Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Guanidinoneomycin-Maleimide Molecular Transporter: Synthesis, Chemistry and Cellular Uptake

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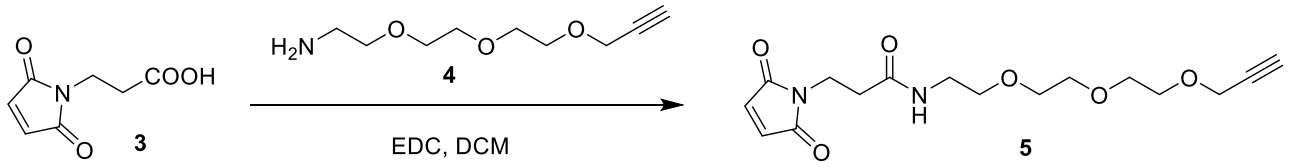
Synthetic procedures and characterization

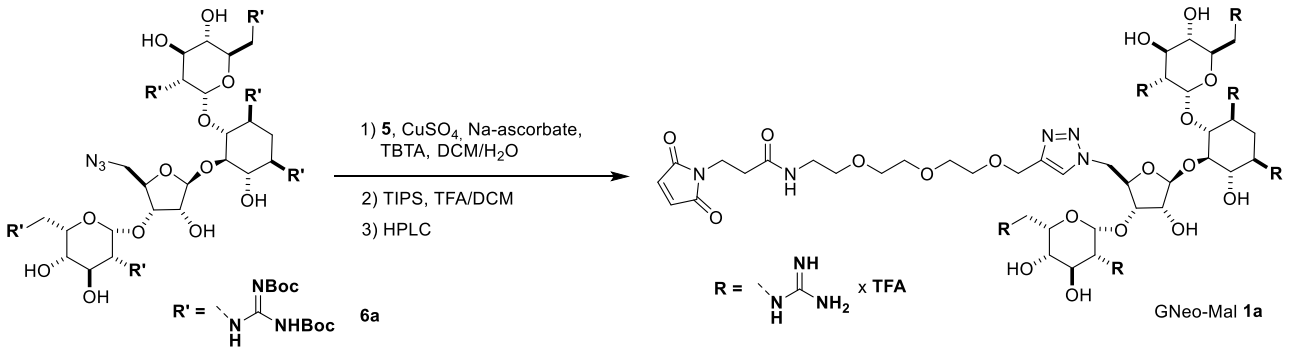
Synthesis of compound 5



To a stirred solution of **3** (500 mg, 2.96 mmol) in dichloromethane (DCM) (10 mL) at 0°C was added solid EDC hydrochloride (737 mg, 3.85 mmol). The solution was stirred for 15 minutes at 0°C and a solution of **4** (461 mg, 2.47 mmol) in DCM (5 mL) was added. The reaction was allowed to stir for 12 h at room temperature. Water was added and the mixture extracted three times with DCM. The collected organic phases were dried over Na_2SO_4, filtered and the concentrated. The crude was purified by flash chromatography (eluent CH_2Cl_2/MeOH from 100:0 to 95:5) affording 701 mg of **5** as a colorless liquid (yield 84 %)

Rf = 0,37 (CH_2Cl_2:MeOH 95:5); ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 6.26 (s, 2H), 6.48 (br s, 1H), 4.09 (d, *J* = 2.1 Hz, 2H), 3.73 (t, *J* = 6.9 Hz, 2H), 3.59-3.58 (m, 4H), 3.53-3.52 (m, 4H), 3.42-3.40 (m, 2H), 3.30-3.27 (m, 2H), 2.43-2.39 (m, 3H); ^{13}C-NMR (75.4 MHz, CD_3Cl): δ = 170.5, 169.8, 134.2, 79.5, 74.8, 70.3, 70.2, 70.0, 69.5, 69.0, 58.2, 39.1, 34.5, 34.2; ESI (*m/z)* 361.22 $[M+Na, (46)]^+$, 339.39 $[M+H, (100)]^+$; HRMS found 361.1367v$[M + Na]^+$, calcd 361.1370

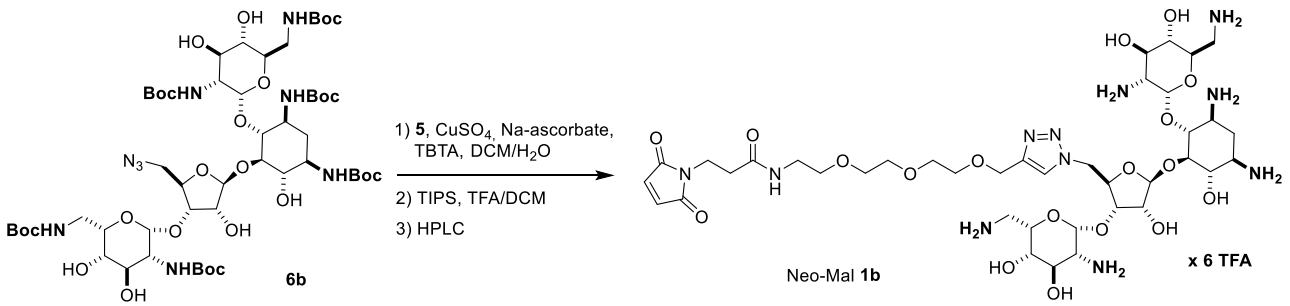
Synthesis of GNeo-Mal 1a



Compounds **6a** (50 mg, 23 μmol) and **5** (10 mg, 27 μmol) were dissolved in DCM (1 mL) and TBTA (0.5 mg, 1.0 μmol) was added. A solution of $CuSO_4$•5 H_2O (0.3 mg, 1.0 μmol) and sodium ascorbate (0.65 mg, 2.9 μmol) in water (500 μL) was added at room temperature and the mixture was vigorously stirred for 18 hours. The mixture was diluted with DCM (10 mL) and water (5 mL) and washed with aqueous EDTA (0.1 M), aqueous KCN (5%) and brine. The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated. The crude was dissolved in DCM (1 mL) and triisopropylsilane (70 μL) was added followed by trifluoracetic acid (1 mL). The solution was stirred at room temperature for 12 hours. The volatiles were evaporated under vacuum and co-evaporated three times with toluene. The residue was purified on reverse phase HPLC to obtain, after lyophilization, 28 mg GNeo-Mal **1a** as a fluffy white solid (overall yield 64%).

^{1}H-NMR (500 MHz, D_2O): δ = 7.88 (s, 1H, *H*-triazole), 6.72 (s, 2H, *H*-maleimide), 5.59 (s, 1H, *H1''*), 4.96 (s, 1H, *H1'''*), 4.94 (s, 1H, *H1'*), 4.56-4.54 (m, 3H), 4.32 (m, 1H), 4.23-4.22 (m, 2H), 4.04 (br s, 1H), 4.01 (br s, 1H), 3.64-3.17 (m, 30H), 2.37-2.36 (m, 2H, -C*HH*CO-), 2.10-2.08 (m, 1H, -C*H*H-desoxystreptamine), 1.53-1.50 (m, 1H, -CH*H*-desoxystreptamine); ^{13}C-NMR (125.7 MHz, D_2O): δ = 173.5, 172.5, 162.9 (q, *J* = 35.3 Hz, CF_3*C*OOH), 157.7, 157.2, 157.15, 157.11, 156.9, 156.4, 134.3, 125.6, 116.6 (q, *J* = 290.4 Hz, *C*F_3COOH), 111.4, 98.0, 95.8, 85.2, 77.9, 77.2, 74.3, 72.9, 72.6, 71.8, 69.5, 69.4, 69.3, 69.2, 69.0, 68.5, 66.7, 63.0, 55.3, 53.3, 51.8, 50.4, 41.8, 41.6, 38.8, 34.6, 34.3, 31.9; ESI (*m/z)* 1230.54 [M+H, (100)]$^+$, 616.02 [M+2H, (60)]$^{2+}$; HRMS found 615.8049 [M+2H]$^{2+}$, calcd 615.8059.

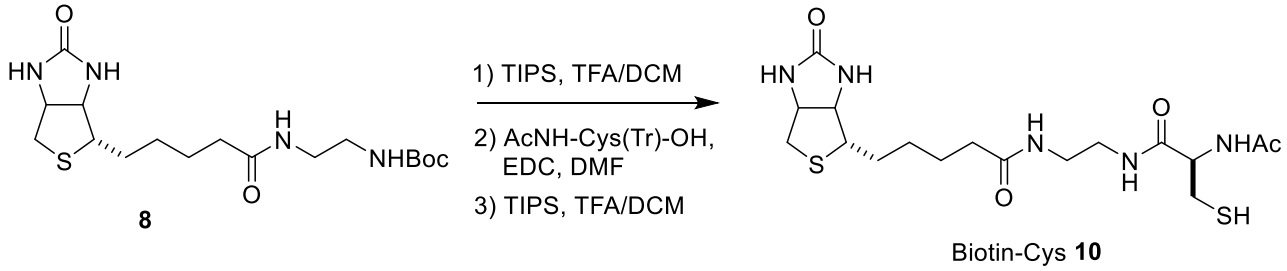
Synthesis of Neo-Mal 1b



The synthesis of Neo-Mal **1b**was performed following the procedure described for the synthesis of GNeo-Mal **1a**, starting from 50 mg (40 μmol) of Boc-Neo-N_3. Obtained 48 mg of Neo-Mal **1b** as a fluffy white solid (overall yield 73%).

^{1}H-NMR (500 MHz, D_2O): δ = 7.98 (s, 1H, *H*-triazole), 6.64 (s, 2H, *H*-maleimide), 5.85 (d, *J* = 3.5 Hz, 1H, *H1''*), 5.24 (s, 1H, *H1'''*), 5.12 (s, 1H, *H1'*), 5.64-5.58 (m, 1H), 51 (s, 2H), 4.38 (br s, 1H), 4.29-4.27 (m, 1H), 4.14 (br s, 1H), 4.04 (s, 1H), 3.91 (t, *J* = 9.5 Hz, 1H), 3.85 (t, *J* = 10.0 Hz, 1H), 3.78-3-77 (m, 2H), 3.63-3.62 (m, 2H), 3.57-3.10 (m, 21H), 2.29-2.28 (m, 3H, -C*HH*CO- and -C*H*H-desoxystreptamine), 1.72-1.70 (m, 1H, -CH*H*-desoxystreptamine); ^{13}C-NMR (125.7 MHz, D_2O): δ = 173.5, 172.4, 162.8 (q, *J* = 35.2 Hz, CF_3*C*OOH), 144.1, 134.3, 125.5, 115.7 (q, *J* = 291.6 Hz, *C*F_3COOH), 109.8, 95.4, 95.1, 84.7, 79.5, 72.2, 70.1, 70.0, 69.4, 69.3, 69.1, 69.0, 68.4, 62.9, 53.1, 51.5, 50.6, 40.4, 48.2, 40.3, 39.7, 38.7, 34.5, 34.3, 27.8; HRMS found 489.7399 $[M+2H]^{2+}$, calcd 489.7406.

Synthesis of biotin-Cys conjugate 10



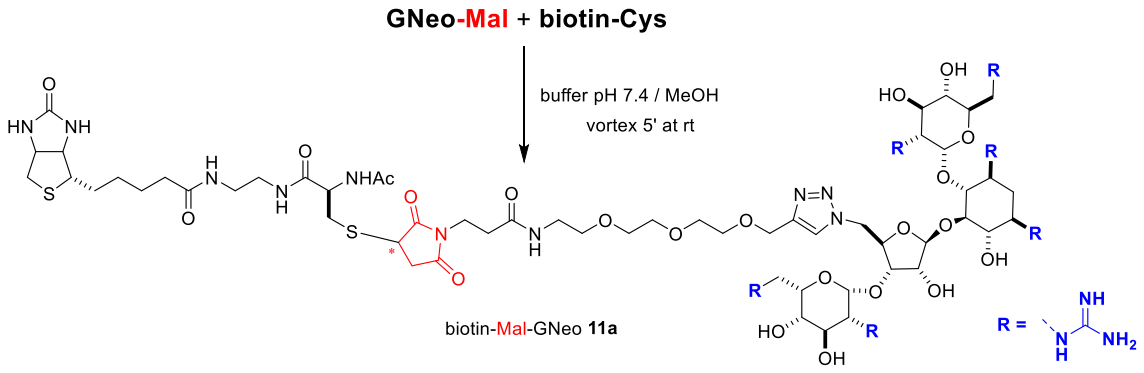
8 Biotin-Cys **10**

To a solution of **8** (100 mg, 0.26 mmol) in DCM (2 mL) was added triisopropylsilane (20 μL) followed by trifluoracetic acid (0.2 mL). The reaction was stirred at room temperature for 2 hours. The solution was diluted with DCM (10 mL) and washed with a saturated aqueous solution of $NaHCO_3$. The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated. The crude was dissolved in DMF (5 mL) and a solution of AcNH-Cys(Tr)-OH (126 mg, 0.31 mmol) and EDC hydrochloride (59 mg, 0.31 mmol) in DMF (5 mL) was added. The reaction was stirred at room temperature overnight. AcOEt (30 mL) was added and the solution was washed with water, saturated

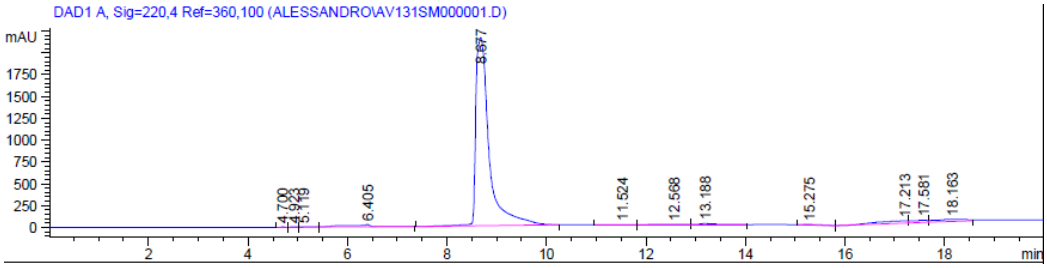
aqueous $NaHCO_3$, 1N aqueous HCl, and three times with brine. The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated. The crude was dissolved in DCM (5 mL) and triisopropylsilane (50 μL) was added followed by trifluoracetic acid (0.5 mL). The solution was stirred for 2 hours at room temperature. The volatiles were evaporated under vacuum and co-evaporated three times with toluene. The residue was purified by flash chromatography (eluent CH_2Cl_2/MeOH from 100:0 to 90:10) to obtain 63 mg biotin-Cys **10** as a yellowish solid (overall yield 57%).

