Electronic Supplementary Information

Site-Specific PEGylation of Proteins by a Staudinger-Phosphite Reaction

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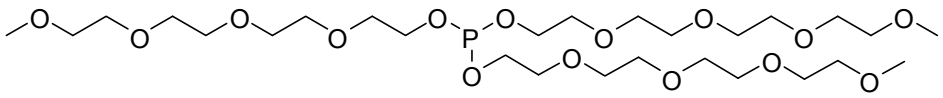
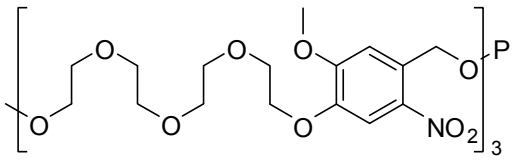
General Methods. α-Methoxy-ω-hydroxy poly(ethylene glycol), mPEG, (Mw 750 Da) was purchased from Iris Biotech, whereas all other reagents and solvents were purchased form Sigma Aldrich or Acros Organics. Phosphite **9** was prepared according to a previous report.[1] C-18 Sep-Pak cartridges were purchased from Waters, whereas C-18 Zip Tips were purchased from Millipore. HRMS (ESI-TOF) was performed on an Agilent 6210 system, Agilent Technologies (Santa Clara, CA). Spray voltage was set to 4 kV, and drying gas flow rate was set to 25 psi. Mass spectrometry of protein **7** was performed using an MALDI-TOF instrument (AB SCIEX TOF/TOF 5800, Applied Biosystems Deutschland GmbH, Darmstadt, Germany) equipped with an Nd:YAG laser (355 nm). Samples were prepared with alpha-cyano-4-hydroxycinnamic acid matrix and measured in the linear mode. The spectrum obtained was the mean of 5000 laser shots. GPC was performed in THF on a LC-RI Agilent 1100 system, Agilent Technologies (Santa Clara, CA), equipped with 3 PLgel 5µm MIXED-C Gel columns, Varian (Lake Forest, CA). The instrument was calibrated with a polystyrene standard mixture (Mp=474, Mp=3250, Mp=8400, Mp=17600, Mp=66000). Melting point was measured on a BÜCHI 510 instrument from BÜCHI Labortechnik (Essen, Germany). ^{1}H-NMR, ^{13}C-NMR, and ^{31}P-NMR spectra were recorded at a Jeol ECX/400, a Bruker 500 AMC, or a Bruker AMC 700 in $CDCl_3$, CD_3CN, or in 1M Tris buffer. The chemical shifts were reported in ppm relatively to the residual solvent peak.

Synthesis of tris(2,5,8,11-tetraoxatridecan-13-yl) phosphite (4).



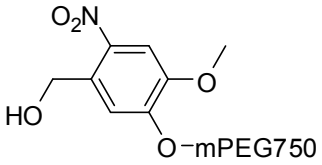
This compound was obtained as colorless oil (96%) according to the procedure applied for the preparation of tri(4-pentenyl) phosphite.[2] ^{1}H NMR (400 MHz, $[D_3]CH_3CN$): δ=3.89-3.83 (m, 6H), 3.56-3.48 (m, 36H), 3.46-3.44 (m, 6H), 3.27 ppm (s, 9H); ^{13}C NMR (101 MHz, $[D_3]CH_3CN$) δ=73.3, 72.49, 72.47, 71.6, 71.5, 71.07, 71.05, 71.03, 71.00, 70.93, 70.87, 70.86, 62.3, 62.2, 61.8, 58.8; ^{31}P NMR (100 MHz, $[D_3]CH_3CN$): δ=140.6 ppm (s); HRMS (ESI-TOF): *m/z*: 691.3303 $[M+O+Na]^+$ (calcd.: *m/z*: 691.3276).

Synthesis of tris(4-(2,5,8,11-tetraoxatridecan-13-yloxy)-5-methoxy-2-nitrobenzyl) phosphite (11).



