Supporting information for:

Solid-Phase Synthesis of PEGylated Lipopeptides Using Click Chemistry

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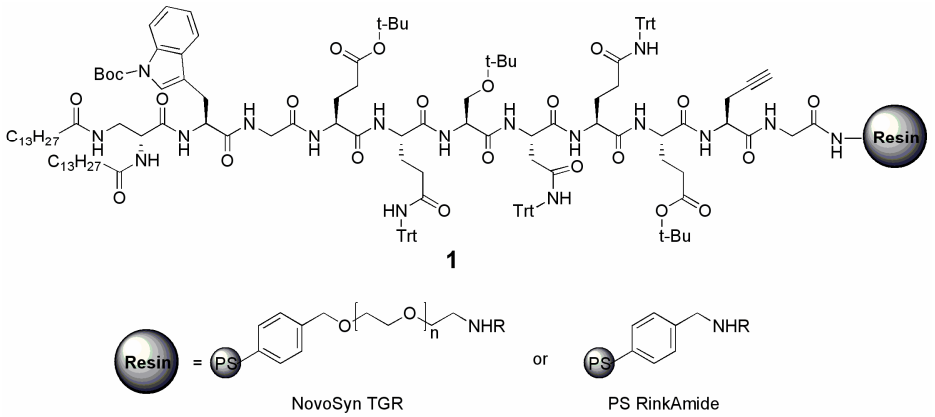
Supporting Information

Synthesis. All Fmoc-protected amino acids, HATU and PS RinkAmide resin (0.83mmol/g) were purchased from GLS China. NovaSyn TGR® resin (0.25mmol/g) was purchased from Merck Chemicals whereas all other chemicals were purchased from Sigma-Aldrich and used without further purification. Thin-layer chromatography was performed using Merck 60 F_{254} aluminium plates precoated with silica gel. Compounds were visualized by heating after dipping in $KMnO_4$ stain solution (1% $KMnO_4$, 6.7% K_2CO_3 and 0.08% NaOH in EtOH). ^{1}H-NMR and ^{13}C-NMR were recorded on Bruker Avance DPS 250 MHz Spectrometer by using the solvent peak as an internal reference ($CDCl_3$: ^{1}H 7.26 ppm, ^{13}C 77.16 ppm). The multiplicity is defined by bs (broad singlet). FT-IR was recorded on a Perkin Elmer Instruments Spectra One FT-IR Spectrometer and MALDI-TOF-MS on a Bruker Reflex IV MALDI-TOF Spectrometer using 2,5-dihydroxybenzoic acid (DHB) spiked with sodium trifluoroacetate in MeOH as matrix.

$(C_{13}H_{27}CO)_2$-dap-Trp(Boc)-Gly-Glu(tBu)-Gln(Trt)-Ser(tBu)-Asn(Trt)-Gln(Trt)-Glu(tBu)-Gly(propargyl)-Gly-CO-Resin (1)



The alkyne functionalized lipopeptide **1** was synthesized on both PS-RinkAmide resin (0.30mmol/g) and NovaSyn TGR® resin (0.25mmol/g) by standard Fmoc methodology. Each coupling was achieved by 4 equiv. Fmoc protected amino acid, 3.98 equiv. HATU and 8 equiv. Colidine in DMF. Acylation with Fmoc-dap(Fmoc)-OH was achieved using 2 equiv. Each acylation and deprotection step was monitored by the Kaiser ninhydrin test. A small portion of each resins were placed in a solid phase reaction vessel and cleaved by a mixture of TFA/TIS/H_2O (95:2.5:2.5) for 3h. The cleaved resin was washed several times with TFA. The filtrates were combined, diluted

with water, reduced *in vacou* and lyophilized from a mixture of water and acetonitrile to give the crude product of the title compound as a white powder. The crude product was analyzed using a Shimadzu LC-2010C analytical HPLC by employing a Waters XTerra® RP18 5µm (4.6*150mm) column. Eluent: (A) 5% CH_3CN + 0.1% TFA in H_2O, (B) 0.1% TFA in CH_3CN. Gradient profile; 0-10min 52%B, 10-40min linear gradient 52-58% B, 40-50min 58% B. Flow rate; 1 mL/min, UV-detection at both 256 and 280nm. Analytical HPLC showed a homogenous peak with retention time of 30.49 min.
MALDI-TOF MS (m/z) (DHB+Na^+):1656.5 (M+Na), 1678.5 (M+2Na), 1700.4 (M+3Na) (Figure 1). No deletions were observed.

