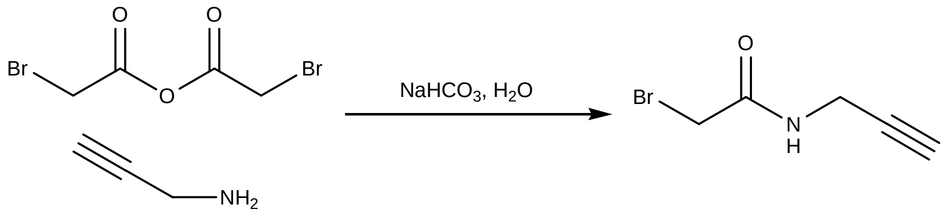
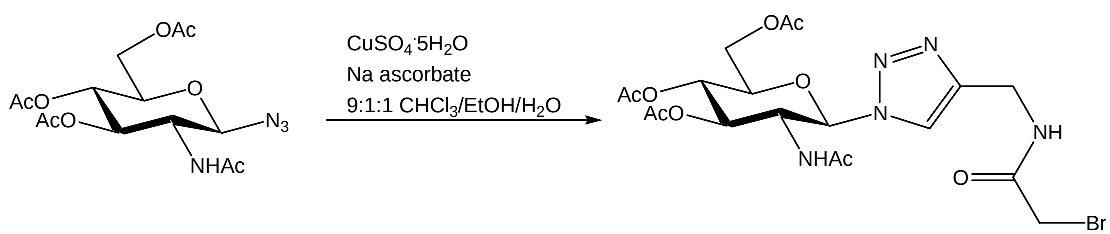
Supporting Information

N-(propargyl)-bromacetamide (**4**)



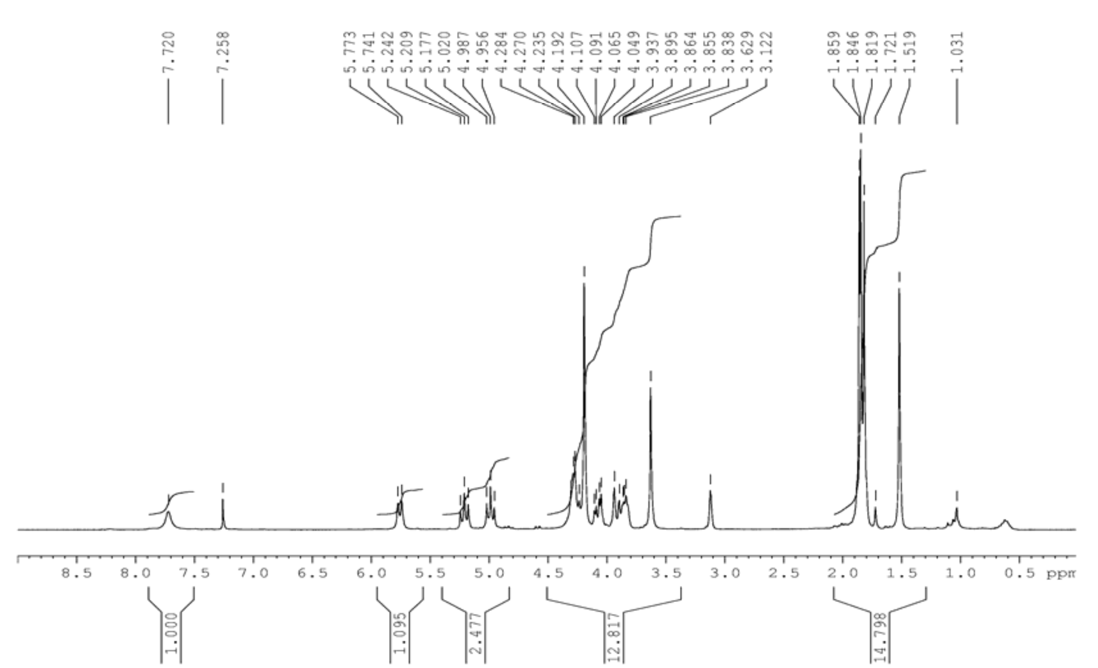
Propargylamine (0.1 ml, 1.45 mmol) was dissolved in water (15.0 ml) and was treated with bromoacetic anhydride (1.85 g, 7.25 mmol) in the presence of $NaHCO_3$ (2.5 g). The reaction was stirred at room temperature for 3 h. The reaction was quenched with HCl 5% (50 ml) and the product was extracted with ethylacetate (3 x 50 ml). The organic phase wash washed with 1 M NaOH (5 x 100 ml) and water (2 x 100 ml). The organic phase was dried with $MgSO_4$, filtered and concentrated under reduced pressure to afford a white crystalline solid (0.11 g, 43 %). R_f = 0.33 (petroleum ether/ethyl acetate 1:1). ^{1}H-NMR (250 MHz, $CDCl_3$) δ (*ppm*): 6.78 (1H, br, NH); 4.07 (2H, q, J= 5.4 Hz, J= 2.6 Hz, CH_2); 3.88 (2H, s, COC<u>H</u>$_2$Br); 2.27 (1H, t, J= 2.6 Hz, C<u>H</u>). ^{13}C-NMR (63 MHz, $CDCl_3$) δ (*ppm*): 165.3 (qC, <u>C</u>O); 78.5 (qC, alkyne); 72.2 (CH), 29.9 and 28.6 (CH_2). FAB-MS calculated for C_5H_6BrNO (M+1) 174.96, found: 197.85 and 199.85 (M+23).