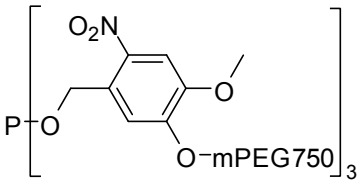
This compound was obtained as pale yellow oil (37% over 3 steps) from 4-(hydroxymethyl)-2-methoxy-5-nitrophenol according to the procedure applied for the preparation of its homologue, tris(4-(2,5,8,11,14-pentaoxahexadecan-16-yloxy)-5-methoxy-2-nitrobenzyl) phosphite.[1] ^{1}H NMR (400 MHz, [D$_3$]CH$_3$CN): δ=7.65 (s, 3H), 7.22 (s, 3H), 5.31 (d, *J*=7.5 Hz, 6H), 4.17-4.15 (m, 6H), 3.86 (s, 9H), 3.82-3.79 (m, 6H), 3.64-3.62 (m, 6H), 3.58-3.56 (m, 6H), 3.56-3.50 (m, 18H), 3.45-3.43 (m, 6H), 3.27 ppm (s, 9H); ^{13}C NMR (62.5 MHz, [D$_3$]CH$_3$CN): δ=155.4, 148.7, 140.6 130.8, 130.7, 111.8, 110.8, 73.1, 71.9, 71.7, 71.63, 71.62, 71.5, 70.5, 70.3, 63.1, 63.0, 59.4, 57.4 ppm; ^{31}P NMR (100 MHz, [D$_3$]CH$_3$CN): δ=139.0 ppm (s); HRMS: *m/z*: 1196.4686 [M+H]$^+$ (calcd.: *m/z*: 1196.4633).

Synthesis of 4-methoxy-5-poly(ethylene glycol) methyl ether-2-nitrobenzyl alcohol [PEG Mw 750 Da] precursor to phosphite 14.



This compound was prepared as pale yellow, low melting solid (29% over 2 steps) from α-methoxy-ω-hydroxy poly(ethylene glycol) [PEG-Mw 750 Da] according to the procedure applied for the preparation of its homologue, 4-(2,5,8,11,14-pentaoxahexadecan-16-yloxy)-5-methoxy-2-nitrobenzyl alcohol.[1] ^{1}H NMR (400 MHz, [D]CHCl$_3$): δ=7.78 (s, 1H), 7.21 (s, 1H), 4.96 (d, *J*=3.8 Hz, 2H), 4.26-4.24 (m, 2H), 3.98 (s, 3H), 3.92-3.90 (m, 2H), 3.73-3.71 (m, 2H), 3.68-3.66 (m, 2H), 3.65-3.59 (m, OCH$_2$), 3.55-3.53 (m, 2H), 3.37 (s, 3H); ^{13}C NMR (62.5 MHz, [D$_3$]CH$_3$CN): δ=155.1, 147.6, 139.9, 134.8, 110.9, 110.3; 72.5, 71.3, 71.1, 71.0, 70.9, 70.0, 69.7, 61.8, 58.8, 56.9.

Synthesis of tris[4-methoxy-5-poly(ethylene glycol) methyl ether-2-nitrobenzyl] phosphite [PEG Mw 750 Da] (14).



To a solution of 4-methoxy-5-poly(ethylene glycol) methyl ether-2-nitrobenzyl alcohol [PEG Mw 750 Da] (165 mg, 171 μmol) in toluene (0.3 mL) was added a solution of tris(dimethylamino)phosphine in toluene (100 μL; 0.57 M), and the mixture was heated at 90 °C for 2 h. Toluene was evaporated under reduced pressure and the residue was purified by C-18 Sep-Pak with a gradient of 0-50% MeCN in water. Upon removal of solvents, phosphate **14** was obtained as pale yellow, low melting solid (93 mg,

56%). ^{1}H NMR (400 MHz, [D$_3$]CH$_3$CN): δ=7.67 (s, 3H), 7.24 (s, 3H), 5.33 (d, *J*=7.6 Hz, 6H), 4.19-4.17 (m, 6H), 3.88 (s, 9H), 3.83-3.81 (m, 6H), 3.66-3.64 (m, 6H), 3.61-3.59 (m, 6H), 3.57-3.52 (m, OCH$_2$), 3.47-3.45 (m, 6H), 3.30 (s, 9H); ^{13}C NMR (62.5 MHz, [D$_3$]CH$_3$CN): δ=155.0, 147.5, 139.9, 134.8, 110.8, 110.3, 72.5, 71.3, 71.1, 71.0, 70.9, 70.0, 69.6, 61.8, 58.8, 56.9; ^{31}P NMR (100 MHz, [D$_3$]CH$_3$CN): δ=139.8 (s); GPC: single peak (Mp=2927 Da, PDI=1.08).

Synthesis of tris[poly(ethylene glycol) methyl ether] phosphite [PEG Mw 2000 Da] (15).