Methoxy poly(ethylene glycol)$_{2000}$ azide (3a)
Methoxy poly(ethylene glycol)$_{2000}$ (2.82g, 1.41mmol) was dissolved in dry CH_2Cl_2 (35mL) and cooled to 0°C in an ice bath. Triethylamine (0.29g, 2.82mmol) was added followed by drop wise addition of methanesulfonyl chloride (8.08g, 70.5mmol) under vicious stirring. The reaction mixture was allowed to heat to rt and was stirred under argon for 72h. After 72h, 2/3 of the solvent was removed *in vacou* and the reaction left another 2h at rt. The crude methoxypolyethylene glycol mesylate was precipitated by addition into cold Et_2O under vicious stirring. The product was filtrated, washed several times with cold ether and dried *in vacou*. Additional salts were removed by passing the crude product solubilized in CH_2Cl_2/MeOH (9:1) over a short silica column. The crude product was dissolved in dry DMF (40mL) and heated to 100°C using an oil bath. Sodium azide (0.83g, 12.8mmol) was added and the reaction was left at 100°C for 60h. After 60h the reaction mixture was filtered through celite and concentrated *in vacou*. The crude product was precipitated by drop wise addition into cold Et_2O. The product was filtrated and dissolved in CH_2Cl_2/MeOH (9:1). Final purification was achieved by column chromatography (CH_2Cl_2/MeOH (9:1)). Fractions with the desired compound were reduced *in vacou* and the product lyophilized from a water/acetonitrile mixture overnight to give 2.18g (84%) of the title compound as a white powder. R_f = 0.55 (CH_2Cl_2/MeOH (9:1)). IR (KBr): ν(cm^{-1}): 2888.9, 2107.8, 1467.4, 1343.4, 1114.9 (Figure 2a). ^{1}H-NMR (250MHz, $CDCl_3$): δ 3.64 (bs, O-C*H*$_2$C*H*$_2$-O), 3.55 (bs, -C*H*$_2$-N_3), 3.37 (bs, C*H*$_3$-O). ^{13}C-NMR (62.9MHz, $CDCl_3$): δ 72.1, 70.7, 70.1, 59.1, 50.8. MALDI-TOF MS (m/z) (DHB+Na^+): 1268.3 (n=27), 1312.3 (n=28), 1356.2 (n=29), 1400.3 (n=30), 1444.3 (n=31), 1488.3 (n=32), 1532.3 (n=33), 1576.3 (n=34), 1620.3 (n=35), 1664.3 (n=36), 1708.3 (n=37), 1752.3 (n=38), 1796.3 (n=39), 1840.3 (n=40), 1884.3 (n=41), 1928.3 (n=42), 1972.3 (n=43), 2016.3 (n=44), 2060.3

(n=45), 2104.3 (n=46), 2148.3 (n=47), 2192.3 (n=48), 2236.3 (n=49), 2280.3 (n=50), 2324.3 (n=51) (Figure 3a).

Methoxy poly(ethylene glycol)$_{1000}$ azide (3b)