1-*N*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-*N*-acetyl-*β*-D-glucopyranosylamide)-4-(*N*'-methylidenyl -2'-bromoacetamido)-4,5-anhydro-triazole (**5**)

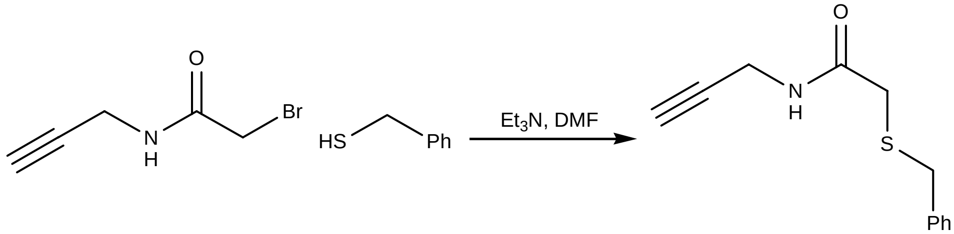


The azido sugar (100 mg, 0.27 mmol) and propargyl bromoacetamide (47 mg, 0.27 mmol) were dissolved in a biphasic solution of $CHCl_3/EtOH/H_2O$ (9:1:1) (1.1 mL). Sodium ascorbate (54 mg, 0.27 mmol) and $CuSO_4 \cdot 5H_2O$ (2 mg, 0.007 mmol) were added. The reaction was stirred at 600 rpm, 50°C overnight. The reaction mixture was then diluted with $CHCl_3$ and washed with saturated aqueous $NaHCO_3$ (3 x 20 mL), and the organic phase was dried with $MgSO_4$, filtered and concentrated under reduced pressure to afford a brown solid (98 mg, 66%). R_f: 0.40 (ethyl acetate). ^{1}H-NMR (300 MHz, $CDCl_3$ and 5% of CD_3OD) δ (*ppm*): 7.72 (1H, bp, C<u>H</u>-triazole); 5.76 (1H, d, J_{1-2}= 9.7 Hz, H1); 5.21 (1H, dd, J_{2-3}= J_{3-4}=9.8 Hz, H3); 4.99 (1H, dd, J_{3-4}= J_{4-5}=9.8 Hz, H4); 4.28 (1H, bp, H2); 4.19 (2H, s, COC<u>H</u>$_2$Br); 4.08 (2H, dd, J_{6a-6b}= 12.7 Hz, J_{5-6a}= 4.8 Hz, H6a); 3.92 (1H, dd, J_{6a-6b}= 12.7 Hz, J_{5-6b}= 1.8 Hz, H6b); 3.87-3.83 (1H, m, H5); 3.63 (2H, s, C<u>H</u>$_2$NH); 1.86, 1.85, 1.82 (9H, 3 x s, C<u>H</u>$_3$CO); 1.52 (3H, s, C<u>H</u>$_3$CONH). ^{13}C-NMR (75 MHz, $CDCl_3$ and 5% of CD_3OD) δ (*ppm*): 171.7, 170.9, 170.4, 169.6 (5 x qC, <u>C</u>O); 82.0, 74.5, 72.0, 68.0, 53.1 (5 x CH; <u>C</u>1-<u>C</u>5); 61.7 (<u>C</u>H$_2$, C6); 35.0 (<u>C</u>H$_2$, Ar-<u>C</u>H$_2$-NH); 28.0 (<u>C</u>H$_2$, CO-<u>C</u>H$_2$-Br); 21.8, 20.2, 20.1. 20.1 (4 x COC<u>H</u>$_3$). FAB-MS calculated for $C_{19}H_{26}BrN_5O_9$ (M+1): 548.09867, found: 548.10034. <u>NOTE</u>: the lower yield stated here (compared to that quoted in table 1) can be attributed to the difficulties associated with the solubility of **5** during column chromatogtaphy

^{1}H-NMR specrum of **5** (300 MHz, $CDCl_3$/5% CD_3OD):

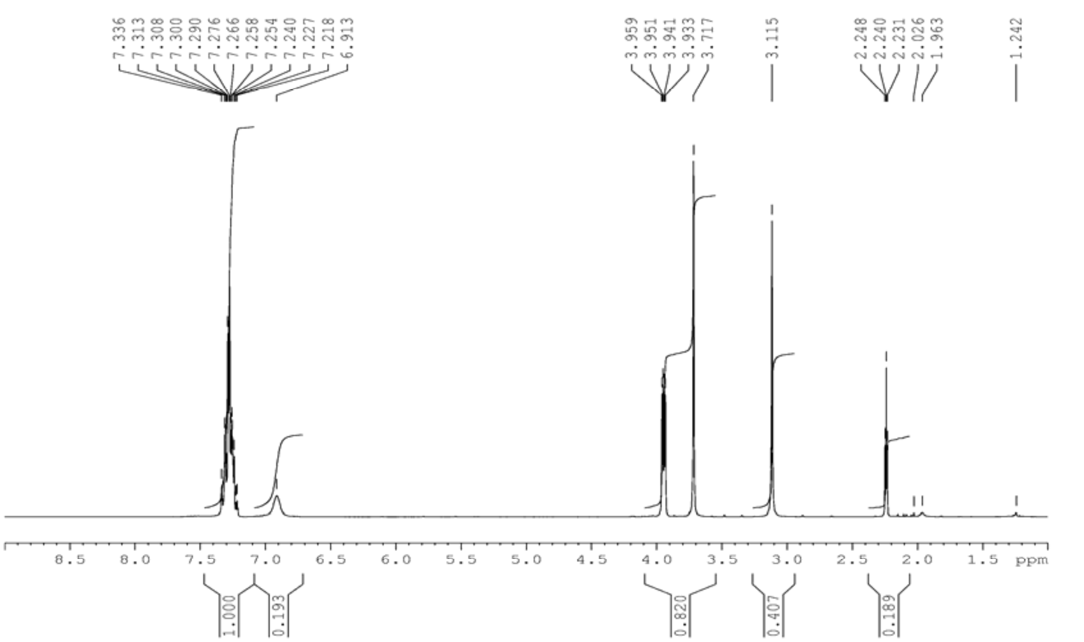


N-propargyl-(2-thiobenzyl)acetamide (**8**)

