN + 0.1% FA as solvent B, detection at 313 nm) of **(6)** (1 mg) with trypsin (42 µL of trypsin-EDTA solution 10x) in water at 0 (black curve) and 120 minutes (blue curve).

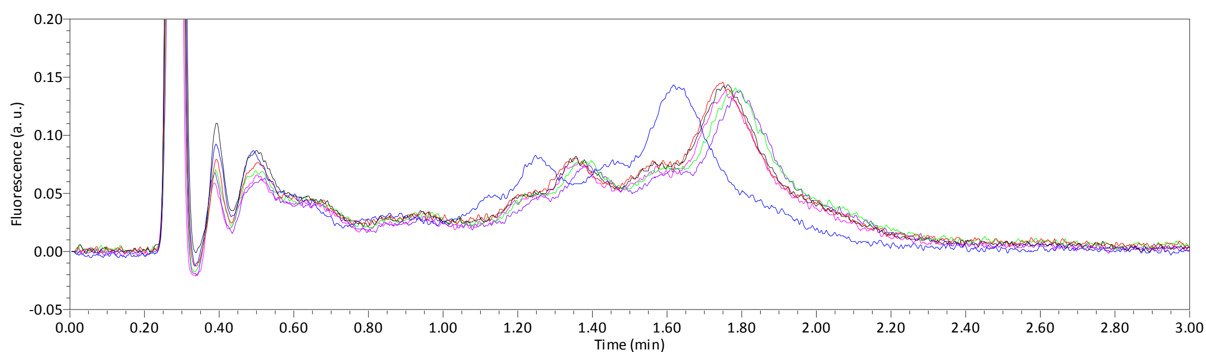



Figure S11 – Follow-up by UPLC (isocratic gradient over 3 minutes, 47.5% solvent B with $H_2O$ + 0.1% FA as solvent A and ACN + 0.1% FA as solvent B, fluorescence detection with $\lambda_{ex}$ = 410 nm and $\lambda_{em}$ = 670 nm, gain of 10) of uPA-cPPP$_{4/5}$ at 30 µM of Pha equivalents alone in water at 0 (black curve), 5 (blue curve), 15 (red curve), 30 (green curve), 60 (purple curve) and 120 minutes (pink curve).

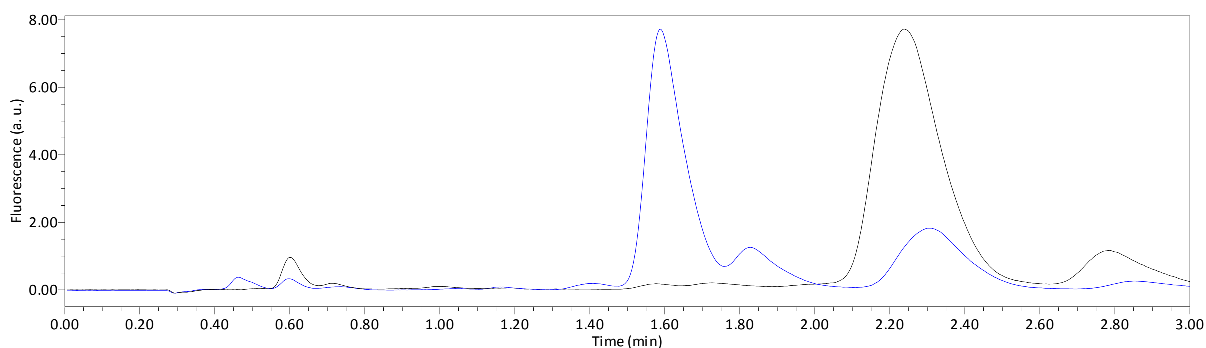



Figure S12 – Digestion by trypsin (42 µL of trypsin-EDTA solution 10x) of Pha-GSGRSAG-DBCO at 30 µM followed by UPLC (isocratic gradient over 3 minutes, 47.5% solvent B with $H_2O$ + 0.1% FA as solvent A and ACN + 0.1% FA as solvent B, fluorescence detection with $\lambda_{ex}$ = 410 nm and $\lambda_{em}$ = 670 nm, gain of 10) at 0 (black curve) and 120 minutes (blue curve).