Methoxy poly(ethylene glycol)$_{1000}$ (10.34g, 10.34mmol) was dissolved in dry CH_2Cl_2 (30mL) after which triethylamine (1.26g, 12.41mmol), *m*-toluenesulfonyl chloride (2.37g, 12.41mmol) and potassium iodide (0.10g, 0.60mmol) was added at rt under vicious stirring. The reaction mixture was stirred 16h at rt under Argon. The reaction mixture was washed with water (3×50mL), the organic phase dried with sodium sulphate, filtered and concentrated in vacou to give methoxy poly(ethylene glycol)$_{1000}$ tosylate. The crude product was dissolved in dry DMF (50mL) and sodium azide (1.34g, 20.68mmol) was added at rt. Full conversion of the tosylate was observed overnight, and the solvent was removed *in vacou*. The crude product was redissolved in CH_2Cl_2 (100mL), washed with water (4×100mL), and dried with sodium sulphate, filtrered and concentrated *in vacou*. The final purification was achieved by column chromatography (CH_2Cl_2/MeOH (20:1)). Fractions with the desired compound were reduced *in vacou* to give 8.29g (78%) of the title compound as an off-white sticky solid. R_f = 0.38 (CH_2Cl_2/MeOH (20:1)). IR (KBr): ν(cm^{-1}): 2872.1, 2103.4, 1438.6, 1349.4, 1110.9 (Figure 2b). ^{1}H-NMR (250MHz, $CDCl_3$): δ 3.62 (bs, O-C*H*$_2$C*H*$_2$-O), 3.53 (bs, -C*H*$_2$-N$_3$), 3.35 (bs, C*H*$_3$-O). ^{13}C-NMR (62.9MHz, $CDCl_3$): δ 72.1, 70.8, 59.1, 50.8. MALDI-TOF MS (m/z) (DHB+Na$^+$): 696.1 (n=14), 740.2 (n=15), 784.1 (n=16), 828.2 (n=17), 872.1 (n=18), 916.2 (n=19), 960.1 (n=20), 1004.1 (n=21), 1048.1 (n=22), 1092.1 (n=23), 1136.1 (n=24), 1180.1 (n=25), 1224.1 (n=26), 1268.1 (n=27), 1312.1 (n=28), 1356.1 (n=29), 1400.0 (n=30) (Figure 3b).

Methoxy poly(ethylene glycol)$_{350}$ azide (3c)

Methoxy poly(ethylene glycol)$_{350}$ (5.57g, 15.91mmol) was dissolved in dry CH_2Cl_2 (30mL) after which triethylamine (1.93g, 19.10mmol), *m*-toluenesulfonyl chloride (3.64g, 19.10mmol) and potassium iodide (0.10g, 0.60mmol) was added at rt under vicious stirring. The reaction mixture was stirred 16h at rt after which the reaction mixture was washed with water (3×50mL), and dried with sodium sulphate, filtered and concentrated *in vacou* to give the corresponding methoxy poly(ethylene glycol)$_{350}$ tosylate. The crude product was dissolved in dry DMF (50mL) and sodium azide (2.07g, 31.82mmol) was added at rt and stirred overnight after which full conversion was observed and the solvent was removed *in vacou*. The crude product was redissolved in CH_2Cl_2

(50mL), washed with water (4×100mL), dried with sodium sulphate, filtered and concentrated *in vacou*. The final purification was achieved by column chromatography (CH_2Cl_2/EtOAc (15:1)). Fractions with the desired compound were reduced *in vacou* to give 0.84g (14%) of the title compound as a slightly yellow viscous oil. R_f = 0.18 (CH_2Cl_2/MeOH (20:1)). IR (KBr): ν(cm^{-1}): 2873.6, 2107.8, 1455.7, 1349.8, 1111.2 (Figure 2c). ^{1}H-NMR (250MHz, $CDCl_3$): δ 3.65 (bs, O-C*H*$_2$C*H*$_2$-O), 3.55 (bs, -C*H*$_2$-N_3), 3.37 (bs, C*H*$_3$-O). ^{13}C-NMR (62.9MHz, $CDCl_3$): δ.72.0, 70.7, 59.0, 50.8. MALDI-TOF MS (m/z) (DHB+Na^+): 344.2 (n=6), 388.2 (n=7), 432.2 (n=8), 476.2 (n=9), 520.2 (n=10) (Figure 3c).

CuAAC Reaction on Solid Phase Support.