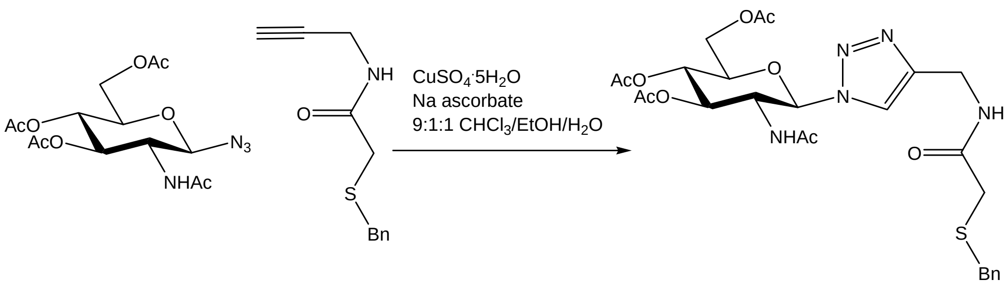


N-(propargyl)-bromacetamide (100 mg, 0.57 mmol) was dissolved in DMF (4.0 ml). Benzylmercaptan (700 µL, 5.77 mmol) and triethylamine (885 µL, 6.35 mmol) was added. The reaction was stirred for 16 h. The reaction mixture was diluted with chloroform (10.0 ml), and washed with a NaOH 1M (10.0 ml), 5% HCl (10.0 ml), saturated aqueous $NaHCO_3$ (10.0 ml) and water (10 ml). The organic phase was dried with $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography over silica (petroleum ether/ethyl acetate, 1:1) to afford the pure product (105 mg, 84 %). R_f: 0.38 (petroleum ether/ethyl acetate 1:1). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (*ppm*): 7.34-7.22 (5H, m, Ph); 6.91 (1H, bp, NH); 3.95 (2H, q, J= 5.4 Hz, J= 2.5 Hz, CH_2); 3.72 (2H, s, COC$\underline{H}_2$S); 3.12 (2H, s, PhC$\underline{H}_2$S); 2.24 (1H, t, J= 2.5 Hz, C$\underline{H}$). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (*ppm*): 168.4 (qC, $\underline{C}$O); 137.0 (qC, Ph); 129.0, 128.8, 127.5 (5 x CH, Ph) 79.3 (qC, alkyne); 71.8 (CH), 37.1, 35.0 and 29.4 (CH_2). FAB-MS calculated for $C_{12}H_{13}NOS$ (M+1) 220.07906, found.220.07910.

^{1}H-NMR spectrum of **8** (300 MHz, $CDCl_3$):



1-*N*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-*N*-acetyl-*β*-D-glucopyranosylamido)-4-(*N*'-methylidenyl -2'-thiobenzylacetamido)-4,5-anhydro-triazole (**9**)