To a solution of mPEG [Mw 2000 Da] (50 mg, 25 μmol) in dry toluene (80 μL) was added a solution of tris(dimethylamino)phosphine in the same solvent (20 μL; 0.40 M), and the mixture was refluxed for 3 h. Toluene was removed and the residue was subjected to a GPC purification in THF, yielding phosphite **15** as a white solid (20 mg, 40%). M.P.=56-58 ºC; ^{1}H NMR (400 MHz, [D$_3$]CH$_3$CN): δ=3.93-3.87 (m, POCH$_2$), 3.66-3.44 (m, OCH$_2$), 3.29 ppm (s, OCH$_3$); ^{31}P NMR (100 MHz, [D$_3$]CH$_3$CN): δ=140.5 (s); GPC: single peak (Mp=6467 Da, PDI=1.04).

NMR studies on the stability of phosphites. Phosphites **2**, **4**, and **9** (20 mM) were dissolved in 1 M Tris buffer (pH 7.4, 7.8 and 8.2) containing trimethyl phosphate as an internal standard (20 mM), and the hydrolytic decay of phosphites at 25 °C was monitored by ^{31}P NMR. The decomposition of **2** and **4** over time is presented in Figure 1. Phosphite **9** was stable for at least 48 h in all the buffers used.

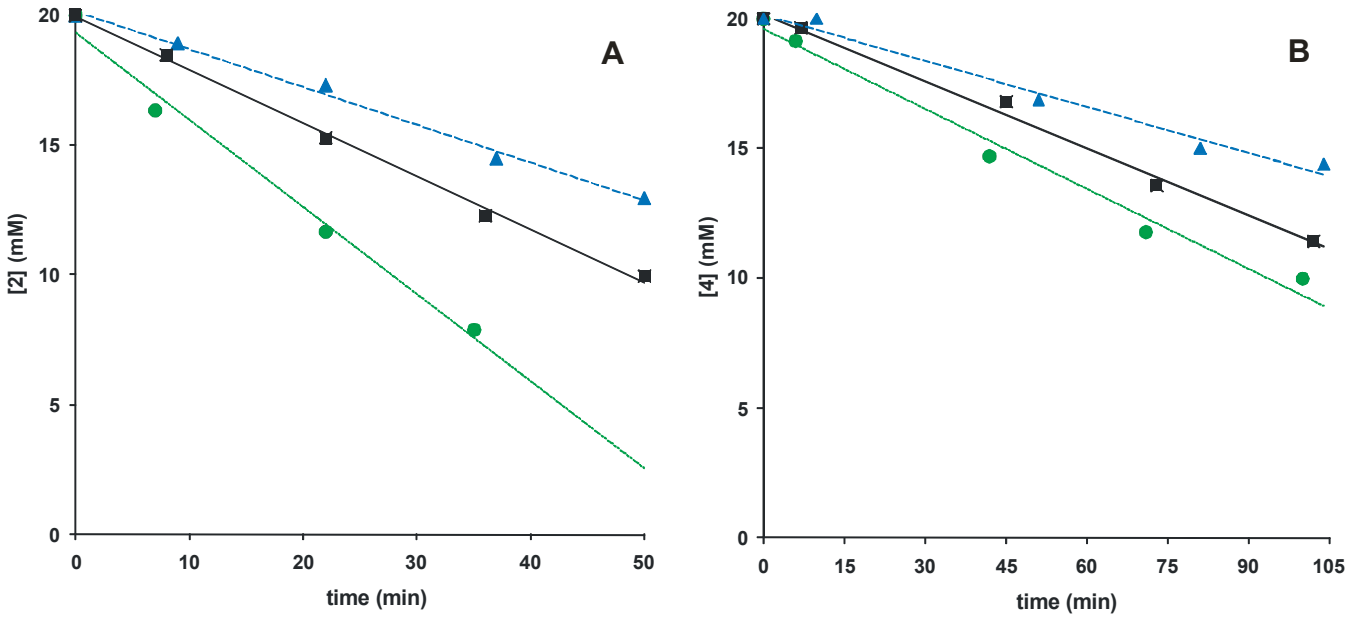


Fig. 1. The decomposition of 20 mM phosphite **2** (A) and 20 mM phosphite **4** (B) at 25 °C in 1 M tris buffer at pH 8.2 (triangles), pH 7.8 (squares), and pH 7.4 (circles).

Water Solubility of phosphites 9 and 11. Homologues **9** (13.3 mg, 10 μmol) and **11** (12 mg, 10 μmol) were mixed vigorously with water (10 mL) for 10 min in 15 mL Falcon tubes at RT, followed by centrifugation (4000 RPM, 5 min) and decantation. Supernatants were lyophilized, and precipitates (if any) were dried under vacuum. A precipitate was not observed upon centrifugation of phosphite **9**, therefore the procedure was repeated 3 times only for compound **11**. Dried fractions were weight out to quantify the solubility (1.53±0.03 mM for **11**) and to ensure high sample recovery (>96%). Phosphite **9** (13.3 mg, 10 μmol) was further mixed vigorously with smaller amounts of water (5 and 2 mL) for 10 min in 15 mL Falcon tubes at RT, followed by centrifugation (4000 RPM, 5 min) and decantation. Again, precipitate was not observed upon centrifugation suggesting water solubility of phosphite **9** to be greater than 50 mM.