Each CuAAC reaction was carried out using 50mg lipopeptide modified resin (12.5-15µmol lipopeptide), 5 equiv. MeO-PEG_X-N_3, 5 equiv. CuI and 5 equiv. sodium ascorbate in either DMF/piperidine (4:1) or CH_2Cl_2/piperidine (4:1). The total reaction volume was adjusted so the final concentration of the above mentioned reagents in all cases was 61.25 mM. Small samples of resin was taken out after 1, 2, 5, 24, 48 and 72h and placed in a solid phase reaction vessel and cleaved by a mixture of TFA/TIS/H_2O (95:2.5:2.5) for 3h. The cleaved resin was filtrated and washed several times with TFA. The combined filtrates was diluted with water, reduced *in vacou* and lyophilized from a mixture of water.

Quantification of PEGylation Efficiency. The lyophilized PEGylated lipopeptides were solubilized in DMSO (1mg/mL) and analyzed on a Shimadzu LC-2010C analytical HPLC by employing a Waters XTerra® RP18 5µm (4.6*150mm) column. Eluent: (A) 5% CH_3CN + 0.1% TFA in H_2O, (B) 0.1% TFA in CH_3CN. Gradient profile; 0-10min 52%B, 10-40min linear gradient 52-58% B, 40-50min 58%B. Flow rate; 1 mL/min, Dual UV-detection at both 256 and 280nm. The coupling efficiency was calculated from the area under the curve (AUC) from the two peaks corresponding to the lipopeptide and the PEGylated lipopeptide respectively at 280nm (only absorbance from the Trp-residue).

($C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{2000})-Gly-$CONH_2$ (4a)

Analytical HPLC showed a retention time of 25.13 min for ($C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{2000})-Gly-$CONH_2$. MALDI-TOF MS (m/z) (DHB+Na^+): 2682.7 (M+Na (n=22)), 2726.7 (M+Na (n=23)), 2770.7 (M+Na (n=24)), 2814.7 (M+Na (n=25)), 2856.8 (M+Na

(n=26)), 2902.7 (M+Na (n=27)), 2946.7 (M+Na (n=28)), 2990.7 (M+Na (n=29)), 3034.6 (M+Na (n=30)), 3078.7 (M+Na (n=31)), 3122.7 (M+Na (n=32)), 3166.7 (M+Na (n=33)), 3210.7 (M+Na (n=34)), 2254.6 (M+Na (n=35)), 3298.6 (M+Na (n=36)), 3342.6 (M+Na (n=37)), 3386.6 (M+Na (n=38)), 3430.5 (M+Na (n=39)) 3474.5 (M+Na (n=40)), 3518.6 (M+Na (n=41)), 3562.6 (M+Na (n=42)), 3606.4 (M+Na (n=43)) 3650.5 (M+Na (n=44)) 3694.4 (M+Na (n=45)), 3738.7 (M+Na (n=46)) (Figure 4a).

$(C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{1000})-Gly-$CONH_2$ (4b)

Analytical HPLC showed a retention time of 27.75 min for $(C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{1000})-Gly-$CONH_2$. MALDI-TOF MS (m/z) (DHB+Na^+): 2154.3 (M+Na (n=10)), 2198.3 (M+Na (n=11)), 2242.3 (M+Na (n=12)), 2286.3 (M+Na (n=13)), 2330.3 (M+Na (n=14)), 2374.3 (M+Na (n=15)), 2418.3 (M+Na (n=16)), 2462.3 (M+Na (n=17)), 2506.3 (M+Na (n=18)), 2550.3 (M+Na (n=19)), 2594.3 (M+Na (n=20)), 2638.3 (M+Na (n=21)), 2682.3 (M+Na (n=22)), 2726.3 (M+Na (n=23)), 2770.3 (M+Na (n=24)), 2814.2 (M+Na (n=25)), 2858.3 (M+Na (n=26)), 2902.3 (M+Na (n=27)) (Figure 4b).

$(C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{350})-Gly-$CONH_2$ (4c)

Analytical HPLC showed a retention time of 29.37 min for $(C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{350})-Gly-$CONH_2$. MALDI-TOF MS (m/z) (DHB+Na^+): 1846.7 (M+Na (n=3)), 1890.7 (M+Na (n=4)), 1934.7 (M+Na (n=5)), 1978.7 (M+Na (n=6)), 2022.7 (M+Na (n=7)), 2066.7 (M+Na (n=8)), 2110.7 (M+Na (n=9)) (Figure 4c).