^{1}H-NMR (300 MHz, CD_3OD): δ = 7.92 (br s, 1H), 4.54-4.50 (m, 1H), 4.42 (t, *J* = 5.7 Hz, 1H), 4.33 (dd, *J* = 7.8 and 4.5 Hz, 1H), 3.31-3.23 (m, 5H), 2.95 (dd, *J* = 12.9 and 5.1 Hz, 1H), 2.93-2.77 (m, 2H), 2.72 (d, *J* = 12.9 Hz, 1H), 2.25-2.23 (m, 2H), 2.05 (s, 3H), 1.68-1.62 (m, 4H), 1.47-1.44 (m, 2H); ^{13}C-NMR (75.4 MHz, CD_3OD/CD_3Cl): δ = 175.1, 172.2, 171.4, 164.7, 61.8, 60.3, 56.0, 55.5, 39.8, 39.2, 38.6, 35.5, 28.2, 28.0, 25.5, 21.5; ESI (*m/z)* 470.09 $[M+K, (24)]^+$, 454.24 $[M+Na, (66)]^+$, 432.14 $[M+H, (100)]^+$; HRMS found 454.1551 $[M+Na]^+$, calcd 454.1553.

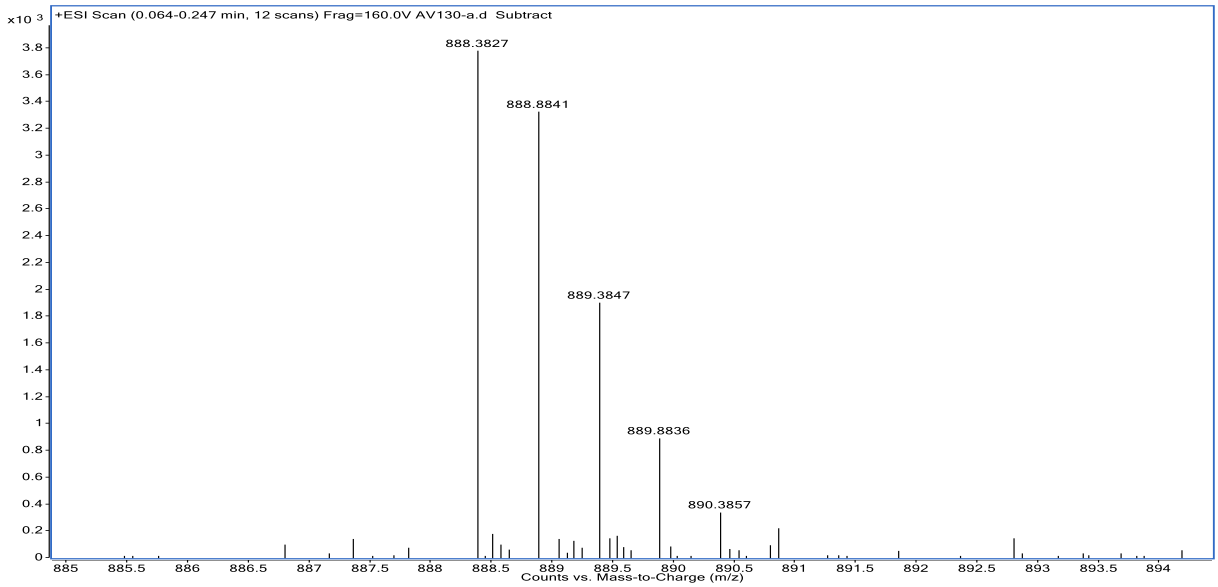
Scheme S1. Thio-Michael addition of biotin-Cys **10** to GNeo-Mal **1a**



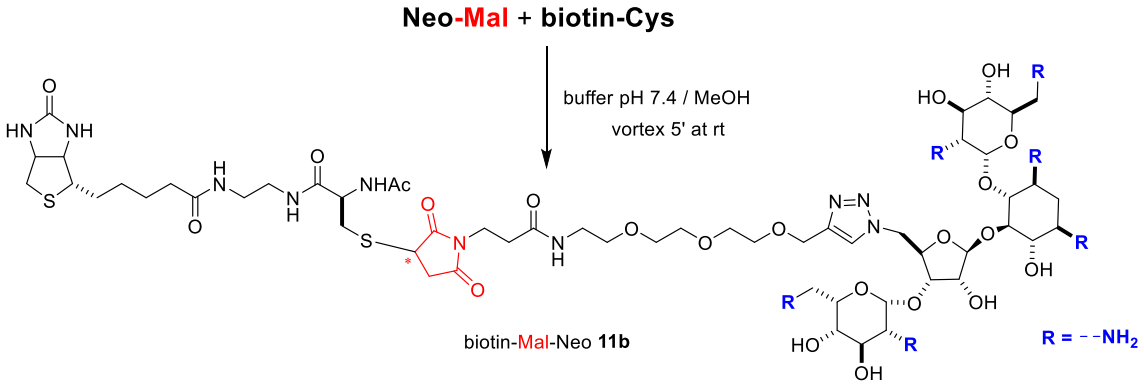
To a solution of GNeo-Mal **1a** (12 mg, 0.010 mmol, 1 eq) in a buffered solution at pH 7.4 (0.5 mL) a solution of biotin-Cys **10** (5 mg, 0.012 mmol, 1.2 eq) in HPLC grade MeOH (0.25 mL) was added. The solution was vortexed at room temperature for 5 minutes and the mixture was lyophilized. The crude conjugate was purified by preparative Reverse Phase High Performance Liquid Chromatography (RP-HPLC) using a Phenomenex Jupiter C18 preparative column (5 µm. 300 Å, 21mm × 250 mm D × L), gradient 0% B to 25% B in 40 min (eluent A Water+0.1%TFA, Eluent B ACN+0.1% TFA). Purity was evaluated by LC-MS using a 0% B to 25% B in 20 min gradient (eluent A Water+0.1%TFA, Eluent B ACN+0.1% TFA).



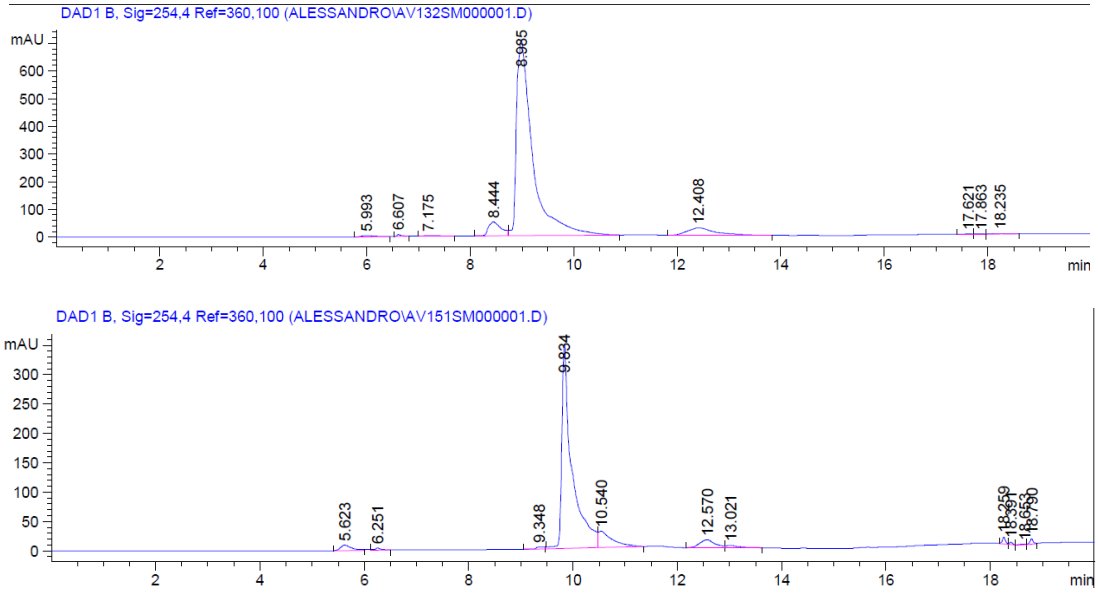
Mass Measured	Theo. Mass	Delta (ppm)	Composition
888.3827	888.3854	-3.0	$[C_{64}H_{111}N_{28}O_{24}S_2F_3]^{2+}$



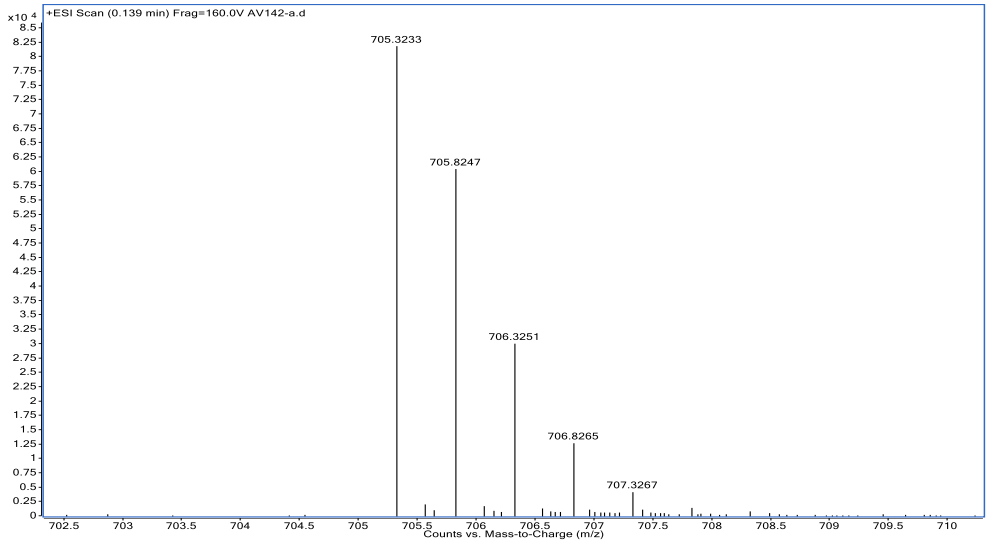
Scheme S2. Thio-Michael addition of biotin-Cys **10** to Neo-Mal **1b**



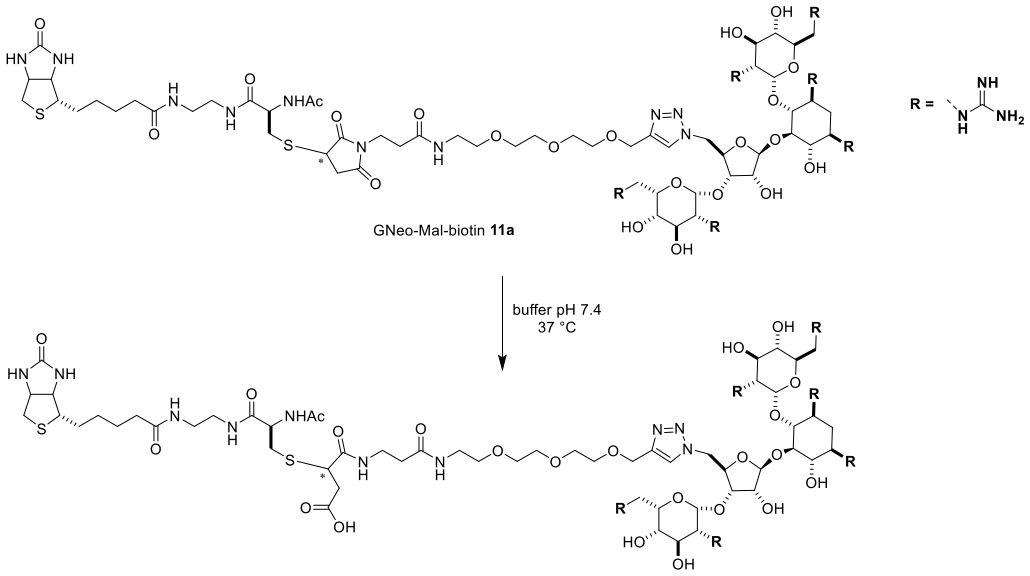
To a solution of GNeo-Mal **1b** (12 mg, 0.010 mmol, 1 eq) in a buffered solution at pH 7.4 (0.5 mL) a solution of biotin-Cys **10** (5 mg, 0.012 mmol, 1.2 eq) in HPLC grade MeOH (0.25 mL) was added. The solution was vortexed at room temperature for 5 minutes and the mixture was lyophilized. The crude conjugate was purified by preparative Reverse Phase High Performance Liquid Chromatography (RP-HPLC) using a Phenomenex Jupiter C18 preparative column (5 µm. 300 Å, 21mm × 250 mm D × L), gradient 0% B to 25% B in 40 min (eluent A Water+0.1%TFA, Eluent B ACN+0.1% TFA). Purity was evaluated by LC-MS using a 0% B to 25% B in 20 min gradient (eluent A Water+0.1%TFA, Eluent B ACN+0.1% TFA).