Consumption of peptide 1 in the presence of phosphites 9 and 11. To a solution of azido-peptide **1** (50 μM) in 1 M phosphate buffer pH 7.8 (1.0 mL) were added phosphites **9** (1.87-15.0 mM) and **11** (15 mM) and the mixture was incubated at 28 ºC. When applicable, the reaction was conducted in 1:1 phosphate buffer pH 7.8/DMSO. Aliquots were withdrawn after 2, 4, 6, and 24 h, and they were immediately analyzed (injection volume: 50 μL) by RP-HPLC connected in line to an ESI-TOF detector. Fractions of peptide **1** in the mixtures were calculated based on signal intensities of extracted ion current m/z=782.347±0.200, which correspond to $[M+H]^+$. A sample graphical representation of the data collected upon incubation of **1** in the presence of **9** (15 mM) or **11** (15 mM) in phosphate buffer is presented in Figure 2.

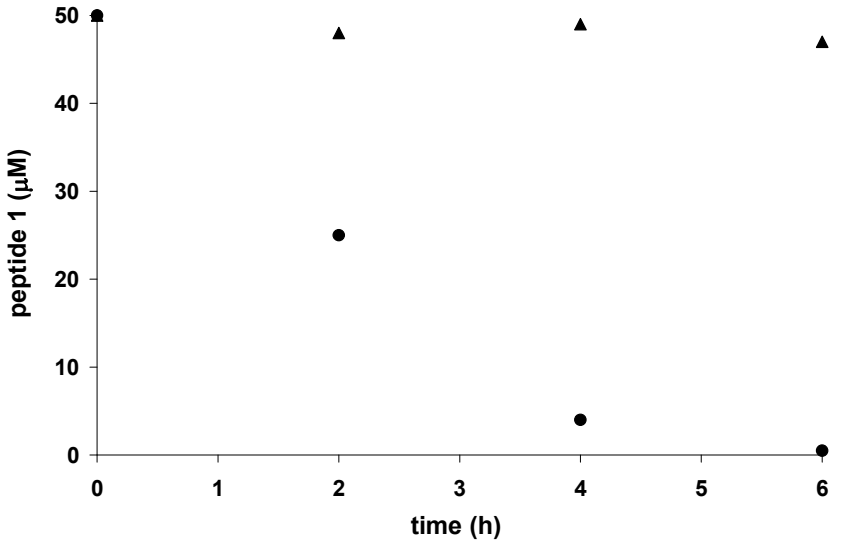


Fig. 2 The disappearance of azido-peptide **1** (50 μM) from buffered solutions contained 15 mM of phosphite **9** (circles) or phosphite **11** (triangles).

Amino acid sequence of model proteins 6 and 8 (SecB from *E.coli*).

MSEQNNTEMT FQIQRIYTKD ISFEAPNAPH VFQKDWQPEV KLDLDTASSQ LADDVYEVVL RVTVTASLGE ETAFLCEVQQ GGIFSIAGIE GTQMAHCLGA YCPNILFPYA RECITSMVSR GTFPQLNLAP VNFDALFMNY LQQQAGEGTE EHQDAXGHHH HHH; X = Phe(*p*-N_3) for **6**, and X = Ser for **8**.

Mass spectrum of the protein mixture obtained upon incubation of 6 with 4. Upon the overnight incubation with phosphite **4**, SecB protein was purified with Ni-NTA magnetic beads followed by ZipTip C18 chromatography. A mixture of compounds **6** and **7** was observed by MALDI-MS, in which the phosphoramidate was the major component (Figure 3B). Identities of polypeptides were supported by MS data, which were obtained in good agreement with the calculated values (MALDI-MS for **6**: m/z=18341/18353 $[M+H]^+$, calcd.: 18347; for **7**: m/z=18806 $[M+Na]^+$, calcd.: 18803.