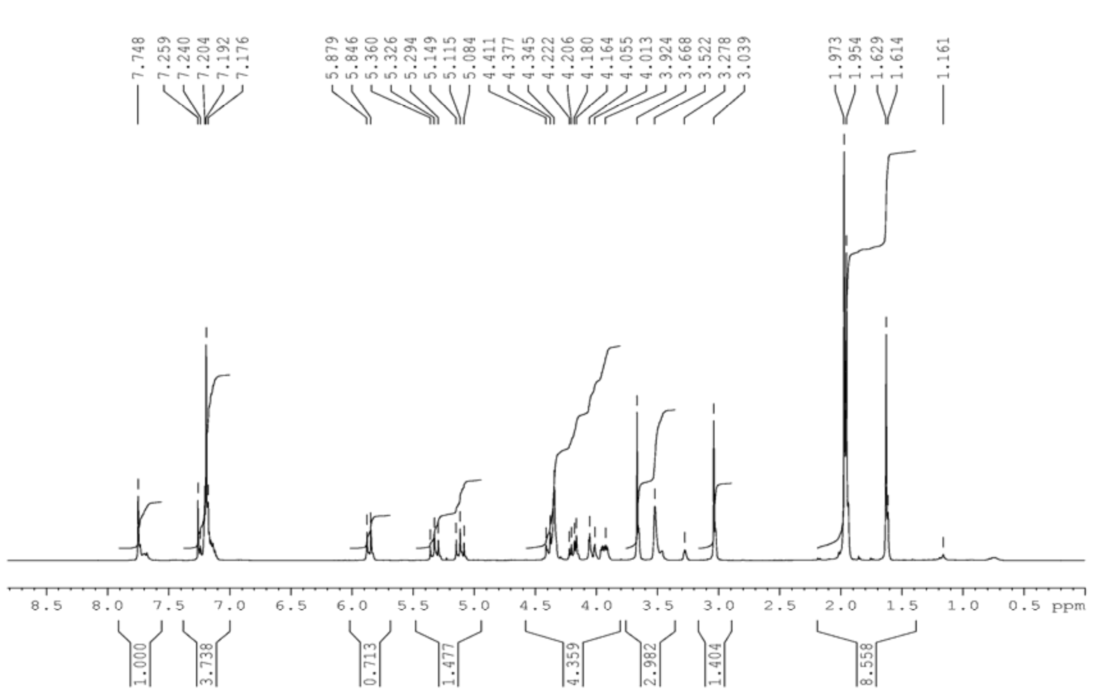


The azido sugar (100 mg, 0.27 mmol) and propargyl derivative (59 mg, 0.27 mmol) were dissolved in a biphasic solution of $CHCl_3/EtOH/H_2O$ (9:1:1) (1.1 mL). Sodium ascorbate (54 mg, 0.27 mmol) and $CuSO_4{\cdot}5H_2O$ (2 mg, 0.007 mmol) were added. The reaction was stirred at 600 rpm, 50°C overnight. Afterwards, the reaction mixture was diluted with $CHCl_3$ and washed with saturated aqueous $NaHCO_3$ (3 x 20 mL), and the organic phase was dried with $MgSO_4$, filtered and concentrated under reduced pressure to afford a pale brown solid (146 mg, 91%). R_f: 0.33 (ethyl acetate). ^{1}H-NMR (300 MHz, $CDCl_3$ and 5% of CD_3OD) δ (*ppm*): 7.75 (1H, s, C<u>H</u>-triazole); 7.24-7.18 (5H, m, Ph); 5.86 (1H, d, $J_{1\text{-}2}$= 9.9 Hz, H1); 5.33 (1H, dd, $J_{2\text{-}3}$= $J_{3\text{-}4}$=9.9 Hz, H3); 5.12 (1H, dd, $J_{3\text{-}4}$= $J_{4\text{-}5}$=9.9 Hz, H4); 4.38 (1H, dd, $J_{1\text{-}2}$= $J_{2\text{-}3}$=9.9 Hz, H2); 4.35 (2H, s, C<u>H</u>$_2$NH); 4.19 (1H, dd, $J_{6a\text{-}6b}$= 12.6 Hz, $J_{5\text{-}6a}$= 4.8 Hz, H6a); 4.03 (1H, dd, $J_{6a\text{-}6b}$= 12.6 Hz, $J_{5\text{-}6b}$= 1.9 Hz, H6b); 3.94 (1H, ddd, $J_{4\text{-}5}$=9.9 Hz, $J_{5\text{-}6a}$= 4.8 Hz, $J_{5\text{-}6b}$= 1.9 Hz, H5); 3.66 (2H, s, COC<u>H</u>$_2$S); 3.03 (2H, s, SC<u>H</u>$_2$Ph); 1.97, 1.95, 1.93 (9H, 3 x s, C<u>H</u>$_3$CO); 1.62 (3H, s, C<u>H</u>$_3$CONH). ^{13}C-NMR (75 MHz, $CDCl_3$ and 5% of CD_3OD) δ (*ppm*): 171.4, 170.9, 170.6, 169.7, 169.6 (5 x qC, <u>C</u>O); 137.1 (qC, triazole); 128.9, 128.5, 127.2 (<u>C</u>H, Ph); 121.2 (qC, Ph); 85.9, 74.7, 72.2, 68.0, 53.3 (5 x CH; <u>C</u>1-<u>C</u>5); 61.7 (<u>C</u>H$_2$, C6); 36.8, 34.8, 34.7 (<u>C</u>H$_2$); 23.6, 22.2, 20.5, 20.4 (4 x COC<u>H</u>$_3$). FAB-MS calculated for $C_{26}H_{33}N_5O_9S$ (M+1): 592.20717, found: 592.20858.

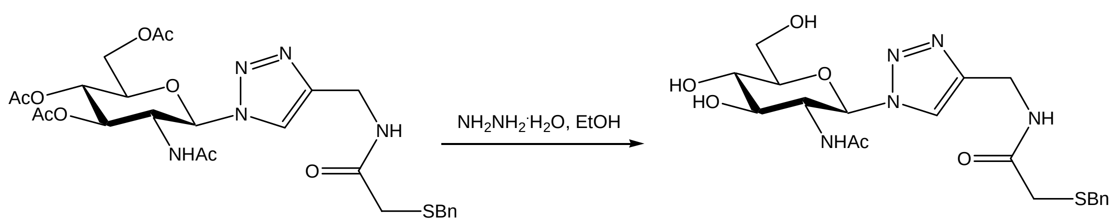
S-Benzyl thioether (**9**)

The sugar (50 mg, 0.09 mmol) was dissolved in DMF (615 µL). Benzylmercaptan (106 µL, 0.9 mmol) and triethylamine (138 µL, 0.726 mmol) was added. The reaction was stirred for 16 h. The reaction mixture was diluted with chloroform (10 mL), and washed with a NaOH (1 M, 10 mL), 5% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL) and water (10 mL). The organic phase was dried with $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography over silica (petroleum ether/ethyl acetate, 9:1→1:9) to afford the pure product (40 mg, 75 %). R_f: 0.33 (ethyl acetate). ^{1}H-NMR data as above.

^{1}H-NMR spectrum of **9** (300 MHz, $CDCl_3$/5% CD_3OD):



Deacetylated S-Benzyl thioether (**10**)



The sugar (137 mg, 0.23 mmol) was dissolved in a 2 % solution of hydrazine monohydrate in ethanol (5 mL). After 3 days, the reaction was complete. The solvent was removed under high vacuum, and the crude product was purified by flash chromatography over silica (10% methanol in DCM) to afford the pure product (71 mg, 66%). R_f 0.01 (10% methanol in DCM). ^{1}H-NMR (300 MHz, D_2O/CD_3OD) δ (*ppm*): 8.04 (1H, s, C<u>H</u>-triazole); 7.30-7.24 (5H, m, Ph); 5.76 (1H, d, $J_{1\text{-}2}$= 9.8 Hz, H1); 4.40 (2H, s, triazole-C$\underline{H}_2$-NH); 4.20 (1H, dd, $J_{1\text{-}2}$= $J_{2\text{-}3}$= 9.8 Hz, H2); 3.90-3.54 (5H, m, H3, H4, H5, H6a, H6b); 3.78 (2H, s, CO-C$\underline{H}_2$-S); 3.12 (2H, s, S-C$\underline{H}_2$-Ph); 1.76 (3H, s, COC$\underline{H}_3$). ^{13}C-NMR (75 MHz, D_2O/CD_3OD) δ (*ppm*): 173.5, 172.2 (2 x qC, <u>C</u>O); 146.0, 139.0 (2 x qC, Ph and triazole); 130.2, 129.5, 128.2, 123.0 (4 x C̲H, Ph and triazole); 88.2, 81.2, 75.6, 71.4, 56.8 (5 x CH; C̲1-C̲5); 62.3 ($\underline{C}H_2$, C6); 37.5, 35.8, 35.5 (3 x $\underline{C}H_2$); 22.6 ($COC\underline{H}_3$). FAB-MS calculated for $C_{20}H_{27}N_5O_6S$ (M+1): 466.17548, found: 466.17335.