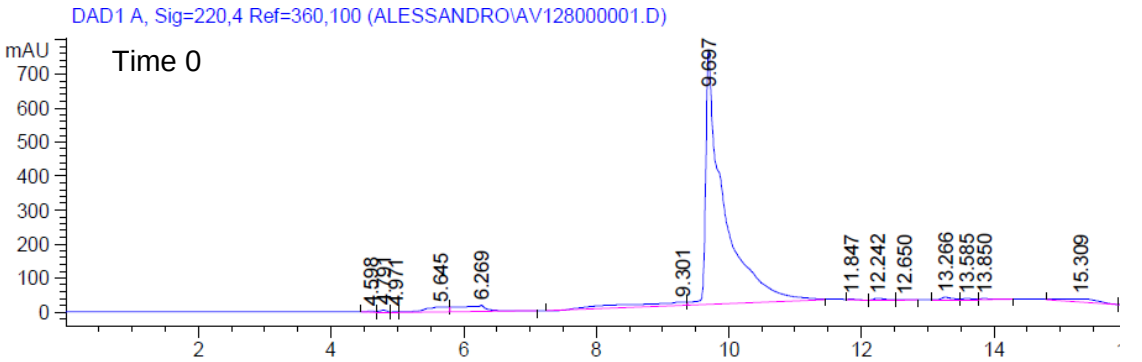


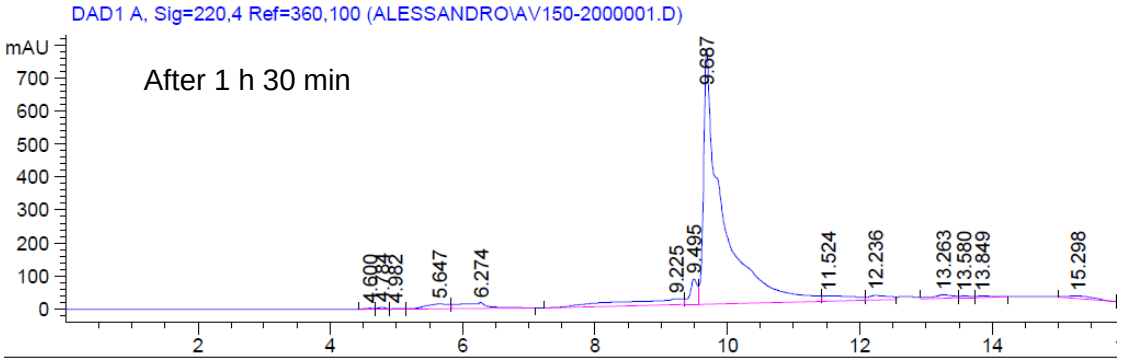
Mass Measured	Theo. Mass	Delta (ppm)	Composition
705.3233	705.3236	-0.4	$[C_{56}H_{98}N_{16}O_{22}S_2]^{2+}$

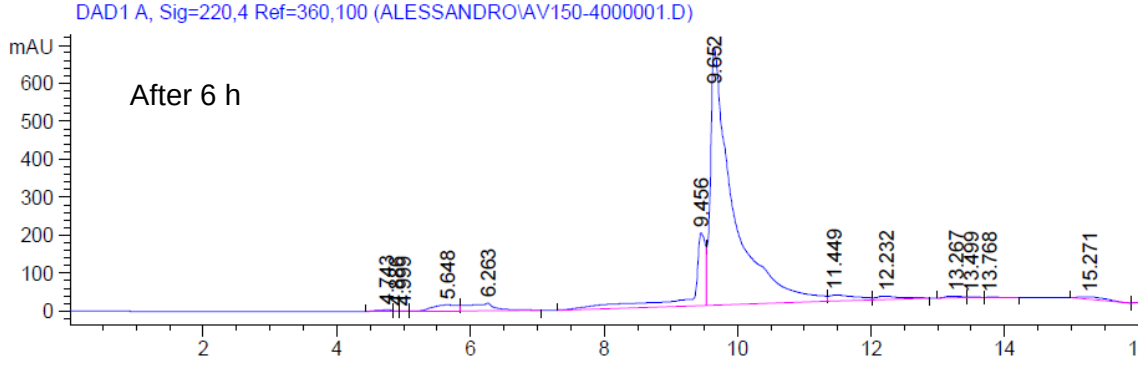


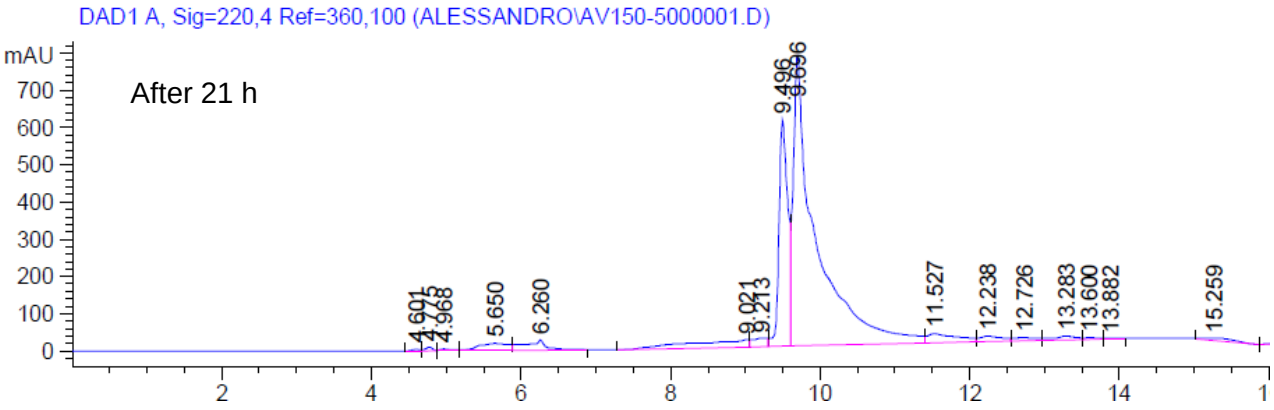
Scheme S3. Stability of GNeo-Mal-biotin conjugate **11a** in buffer solution at pH 7.4 and 37 °C

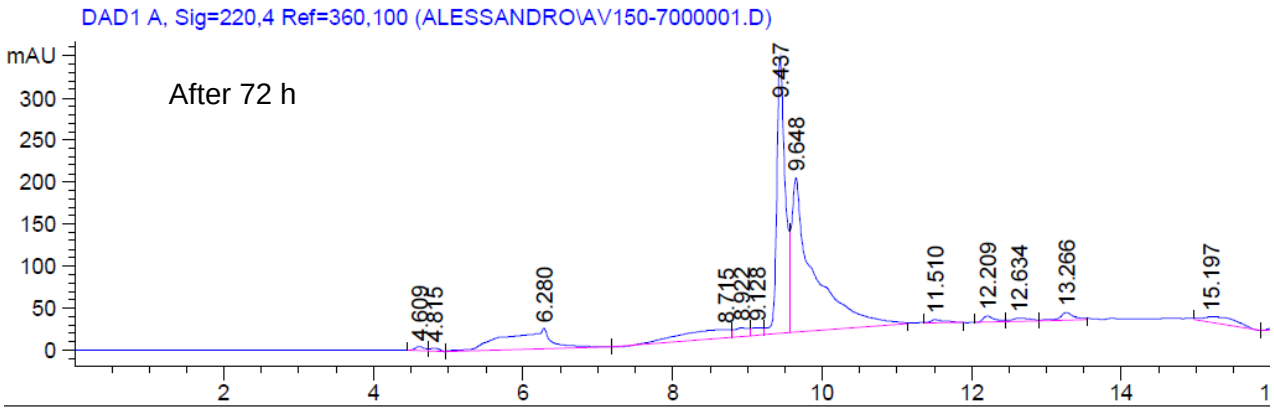


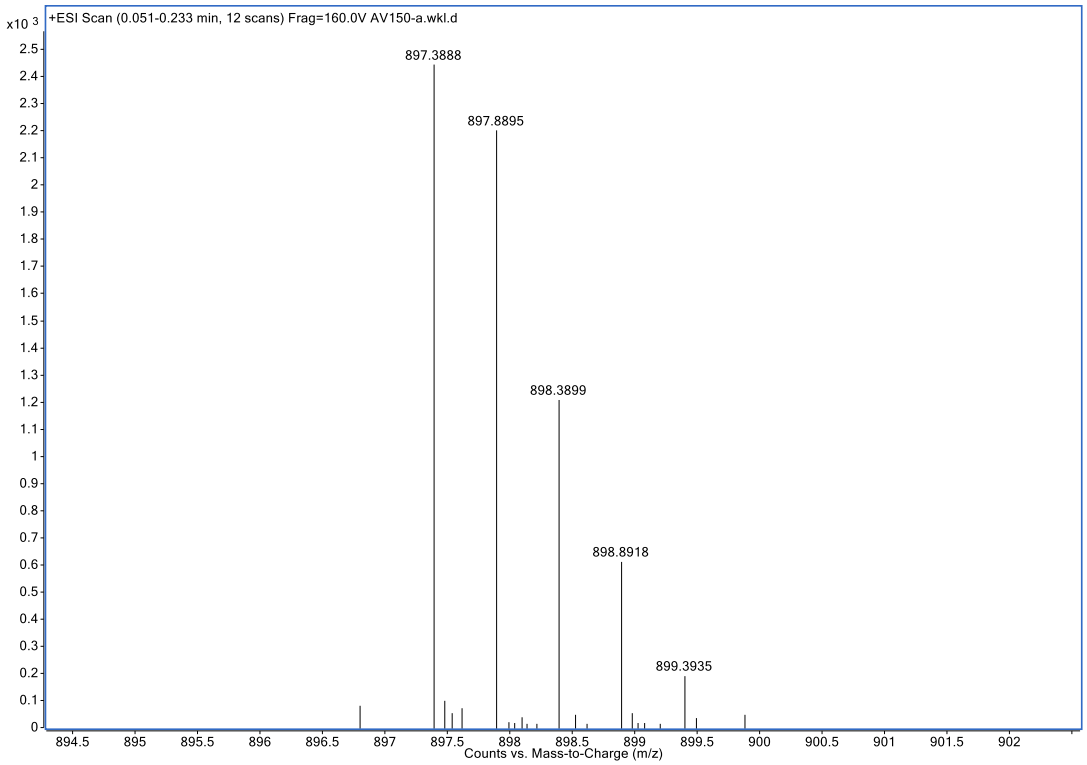






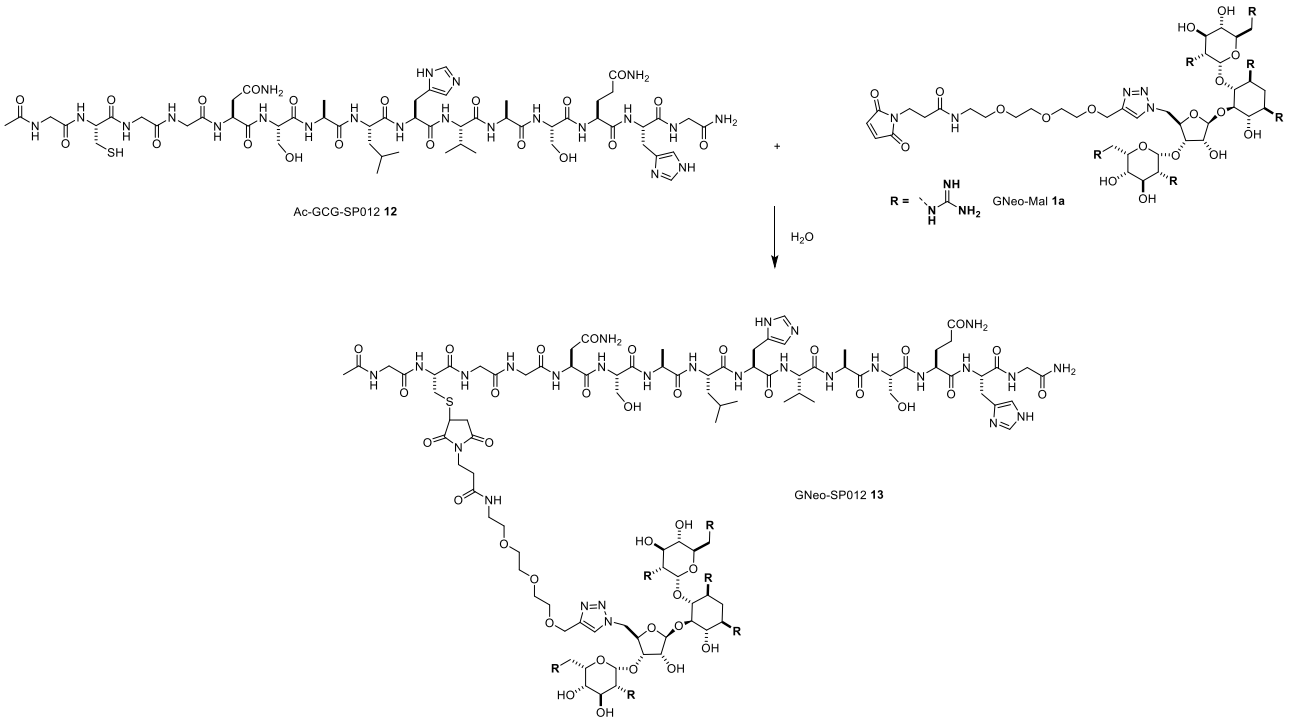






Mass Measured	Theo. Mass	Delta (ppm)	Composition
897.3888	897.3907	-2.1	$[C_{62}H_{113}N_{28}O_{23}S_2\ C_2F_3O_2H]^{2+}$