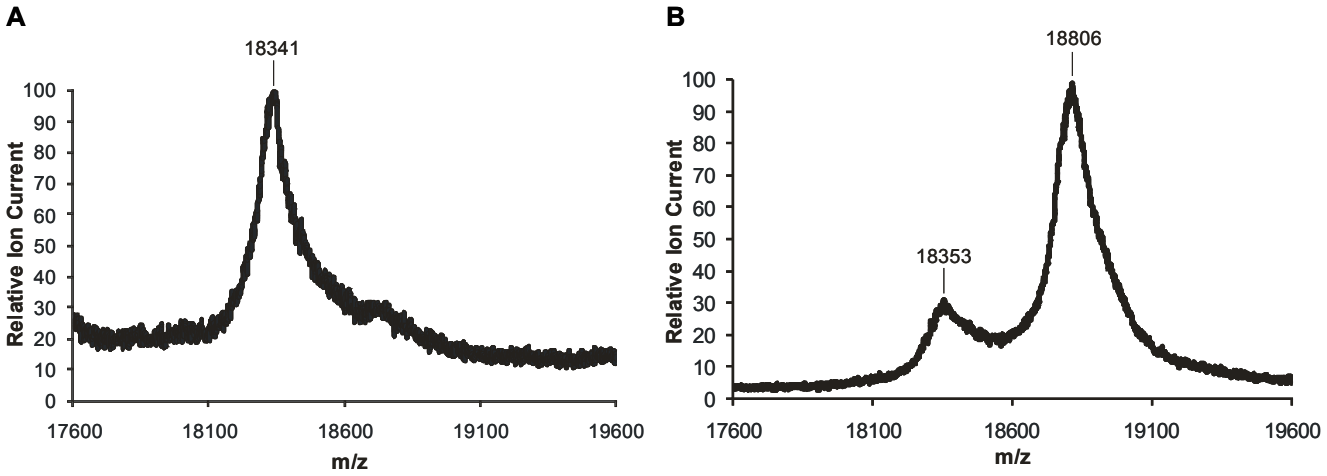


Fig. 3 MALDI-MS spectra of unmodified protein **6** (A) and the mixture obtained upon overnight incubation of **6** with phosphite **4** (B).

The influence of GdnCl and DMSO on the PEGylation of protein 6 in the presence of phosphite 15. To a suspension of protein **6** (20 μM) and phosphite **15** (0.39 mg, 65 nmol) in phosphate buffer pH 8.0 (10 μL) was added, when applicable, DMSO (10 μL), 6 M GdnCl in the buffer (10 μL), or buffer (10 μL); and the sample was shaken (2000 RPM) for 36 h at 28°C, during which time two extra portions of phosphite **15** (65 nmol each) were added. Following the incubation, the mixture was diluted with water (60 μL) and 4× sample loading buffer (20 μL), and it was incubated at 35 °C (10 min) prior to loading it (30 μL) on a 15% SDS-PAGE gel. Upon electrophoresis, the gel was stained with Coomassie Brilliant Blue (Figure 4).

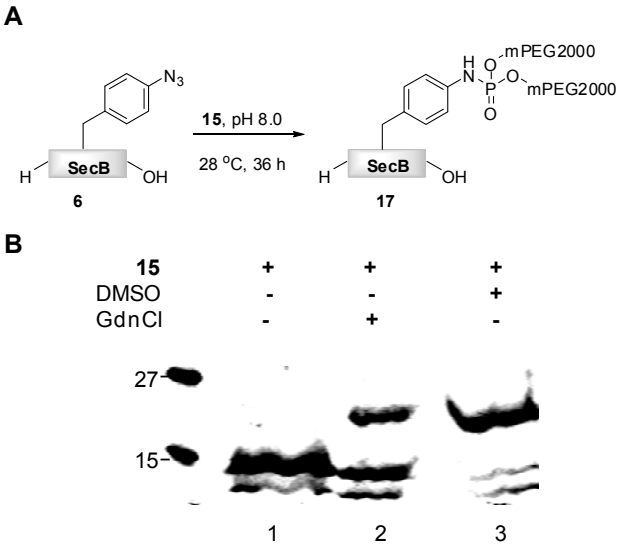


Fig. 4 PEGylation of protein **6** (10 μM) with phosphite **15** (3.3-10 mM) at 28 °C, in phosphate buffer pH 8.0 (Lane 1) in the presence of additives: 3 M GdnCl (Lane 2) and 50% DMSO (Lane 3); A. Reaction scheme; B. Coomassie stained SDS-PAGE gel.

References

1. R. Serwa, I. Wilkening, G. Del Signore, M. Mühlberg, I. Claußnitzer, C. Weise, M. Gerrits, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* 2009, **48**, 8234-8239.
2. K. Skopek, J. A. Gladysz, J. Organomet. Chem. 2008, **693**, 857-866.