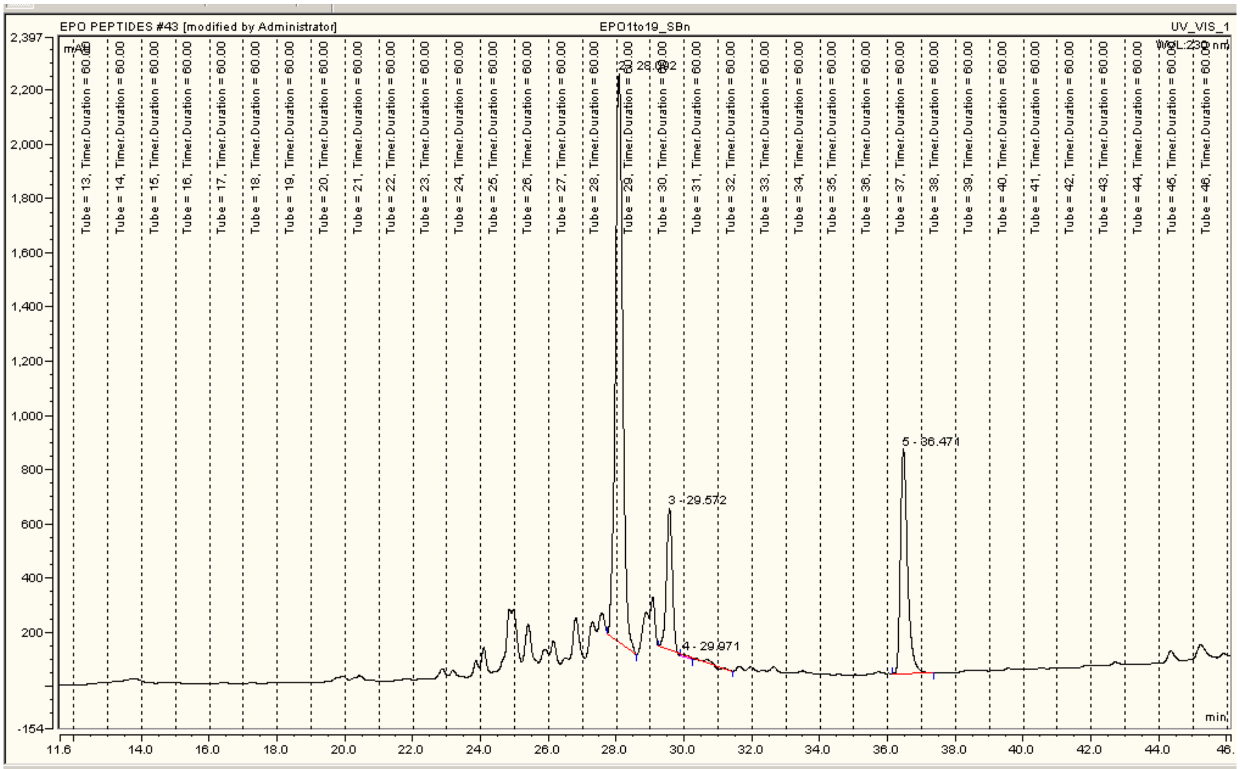
Peptide synthesis

Peptide synthesis was carried out using Rink amide-MBHA resin for the production of peptide thioesters (loading = 0.67 mmol/g). All resins and Fmoc amino acids were purchased from Novabiochem. Mass spectra were obtained on a Micromass Quattro LC series electrospray mass spectrometer. Semi-preparative HPLC was performed using a Phenomenex LUNA C_{18} column and a gradient of 5-95% acetonitrile containing 0.1% TFA over 45 minutes (flow rate of 3.0 mL/min). All other chemical reagents were obtained form Aldrich.

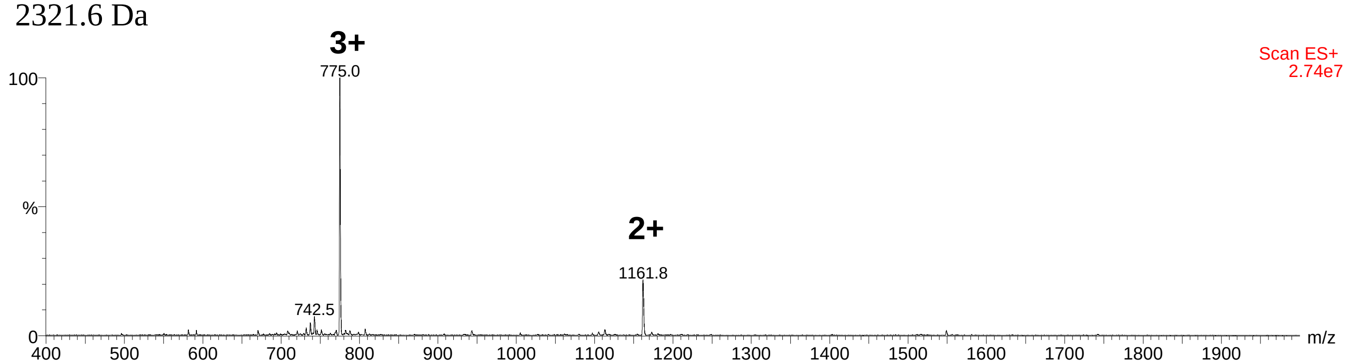
Peptide thioester synthesis (EPO residues 1-19).

The peptide thioesters were prepared using the dual linker strategy recently described by the Unverzagt group.[1] Briefly, rink amide resin (0.1 mmol) was deprotected by exposure to 20 % piperidine in DMF. Fmoc-Phe-OH (5 equiv) was coupled using HBTU/HOBt as coupling reagents. The coupling time was 4 h. After Fmoc removal with 20 % piperidine in DMF the sulfonamide linker was coupled through exposure of the resin to 3-carboxypropanesulfonic acid (50 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIC (47µL, 0.3 mmol) for 5 h. The first amino acid (Fmoc-Ser(tBu)-OH, 5 equivalents per coupling) was then double coupled employing N-methylimidazole (40 µL, 0.5 mmol), DIC (78 µL, 0.5 mmol) as coupling reagents in 4:1 DCM/DMF for 16 h. The peptide was extended (target sequence: APPRLICDSRVLE RYLLEA-SBn) and cleaved with benzylmercaptan, after ICH_2CN activation, using well established procedures.[2] The crude fully deprotected and precipitated peptide was redissolved in 25 % aqueous MeCN and purified by semi-prep HPLC. The major peak (retention time = 29 mins) was analysed by ESI-MS and was found to correspond to the desired product. This fraction was lyophilized to obtain approximately 1mg of the product that was used in subsequent NCL reactions. NOTE: use of the double linker strategy indicated that although the resin activation with ICH_2CN had been near quantitative the subsequent release of the thioester was particularly sluggish as significant quantities of the activated resin-bound peptide remained attached to the solid support. Further peptide could be released by re-exposure of the resin to benzyl mercaptan and by conducting the cleavage reaction at 40 °C.