Scheme S4. Synthesis of GNeo-SP012 peptide conjugate 13

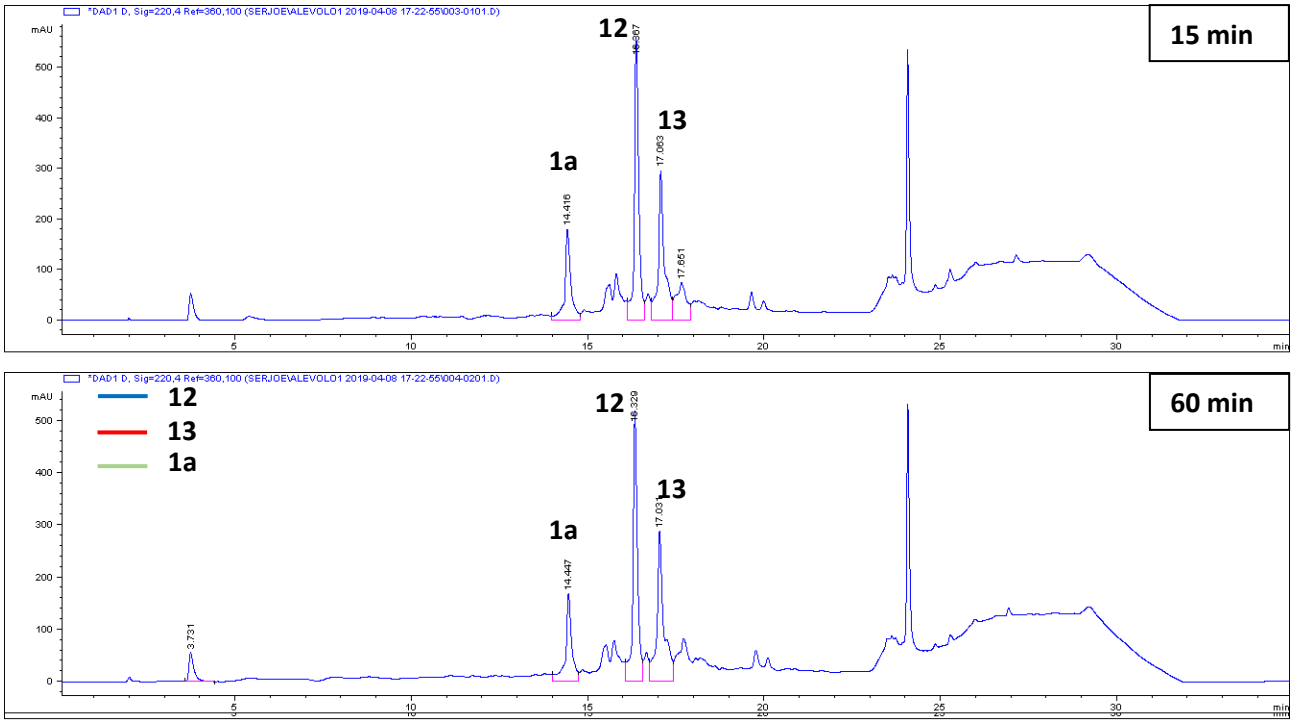


Peptide **12** was prepared through 9-Fluorenylmethoxycarbonyl (Fmoc) solid phase microwave assisted peptide synthesis (SPPS) using a Liberty Blue™ Automated Microwave Peptide Synthesizer (CEM) using the standard protocol of the instrument. The Fmoc-protected amino acids were coupled in sequence using a 1 M solution of N,N′-diisopropylcarbodiimide (DIC) in dimethylformamide (DMF) as coupling agent and a 1 M solution of ethyl cyano(hydroxyimino)acetate (Oxyma pure) in dimethylformamide as additive. Fmoc deprotection was performed by a 20% piperidine solution in DMF. Final acetylation was performed using a solution of 20% acetic anhydride and 5 % N,N-diisopropylethylamine in DMF.

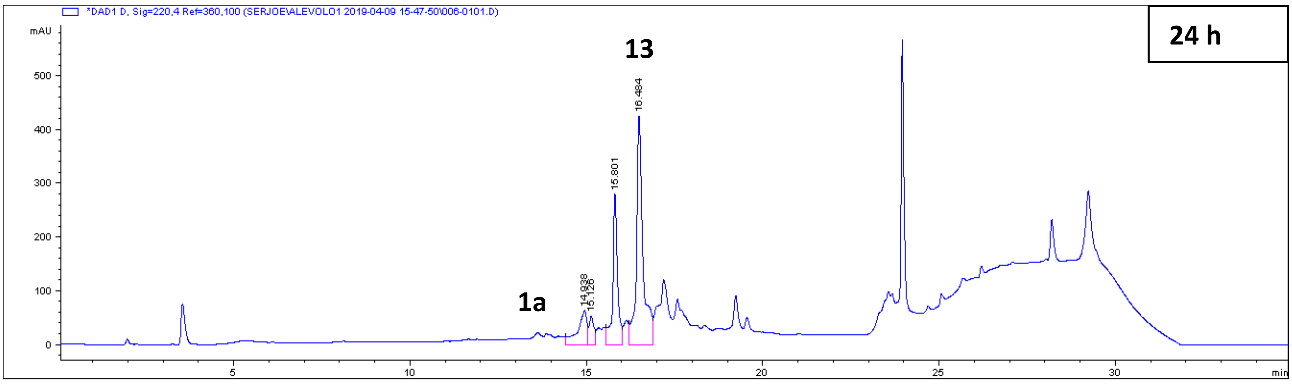
Cleavage of the peptide **12** from the resin and removal of side protecting groups was performed by treating the resin with a cleavage solution of 92.5% trifluoroacetic acid (TFA), 2.5 % 1,2-Ethanedithiol, 2.5% triisopropylsilane (TIS) and 2.5% of water for 3 h at room temperature. TFA was removed in a stream of nitrogen and crude peptide was obtained by precipitation with 12 mL of cold diethyl ether and centrifugation at 1000 g for 5 min (3 times). The supernatant diethyl ether was decanted from the centrifuge tube and the crude peptide was dried by a stream of nitrogen and under

vacuum. Crude peptide **12** was submitted to conjugation with GNeo-Mal **1a** without further purification.

To a solution of **12** (15 mg, 0.01 mmol, 1 eq) in HPLC grade water (0.5 mL) a solution of GNeo-Mal **1a** (20 mg, 0.012 mmol, 1.2 eq) in HPLC grade water (0.25 mL) was added. The solution was stirred at room temperature for 24 hours and the mixture was lyophilized. The crude conjugate was purified by preparative Reverse Phase High Performance Liquid Chromatography (RP-HPLC) using a Phenomenex Jupiter C18 preparative column (5 µm. 300 Å, 21mm × 250 mm D × L), gradient 0% B to 25% B in 40 min (eluent A Water+0.1%TFA, Eluent B ACN+0.1% TFA). Purity was evaluated by LC-MS using a 0% B to 25% B in 20 min gradient (eluent A Water+0.1%TFA, Eluent B ACN+0.1% TFA).



12



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24 h
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1a
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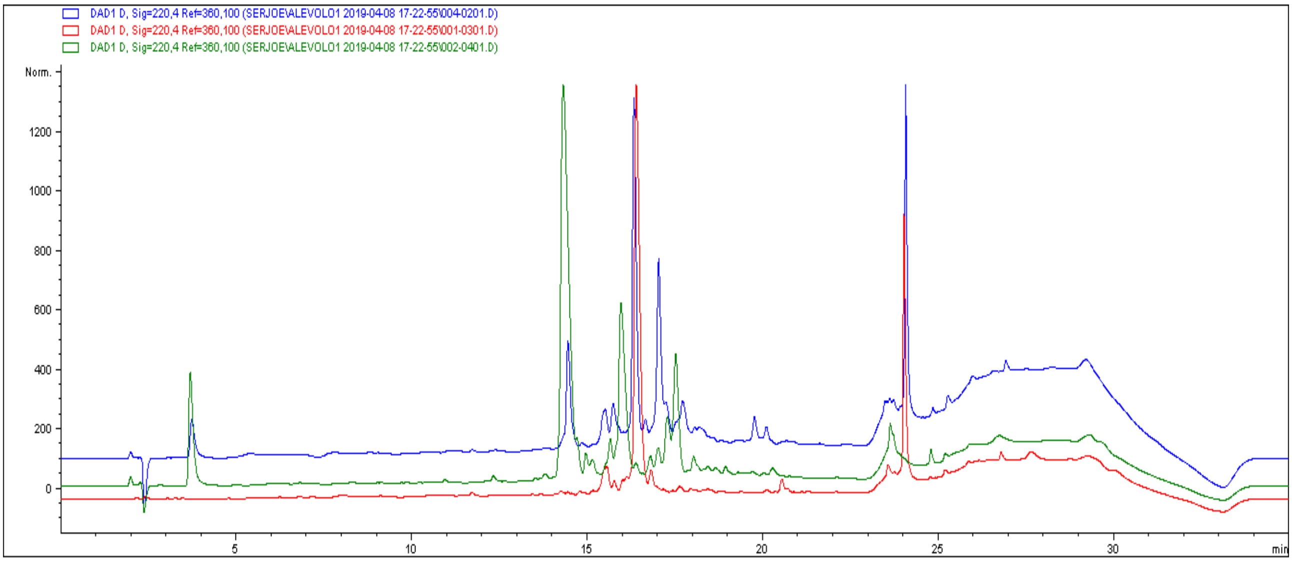
15.801
16.484
mAU
500
400
300
200
100
0
5
10
15
20
25
30
min



DAD1 D, Sig=220,4 Ref=360,100 (SERJOE\ALEVOLO1 2019-04-08 17-22-55\004-0201.D)
DAD1 D, Sig=220,4 Ref=360,100 (SERJOE\ALEVOLO1 2019-04-08 17-22-55\001-0301.D)
DAD1 D, Sig=220,4 Ref=360,100 (SERJOE\ALEVOLO1 2019-04-08 17-22-55\002-0401.D)
Norm.
1200
1000
800
600
400
200
0
5
10
15
20
25
30
min

Figure S1. FACS histograms following cells incubation with (G)Neo-Mal-biotin + SCy5 complexes