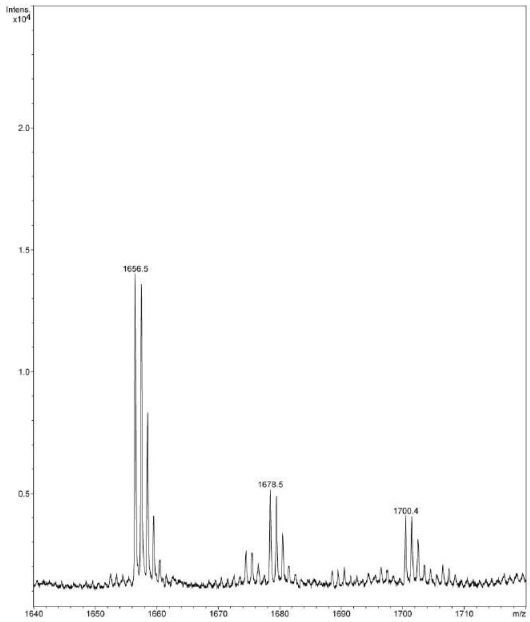


Figure 1. MALDI-TOF MS of the diacylated lipopeptide **1** used for the on-resin PEGylation by the CuAAC reaction.

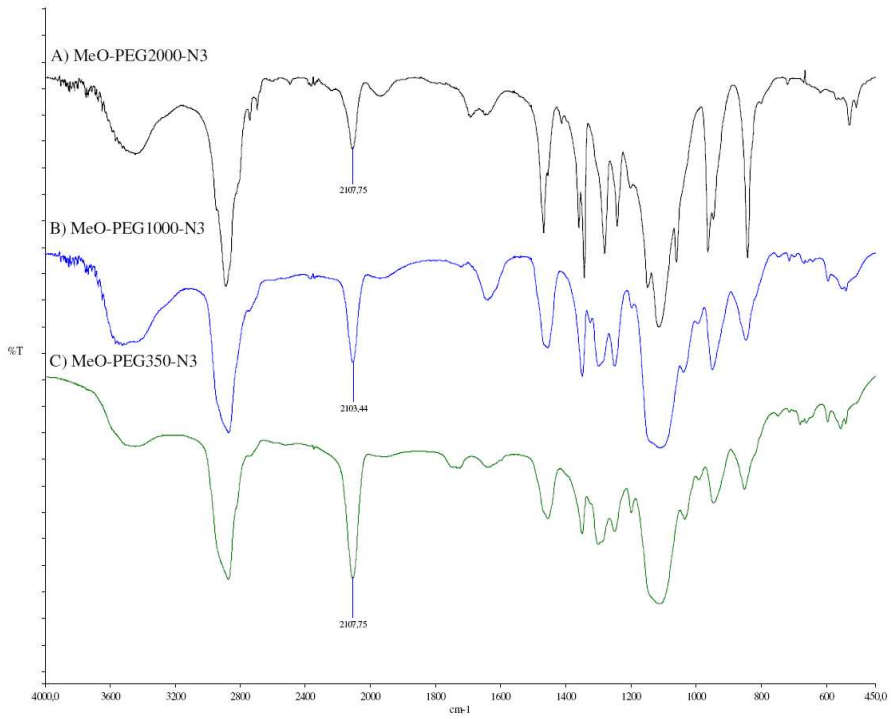


Figure 2. FT-IR (KBr) of the synthesized PEG-azides used for on resin PEGylation reaction. Azide formation confirmed by azide stretching frequency observed at approx. 2100 cm^{-1}. a) MeO-PEG_{2000}-N_3 (**3a**), b) MeO-PEG_{1000}-N_3 (**3b**) and c) MeO-PEG_{350}-N_3 (**3c**).