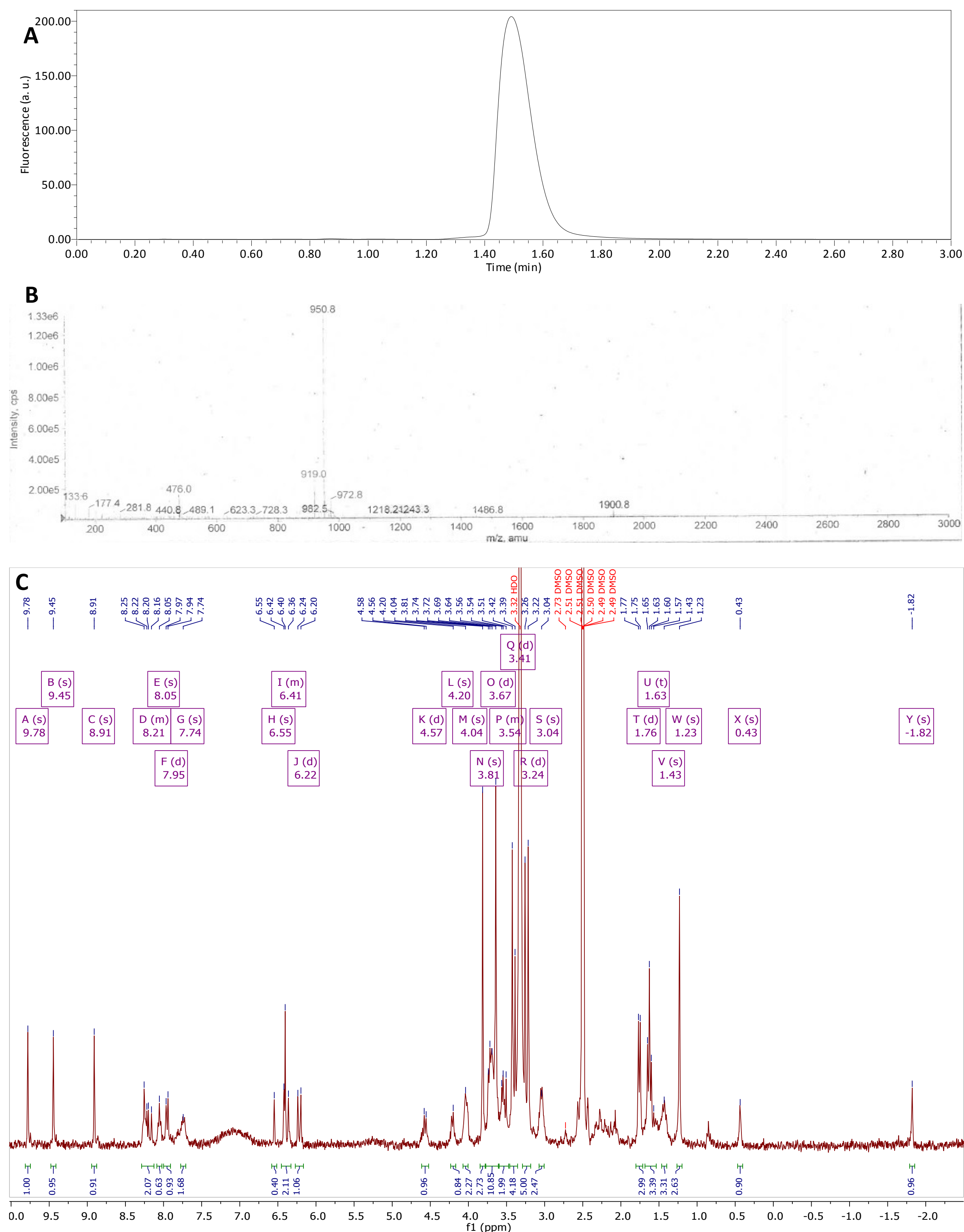


Figure S13 – Isolated peak of the fragment Pha-GSGR analysed by UPLC (A) (isocratic gradient over 3 minutes, 47.5% solvent B with $H_2O$ + 0.1% FA as solvent A and ACN + 0.1% FA as solvent B, fluorescence detection with $\lambda_{ex}$ = 410 nm and $\lambda_{em}$ = 670 nm, gain of 10), by ESI-MS (B) (soft + detection, in $CH_3COONH_4$) and by $^1H$ NMR (C) (300 MHz, DMSO-$d_6$).