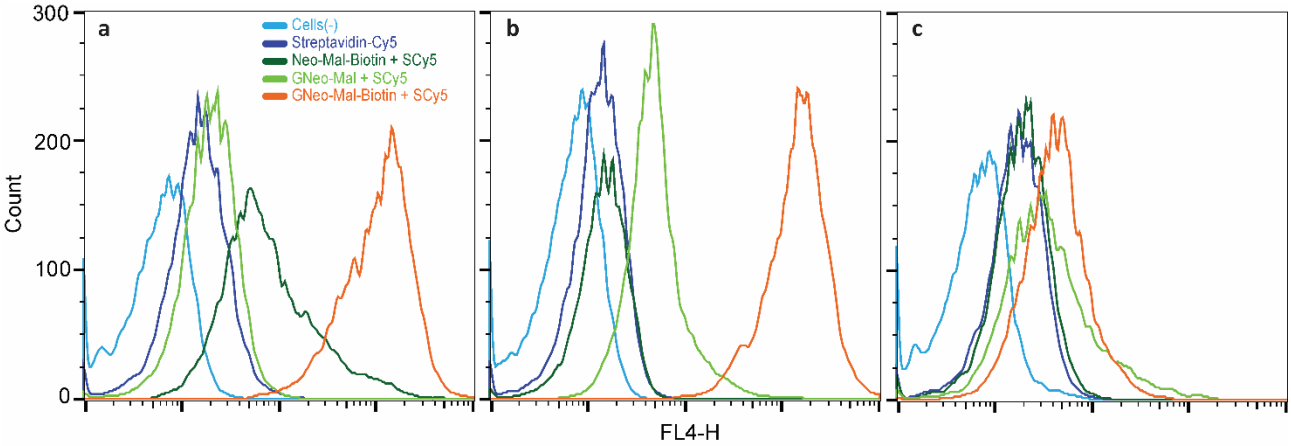
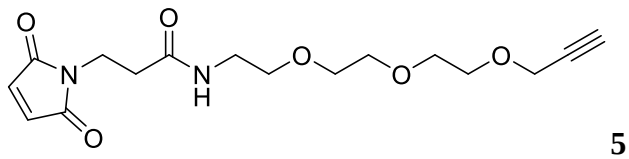
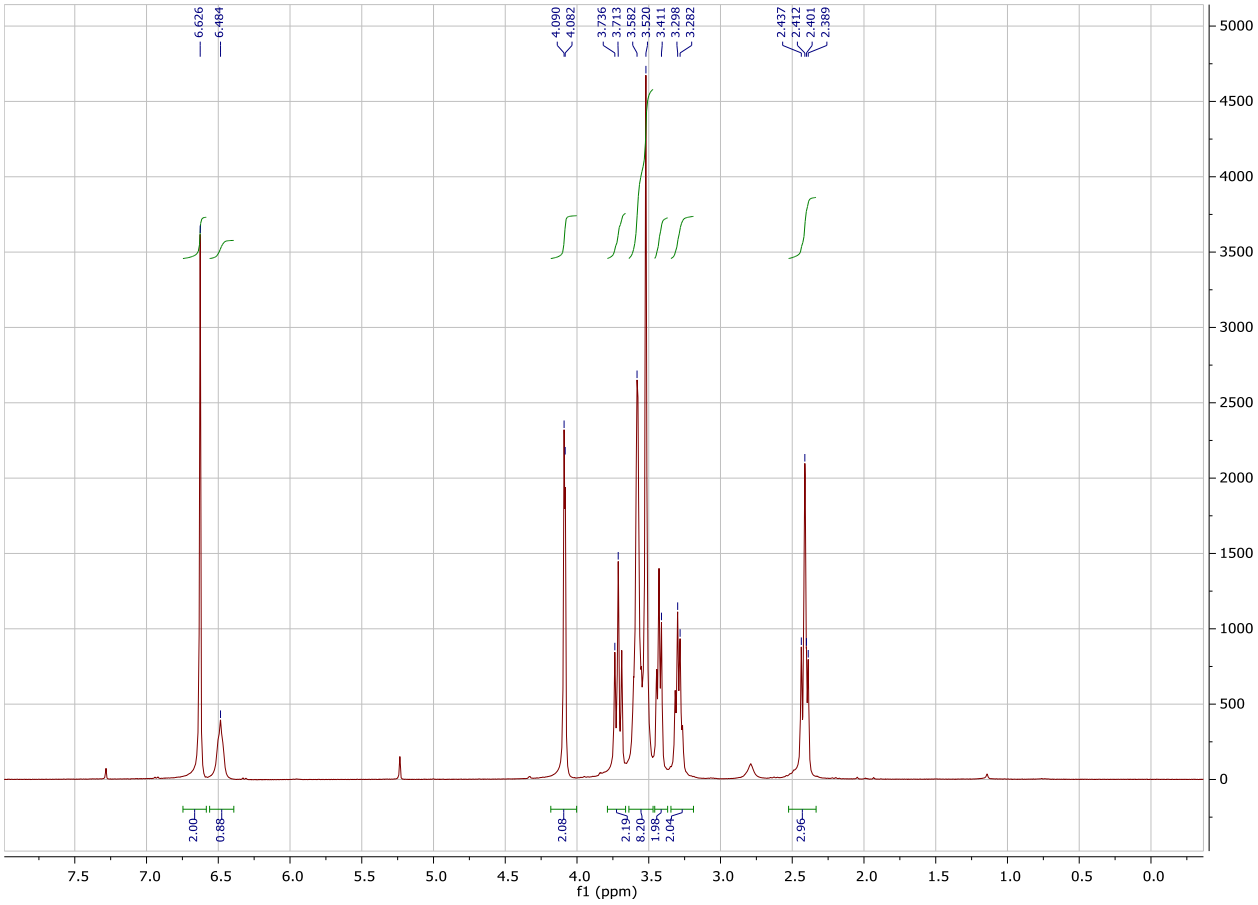
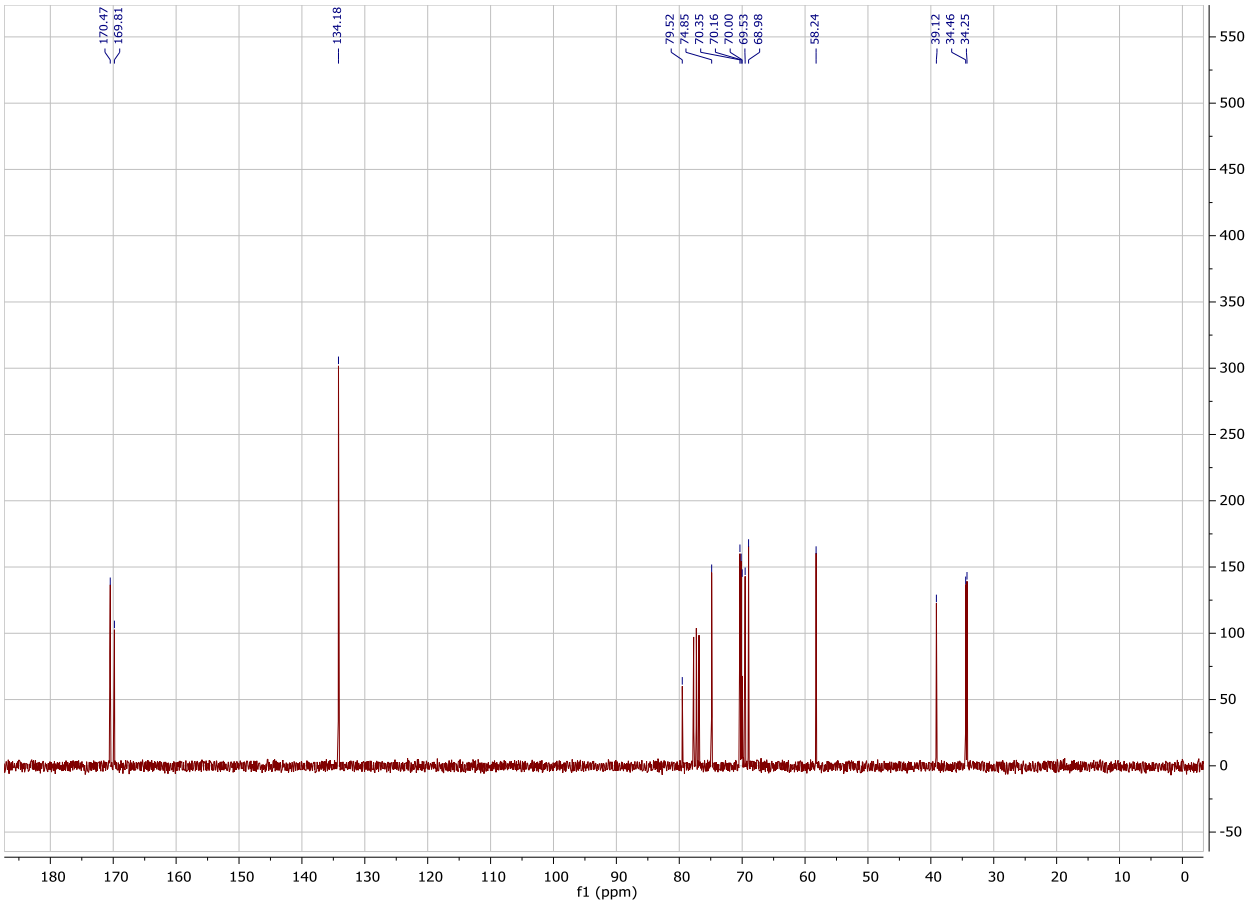


Figure S1. FACS histograms of maleimide-modified neomycin and guanidinoneoymcin. Wild-type CHOK1 cells were incubated with the streptavidin complexes in the presence (a) and absence (b) of FBS. c) pgsA745 cells incubated with the streptavidin complexes.

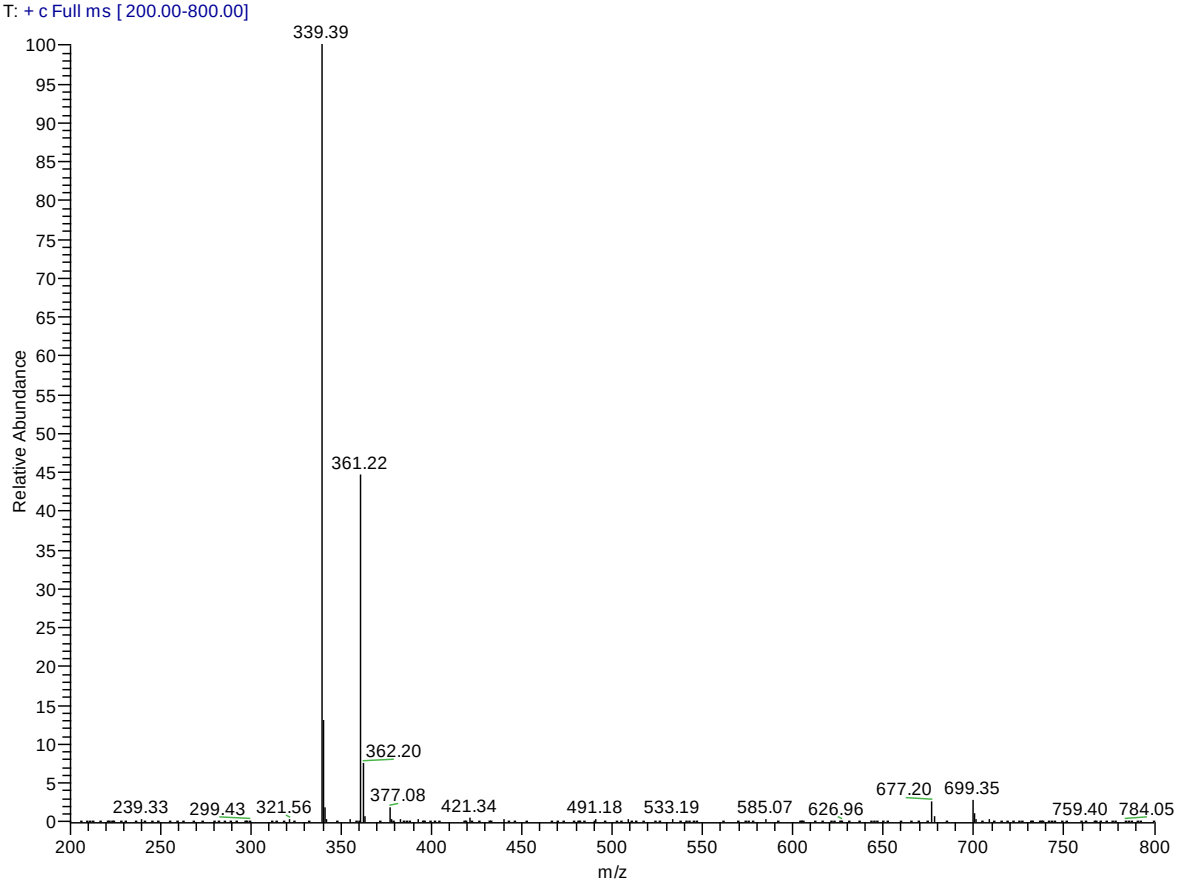


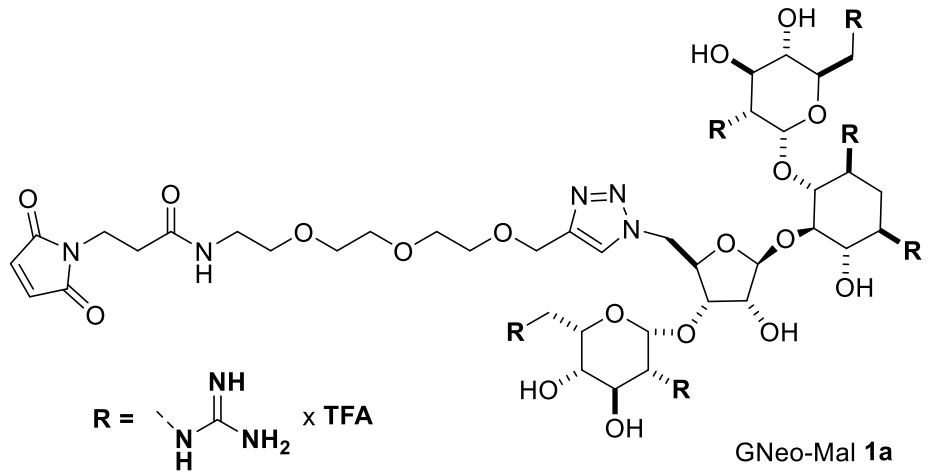
5


6.626
6.484
4.090
4.082
3.736
3.713
3.582
3.520
3.411
3.298
3.282
2.437
2.412
2.401
2.389
2.00
0.88
2.08
2.19
8.20
1.98
2.04
2.96
f1 (ppm)