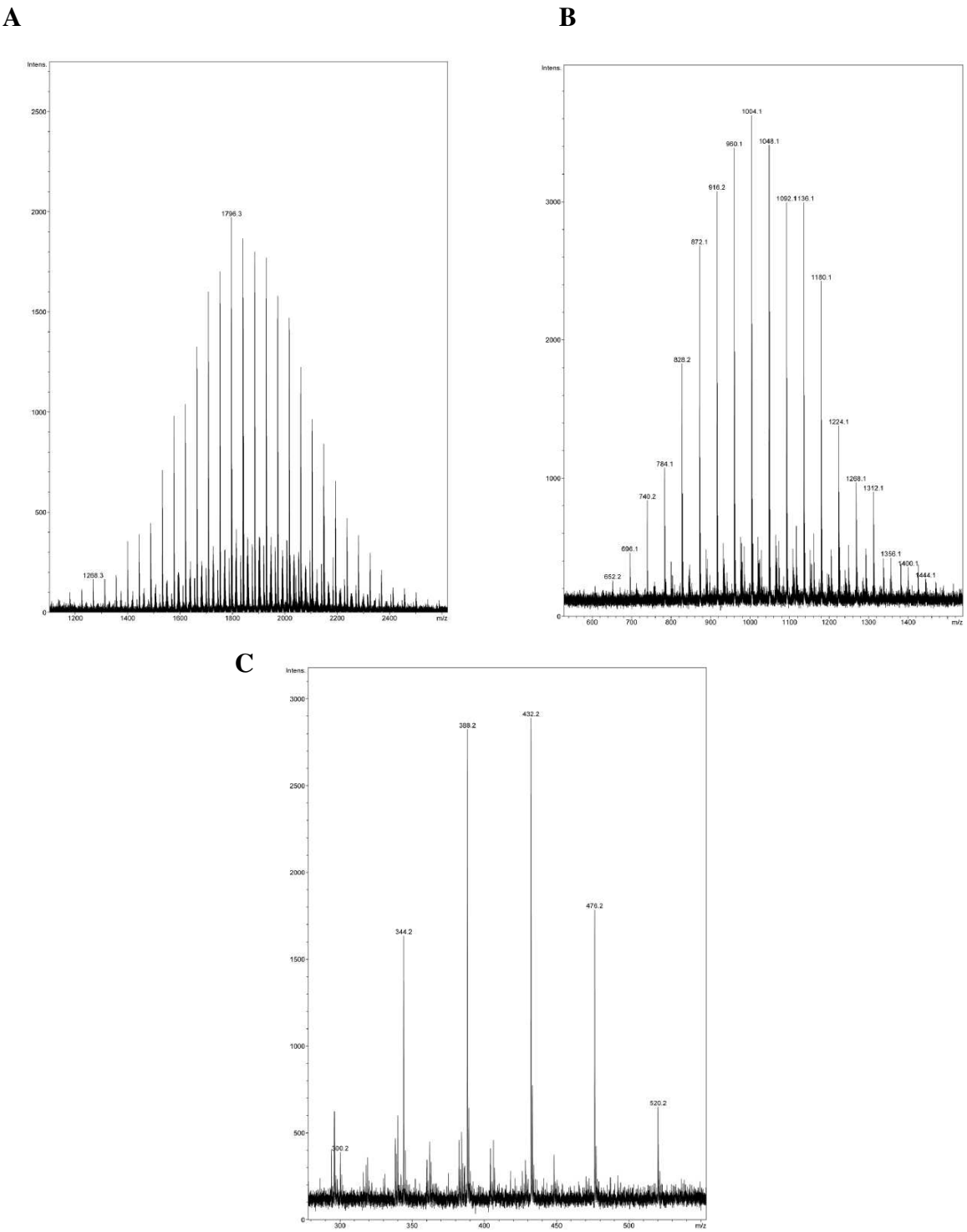


Figure 3. MALDI-TOF MS of the synthesized PEG-azides used for on resin PEGylation reaction. a) MeO-PEG_{2000}-N_3 (**3a**), b) MeO-PEG_{1000}-N_3 (**3b**) and b) MeO-PEG_{350}-N_3 (**3c**).