HPLC of the crude peptide thioester:



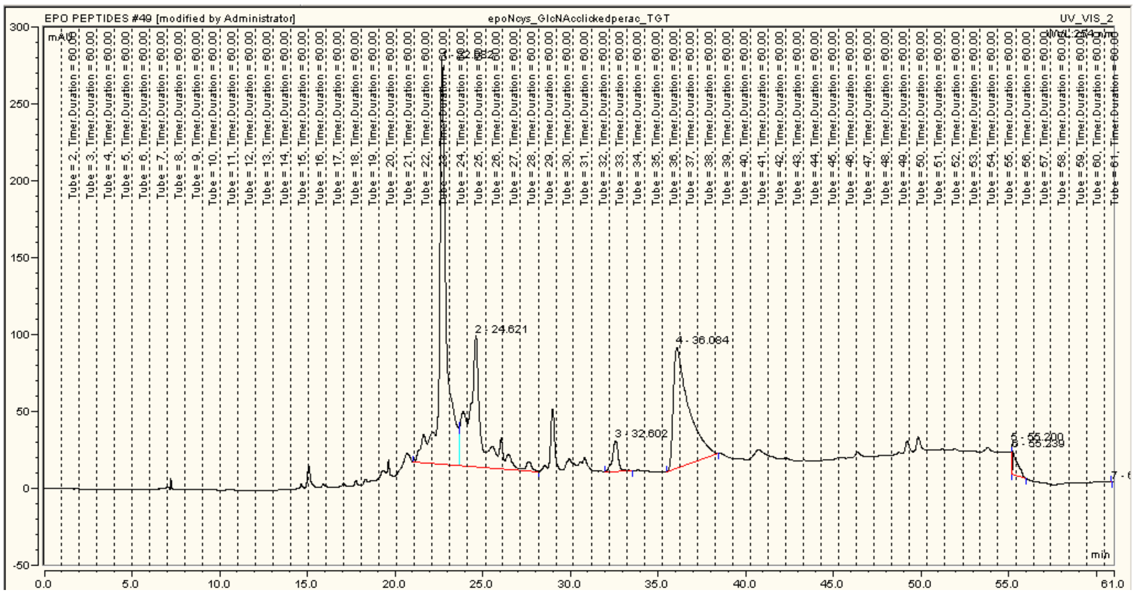
ESI-MS of 29 min fraction (residues 1-19-SBn) Calcd mass= 2320.7 Da , Observed mass = 2321.6 Da



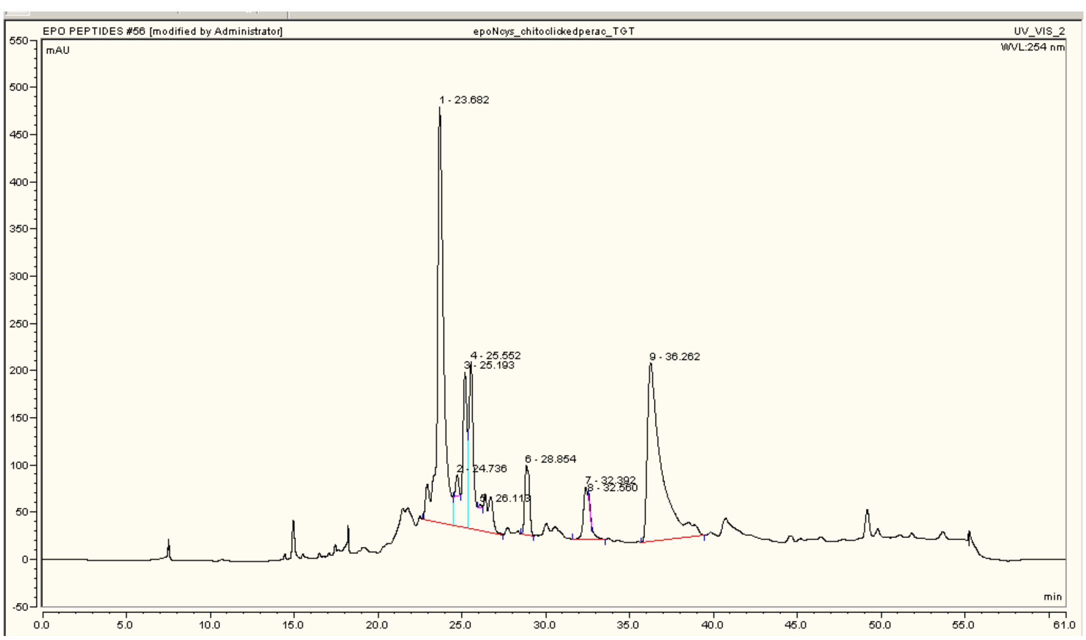
*Reaction of bromoacetamides **4-7** with solid-supported peptide **11***

After MS verification that the desired peptide had been prepared the SStBu protecting groups were cleaved on solid-phase by exposure to fresh 10 % w/v dithiothreitol in dry DMF containing 2.5 % v/v DIPEA for 2 x 24 h . The thiols were capped by treatment with the desired bromoacetamide (3 equivalents per thiol) in DMF containing 2.5 % pyridine (or Et_3N) for 24 h.

HPLC of crude **12** (prior to hydrazine deprotection). The peak in fraction 23 is the desired product:

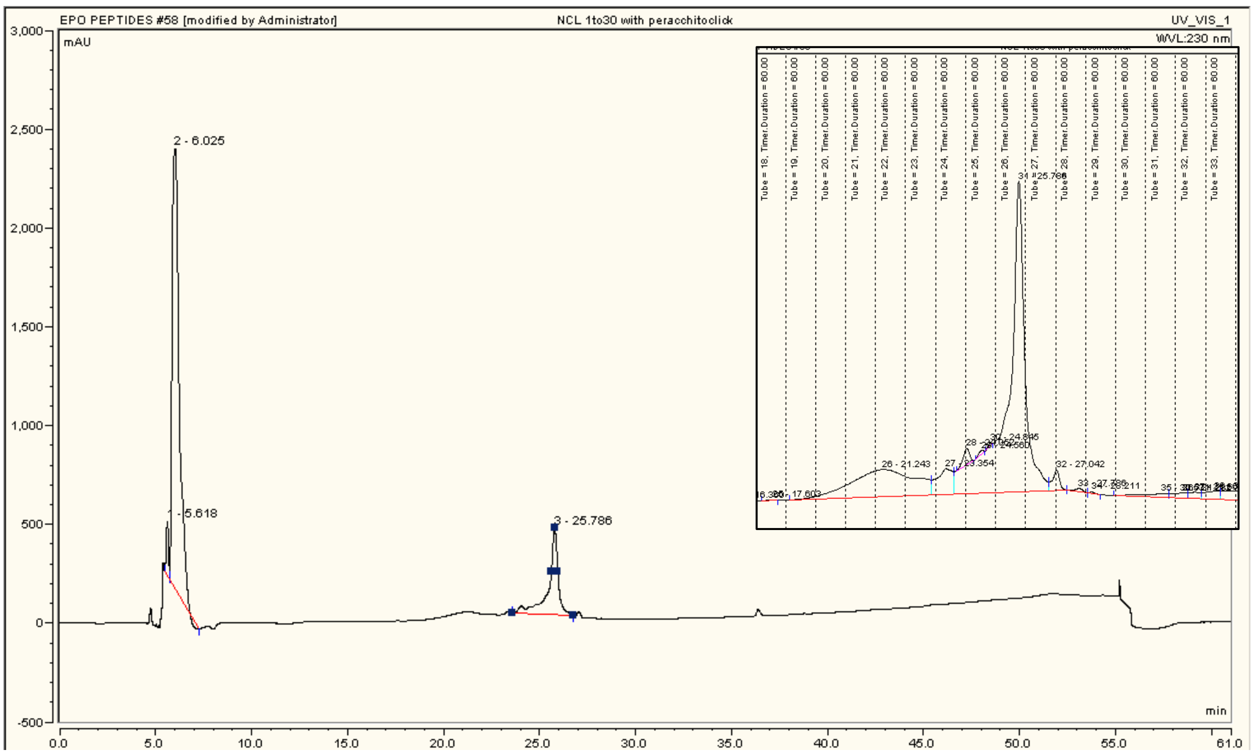


HPLC of crude **13** (prior to hydrazine deprotection). The major peak (retention time = 23.7 min) is the desired product:

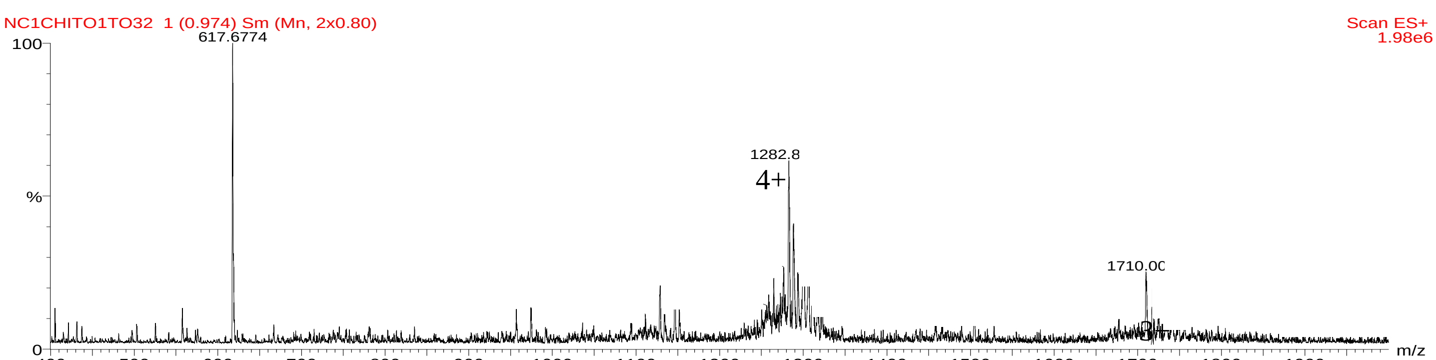


Native chemical ligation:

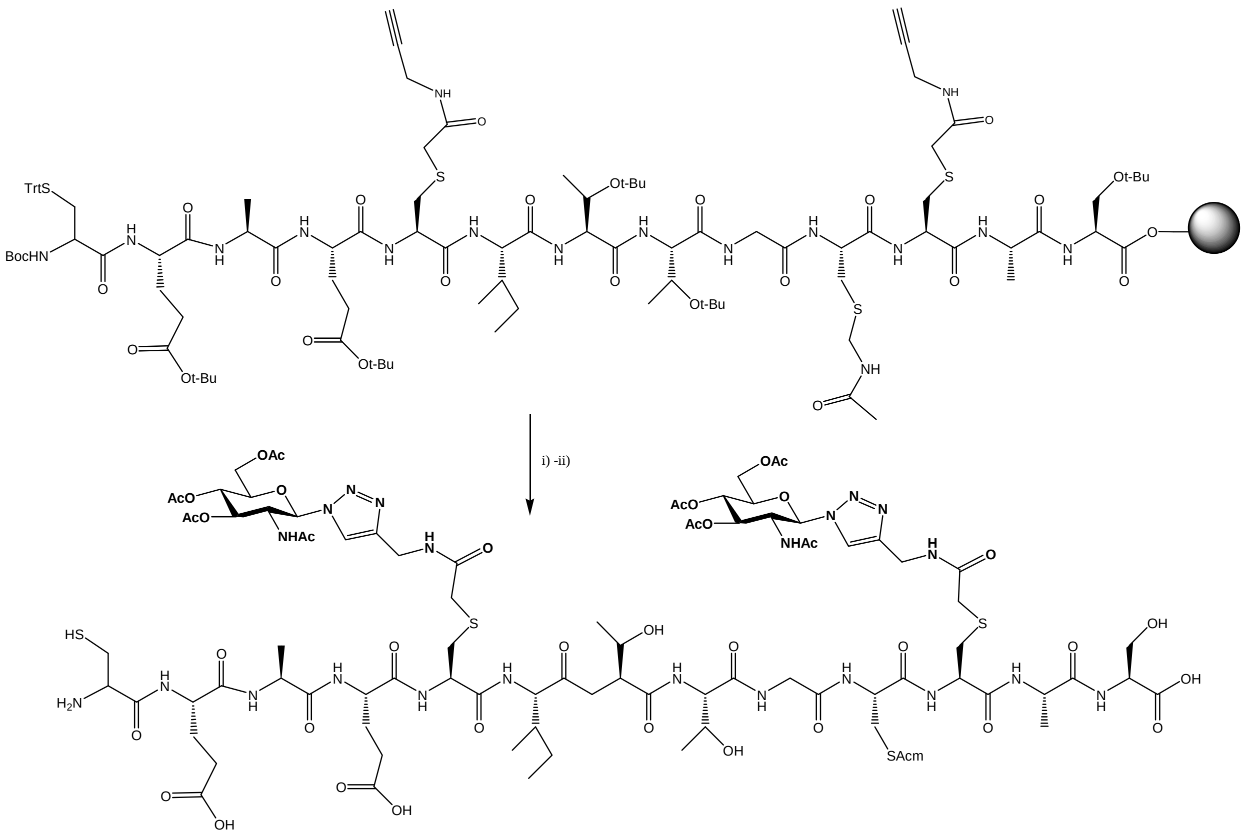
The NCL reaction was conducted under standard conditions. The peptides (1 mg each thioester and purified **13**) were dissolved in 250 µL of 6M guanidine HCl, containing 300 mM Na phosphate buffer; pH 8.0, 1 % w/v MESNA and 10 mM TCEP. The reaction was incubated at room temperature for 36 h. and loaded directly onto a semi-prep HPLC column. The HPLC showed a single major species with a retention time of 25.8 mins which was confirmed as the desired product by ESI-MS (calculated Mwt = 5125.6 Da, Obs MWt = 5127.0 Da.