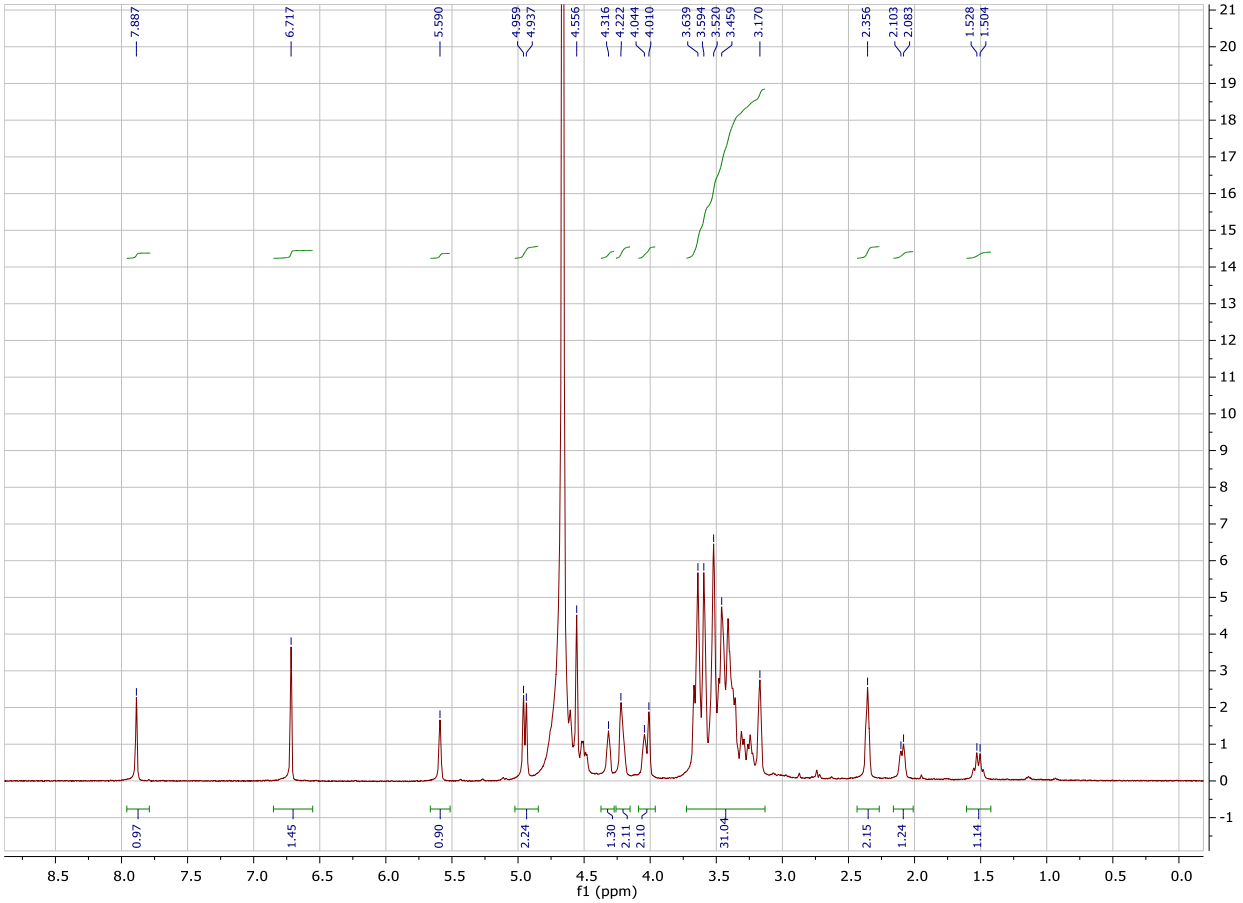


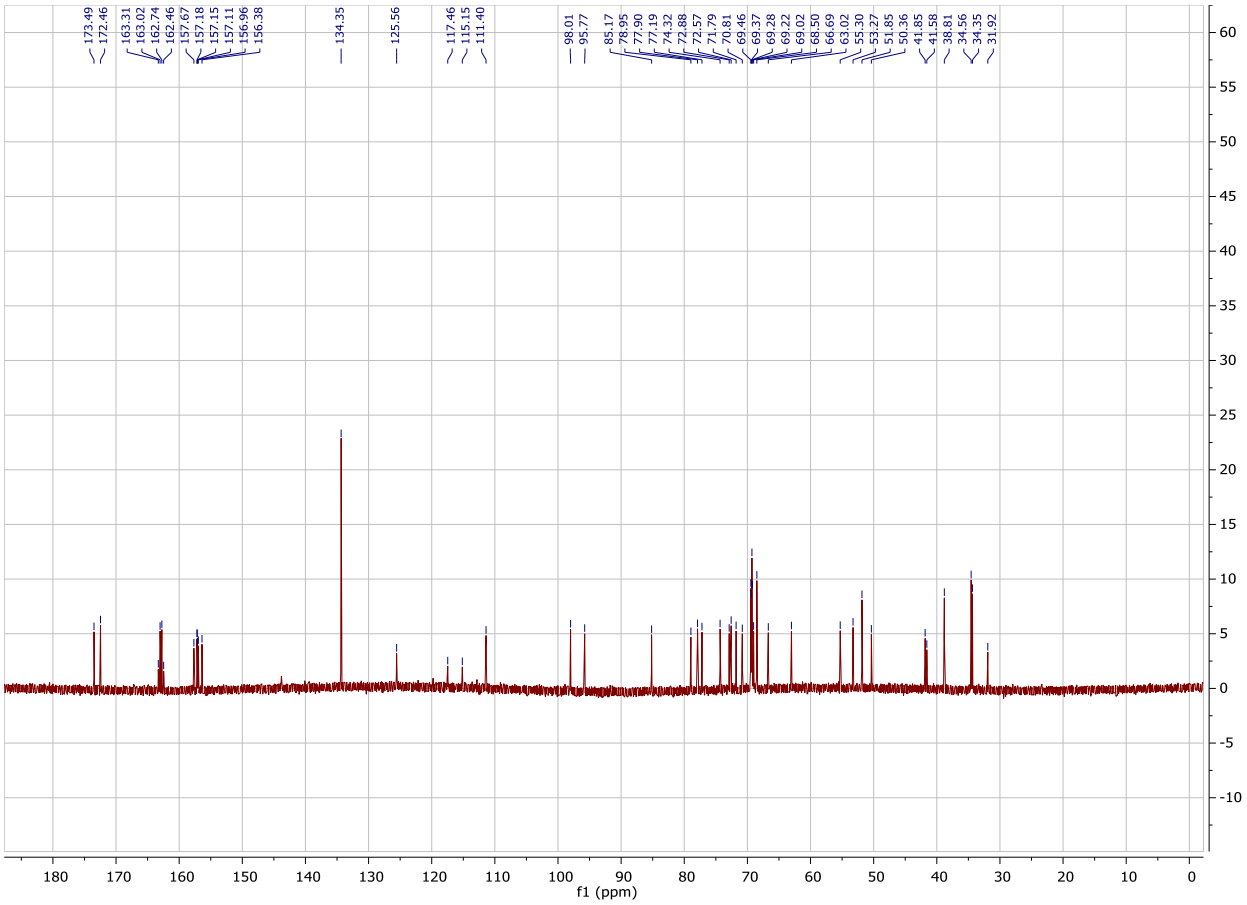
AV106-a #104-114 RT: 1.25-1.38 AV: 11 SB: 6 0.10-0.16 NL: 6.01E7
T: + c Full ms [200.00-800.00]

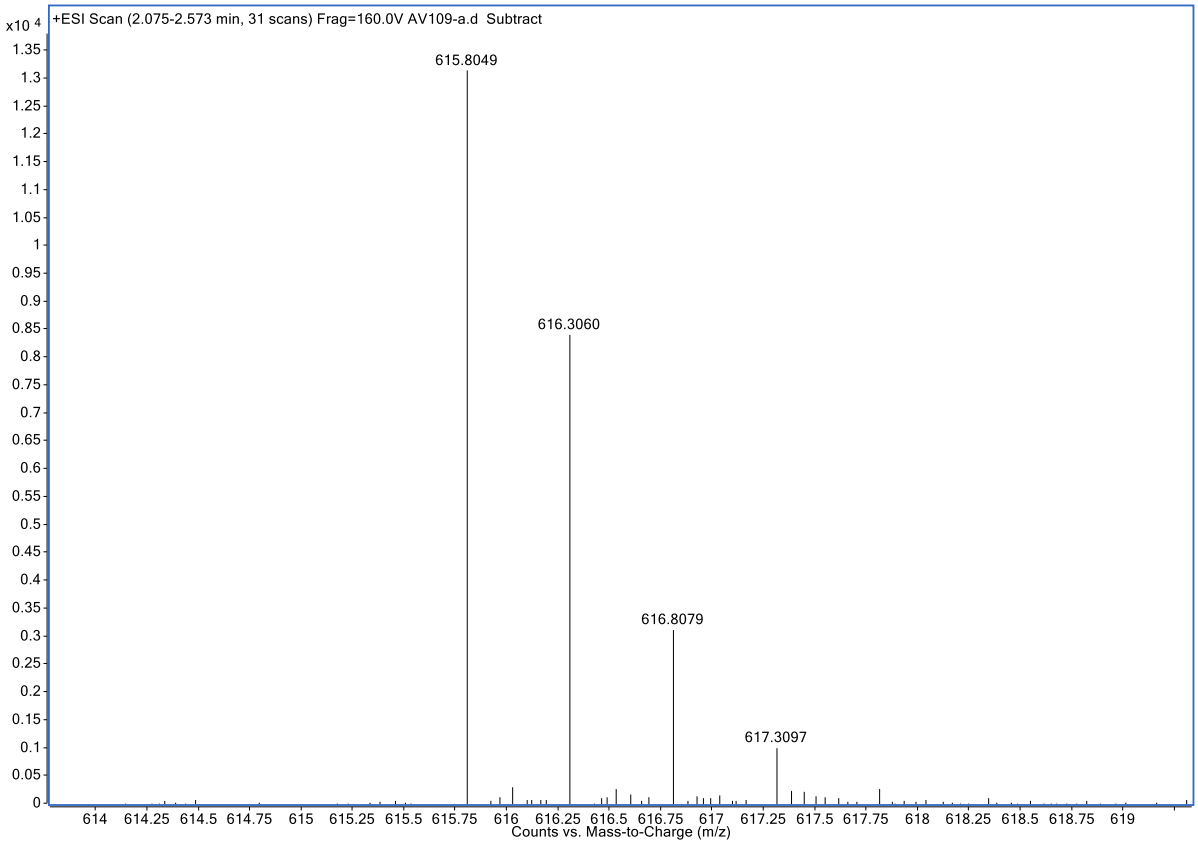


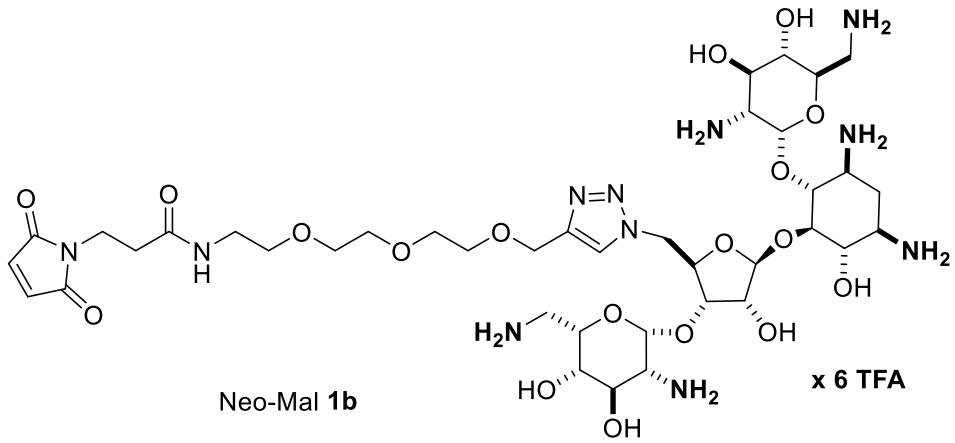

R = NH NH2 x TFA

GNeo-Mal **1a**

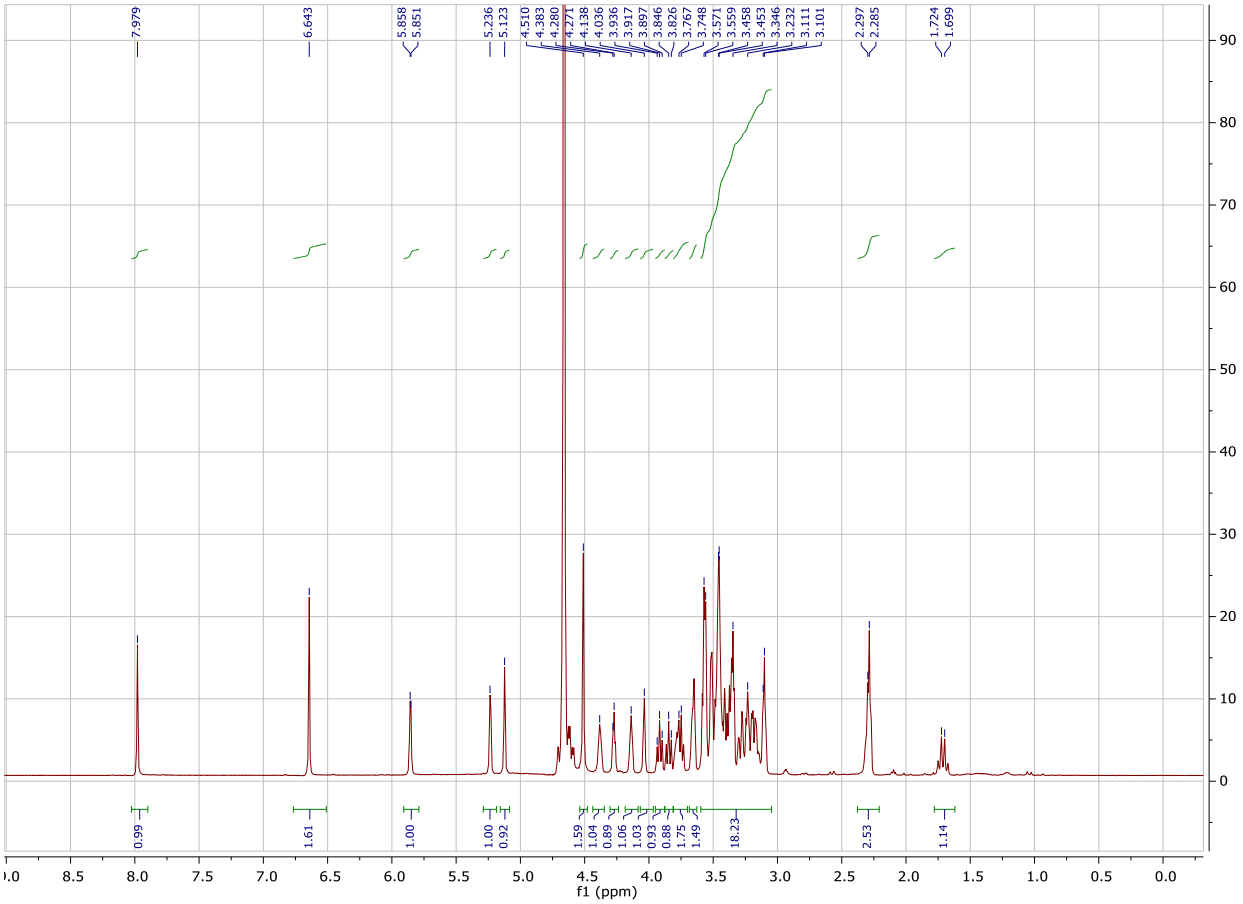

7.887
6.717
5.590
4.959
4.937
4.556
4.316
4.222
4.044
4.010
3.639
3.594
3.520
3.459
3.170
2.356
2.103
2.083
1.528
1.504
0.97
1.45
0.90
2.24
1.30
2.11
2.10
31.04
2.15
1.24
1.14
f1 (ppm)