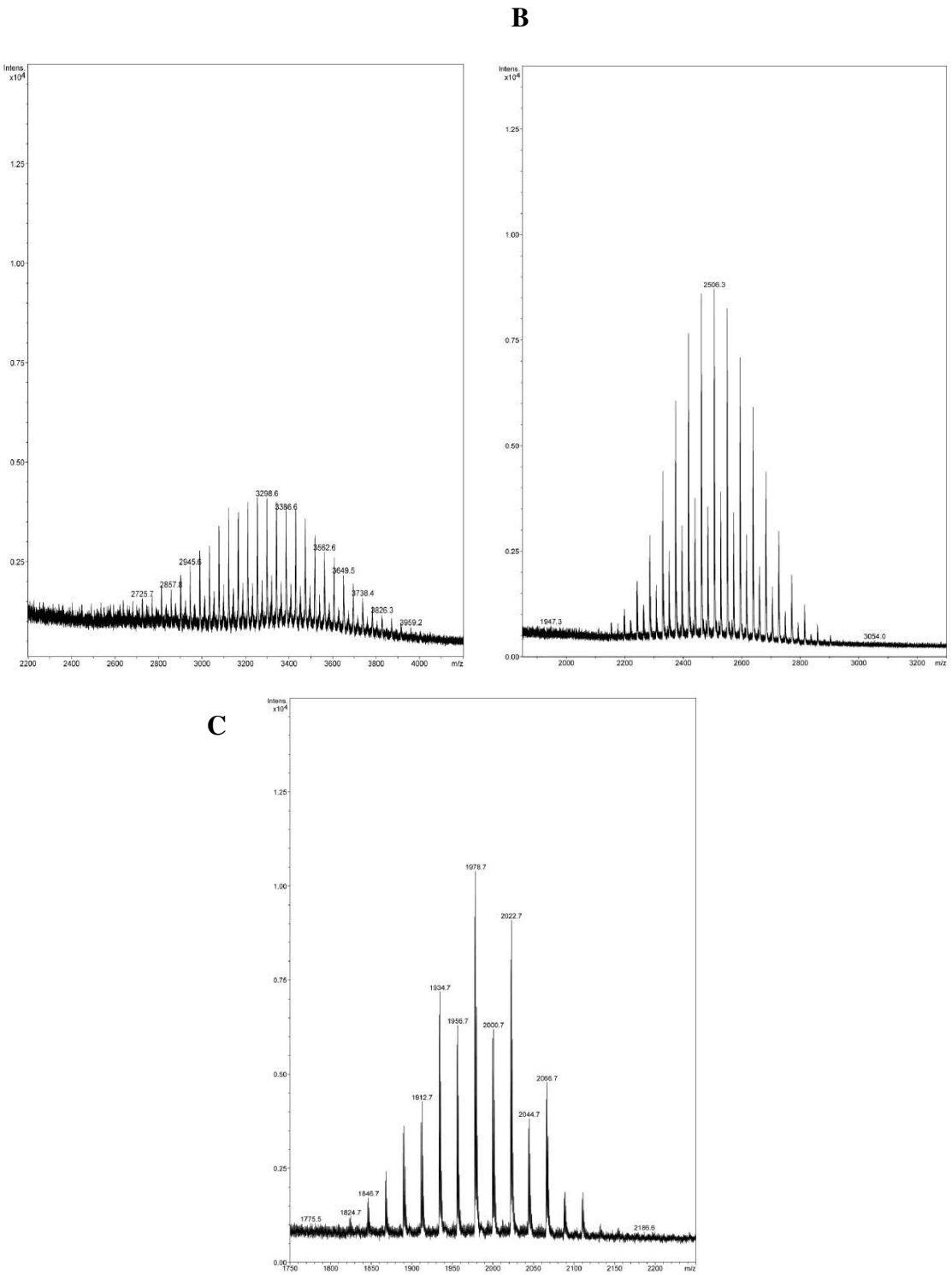


Figure 4. MALDI-TOF MS of the PEGylated lipopeptides achieved by on resin PEGylation using Click Chemistry. a) $(C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{2000})-Gly-$CONH_2$ (**4a**). b) $(C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{1000})-Gly-$CONH_2$ (**4b**). c) $(C_{13}H_{27}CO)_2$-dap-Trp-Gly-Glu-Gln-Ser-Asn-Gln-Glu-Gly(PEG_{350})-Gly-$CONH_2$ (**4c**)