ESI MS (before hydrazine deprotection)

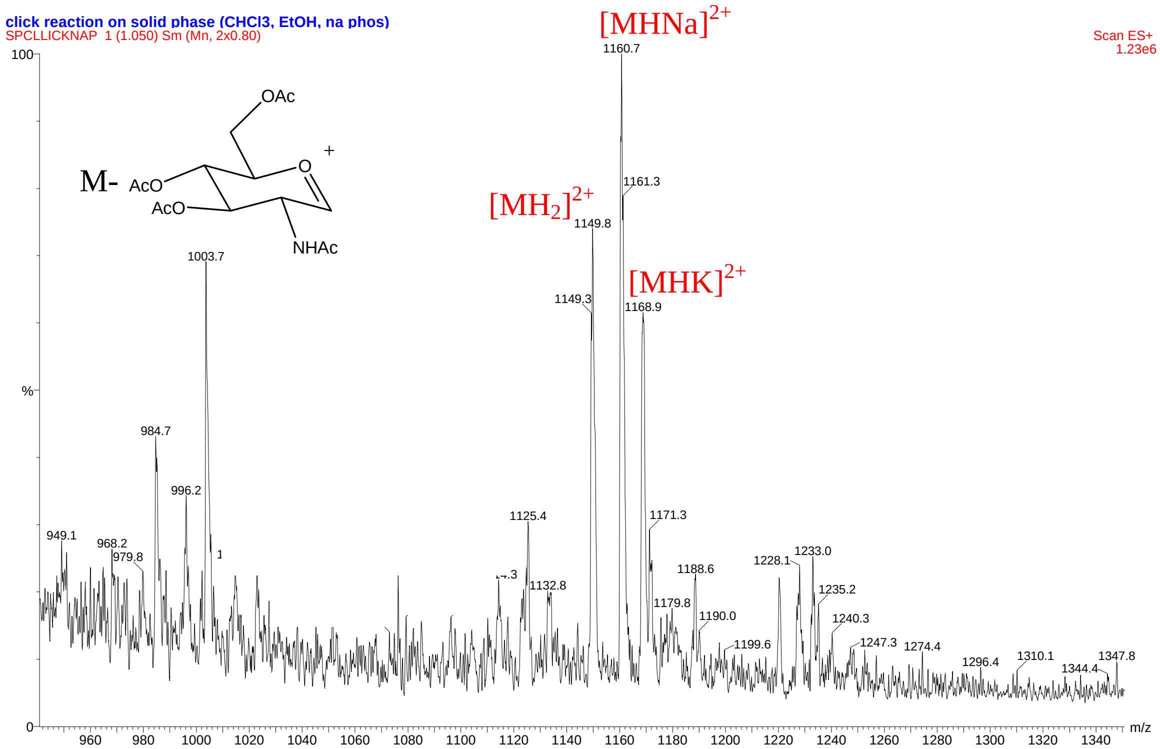


Finally, the click reaction also works when acetylenes are loaded first onto solid phase (scheme 1). The resin (42 mg) containing the peptide shown in scheme 1 (9.16×10^{-6} mol peptide modified with **4** as described in the manuscript) was suspended in 9:1:1 $CHCl_3$/ EtOH/ 50mM sodium phosphate buffer (1.1 ml) and **1** (20 mg, 0.055 $\times10^{-3}$ mol, 3equiv per thiol) and sodium ascorbate (7mg, 0.055 $\times10^{-3}$ mol) were added followed by $Cu(SO_4).5H_2O$ (0.5 mg). The reaction was incubated at 37 ^oC with shaking at 500rpm in a 1.5 ml eppendorf tube, in an eppendorf thermomixer. The resin was then filtered and washed with water, NMP, and then DCM. The product was cleaved from the solid support by exposure to 95 % TFA, 2.5 % water, 2.5 % EDT for 3 h and analyzed by mass spectrometry.



Scheme 1: *Reagents and conditions*: i) 1 (3, equivs), Na ascorbate (3 equivs), $CHCl_3$/EtOH, 50 mM Na phosphate buffer (pH 8.0), cat $CuSO_4.5H_2O$, 37 ^oC, 16 h. ii) 95 % TFA, 2.5 % EDT, 2.5 % H_2O.

ESI MS of the crude product calculated mass = 2296.4, observed mass = 2297.6



References:

1. Mezzato, S.; Schaffrath, M.; Unverzagt, C., An orthogonal double-linker resin facilitates the efficient solid-phase synthesis of complex-type N-glycopeptide thioesters suitable for native chemical ligation. *Angew. Chem. Int. Ed.* **2005,** 44, 1650-1654.

2. Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R., Fmoc-based synthesis of peptide-(alpha)thioesters: Application to the total chemical synthesis of a glycoprotein by native chemical ligation. *J. Am. Chem. Soc.* **1999,** 121, (50), 11684-11689.