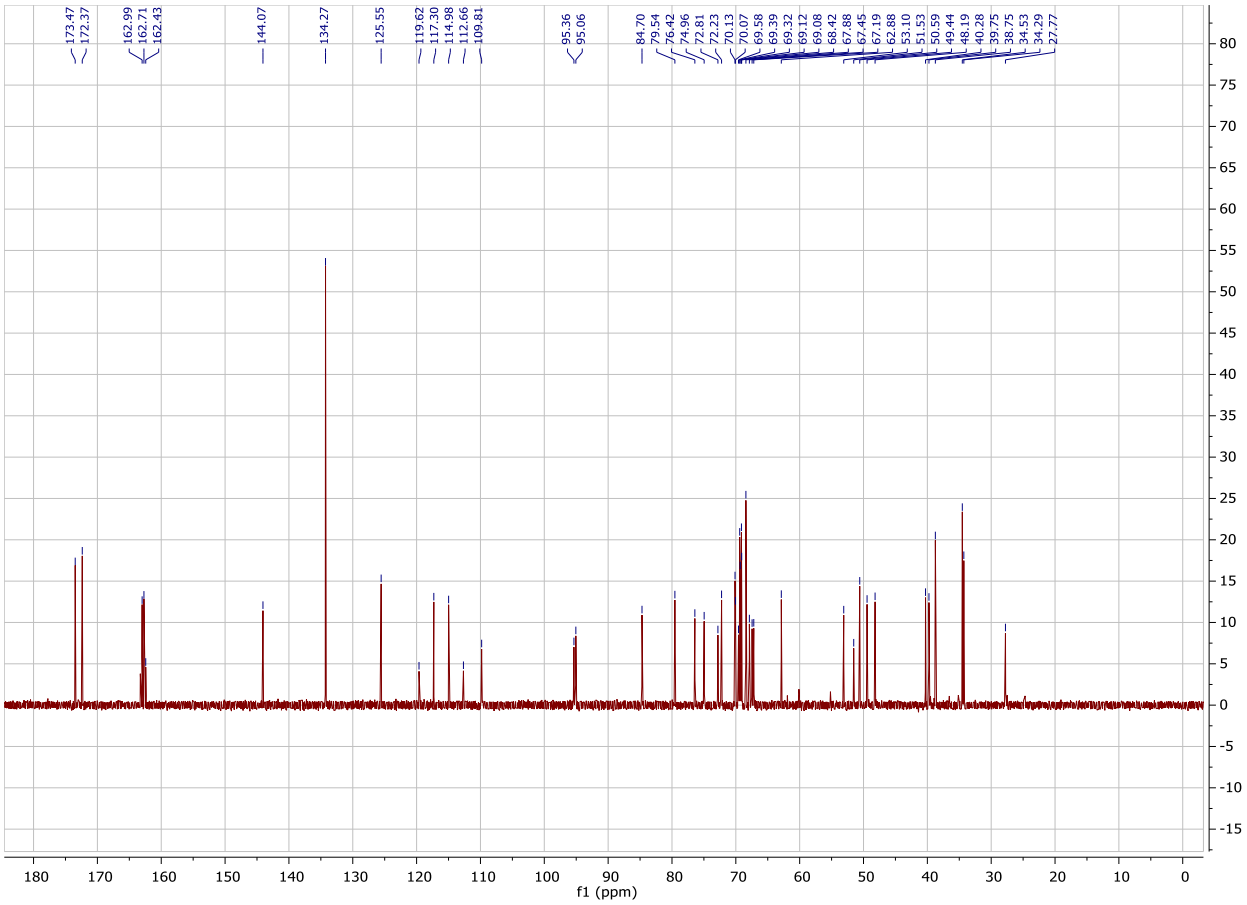

173.49
172.46
163.31
163.02
162.74
162.46
157.67
157.18
157.15
157.11
156.96
156.38
134.35
125.56
117.46
115.15
111.40
98.01
95.77
85.17
78.95
77.90
77.19
74.32
72.88
72.57
71.79
70.81
69.46
69.37
69.28
69.22
69.02
68.50
66.69
63.02
55.30
53.27
51.85
50.36
41.85
41.58
38.81
34.56
34.35
31.92
f1 (ppm)

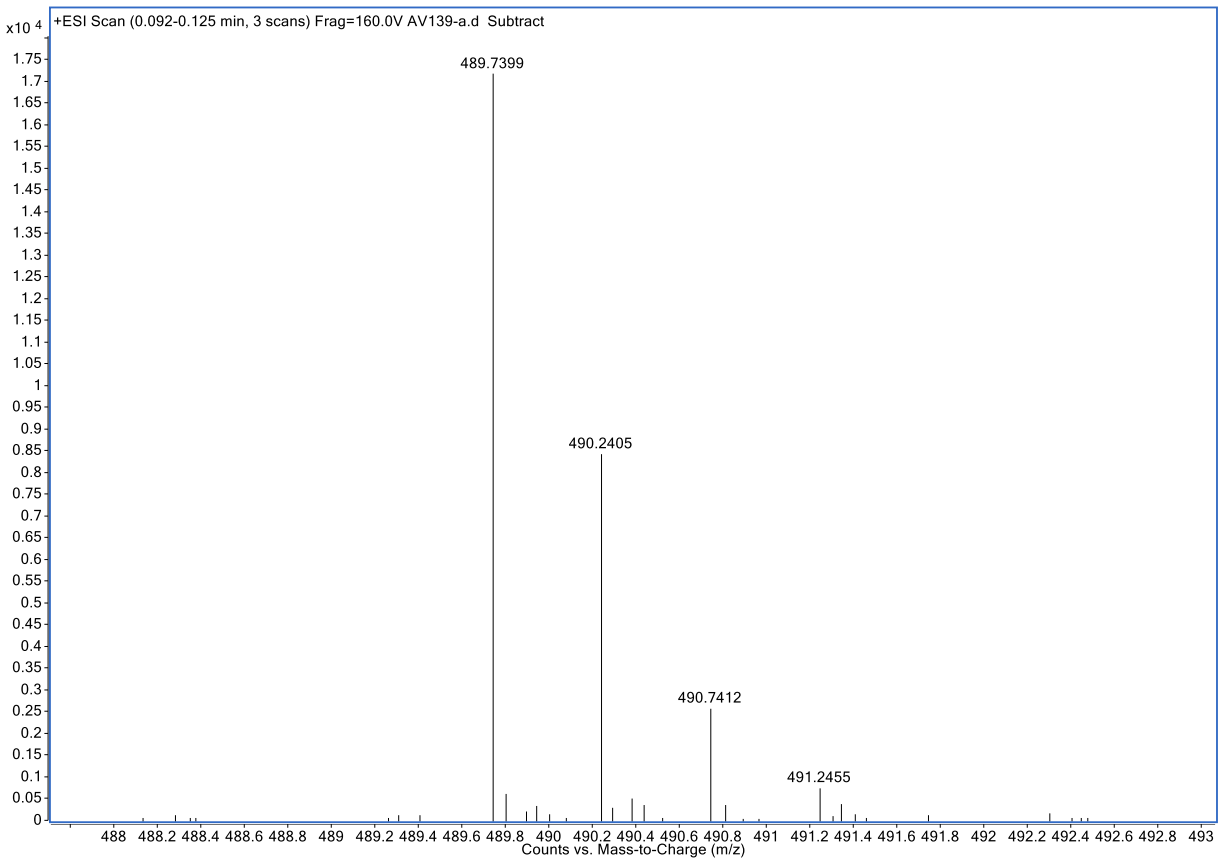

+ESI Scan (2.075-2.573 min, 31 scans) Frag=160.0V AV109-a.d Subtract
x10 4
615.8049
616.3060
616.8079
617.3097
Counts vs. Mass-to-Charge (m/z)

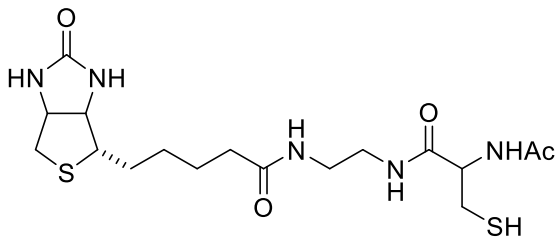


Neo-Mal **1b** **x 6 TFA**

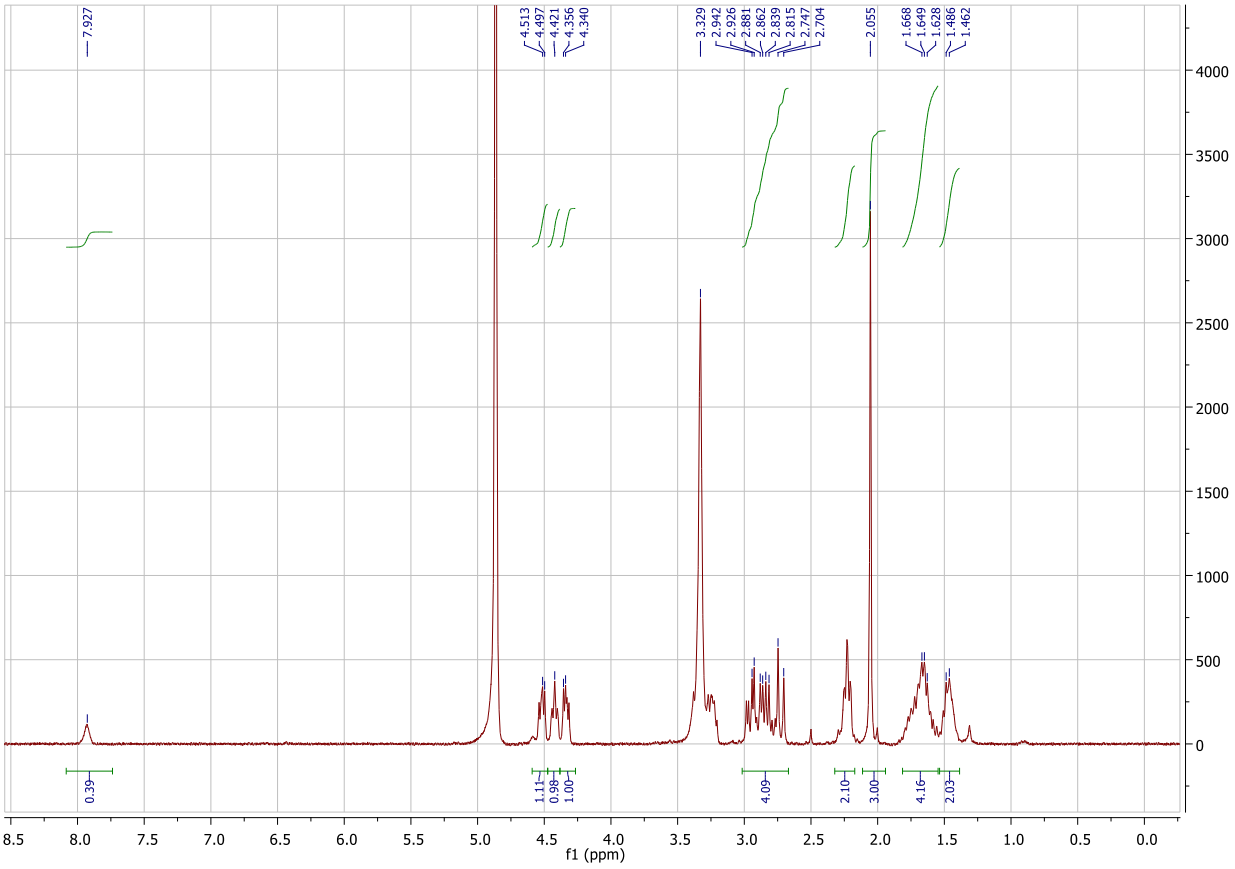


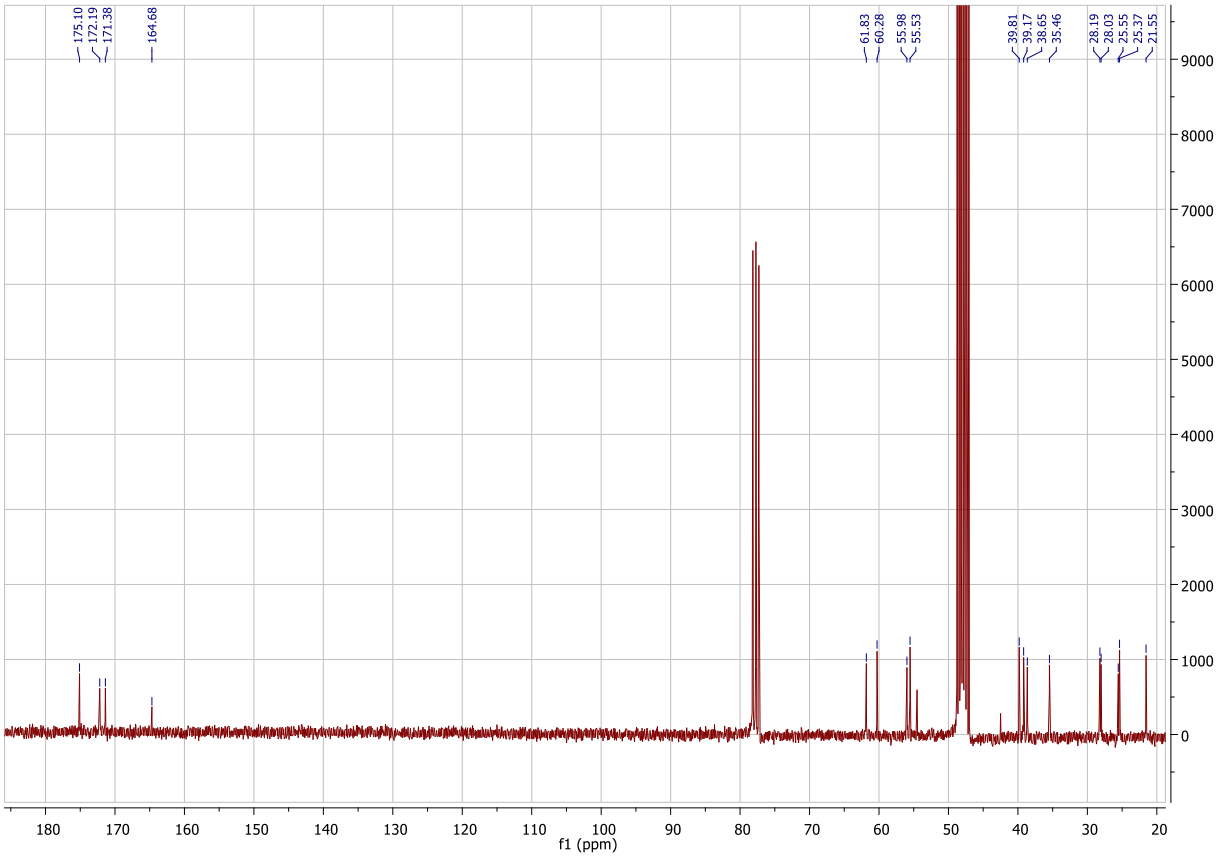

173.47
172.37
162.99
162.71
162.43
144.07
134.27
125.55
119.62
117.30
114.98
112.66
109.81
95.36
95.06
84.70
79.54
76.42
74.96
72.81
72.23
70.13
70.07
69.58
69.39
69.32
69.12
69.08
68.42
67.88
67.45
67.19
62.88
53.10
51.53
50.59
49.44
48.19
40.28
39.75
38.75
34.53
34.29
27.77
f1 (ppm)

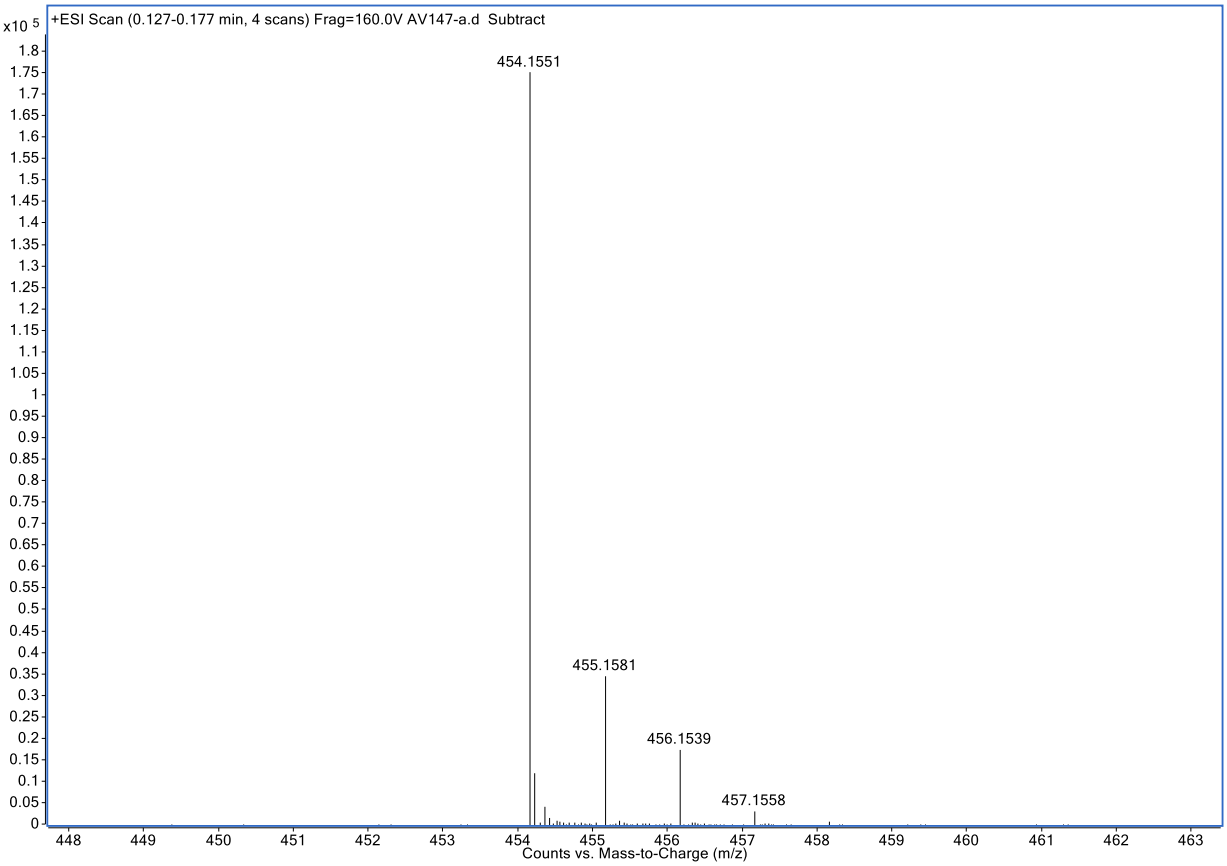

+ESI Scan (0.092-0.125 min, 3 scans) Frag=160.0V AV139-a.d Subtract
x10 4
489.7399
490.2405
490.7412
491.2455
Counts vs. Mass-to-Charge (m/z)

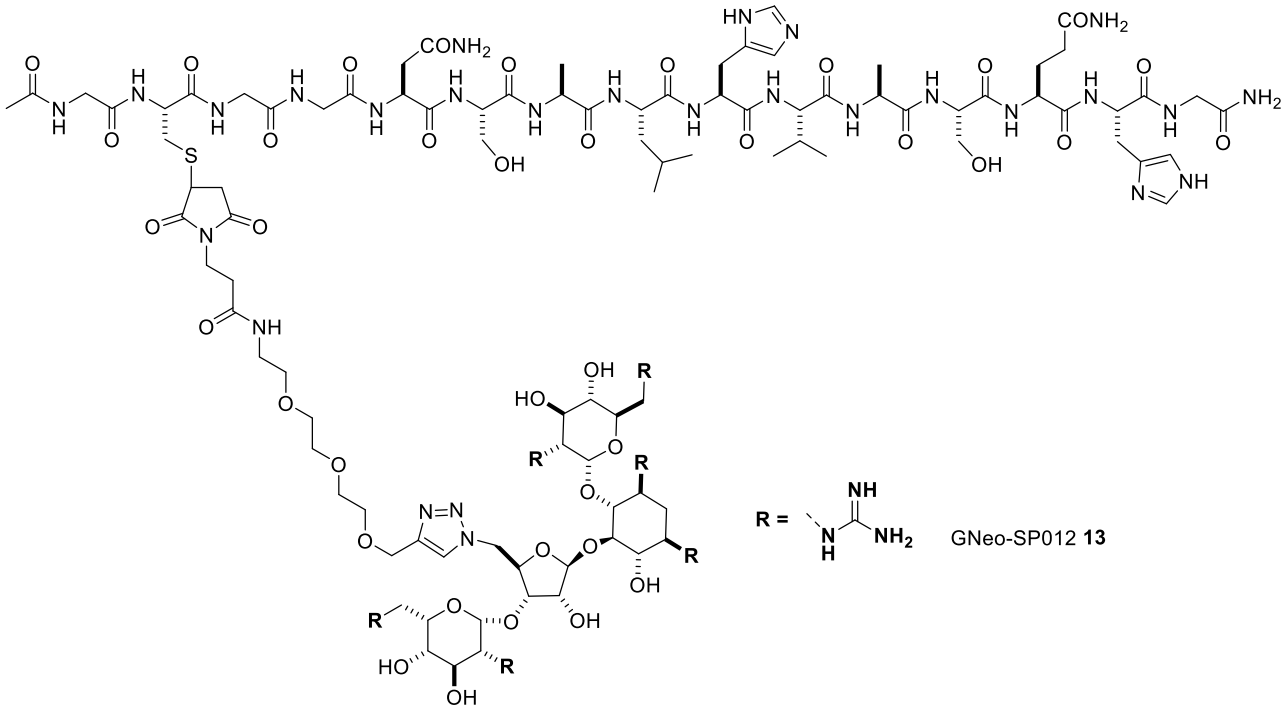

O
HN
NH
S
H
N
O
N
H
O
NHAc
SH

10

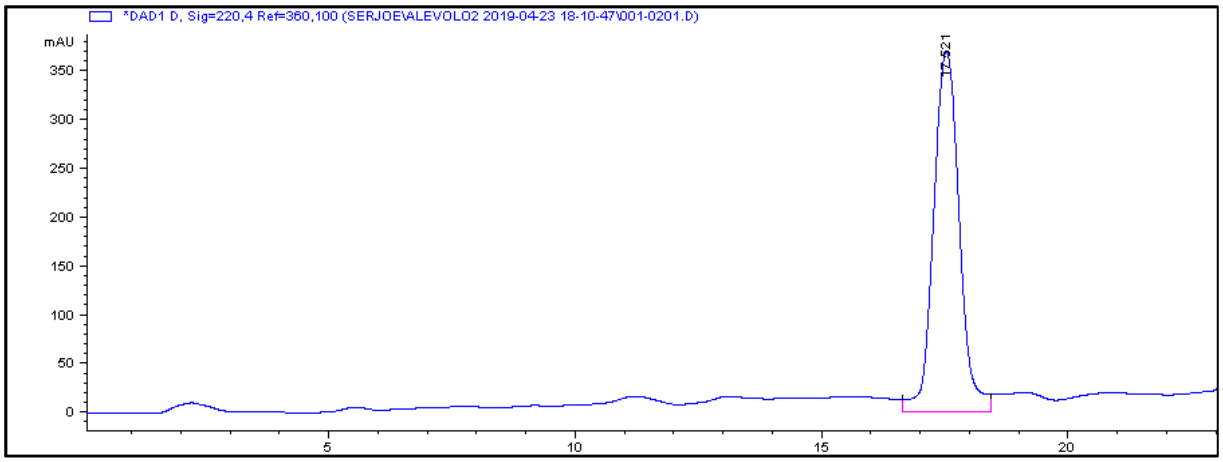

7.927
4.513
4.497
4.421
4.356
4.340
3.329
2.942
2.926
2.881
2.862
2.839
2.815
2.747
2.704
2.055
1.668
1.649
1.628
1.486
1.462
0.39
1.11
0.98
1.00
4.09
2.10
3.00
4.16
2.03
f1 (ppm)

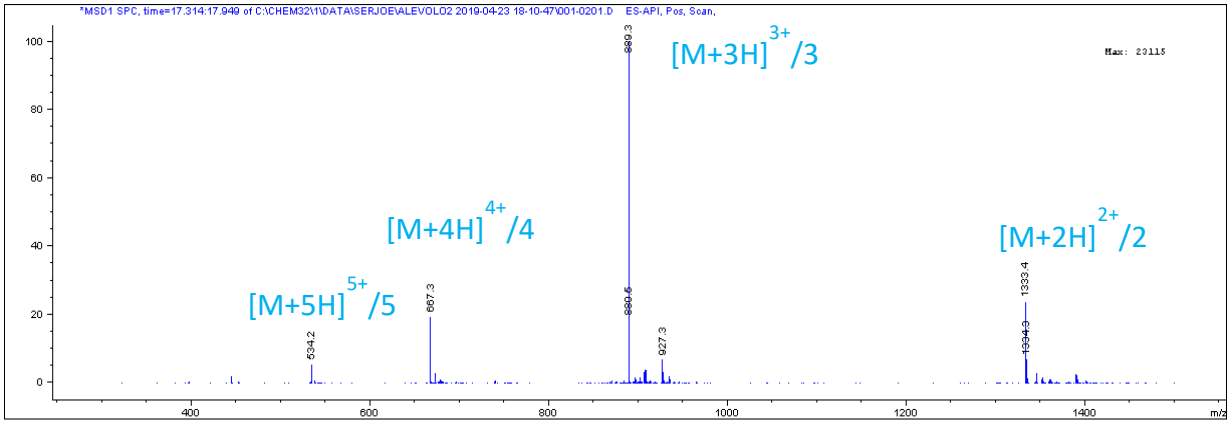

175.10
172.19
171.38
164.68
61.83
60.28
55.98
55.53
39.81
39.17
38.65
35.46
28.19
28.03
25.55
25.37
21.55
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

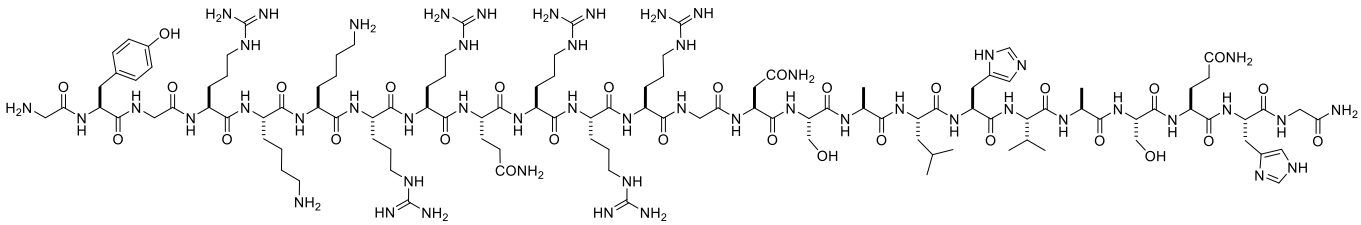

x10 5
+ESI Scan (0.127-0.177 min, 4 scans) Frag=160.0V AV147-a.d Subtract
454.1551
455.1581
456.1539
457.1558
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
Counts vs. Mass-to-Charge (m/z)



Purity was evaluated by LC-MS using a 0% B to 25% B in 20 min gradient (eluent A Water+0.1%TFA, Eluent B ACN+0.1% TFA).

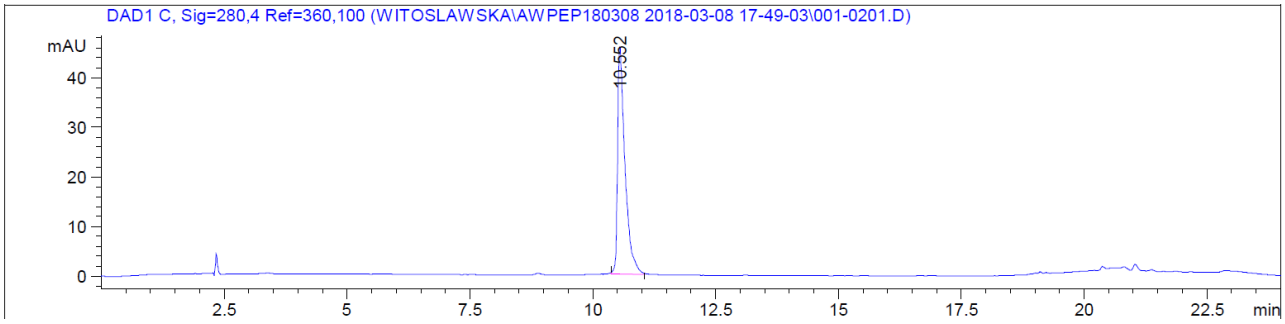




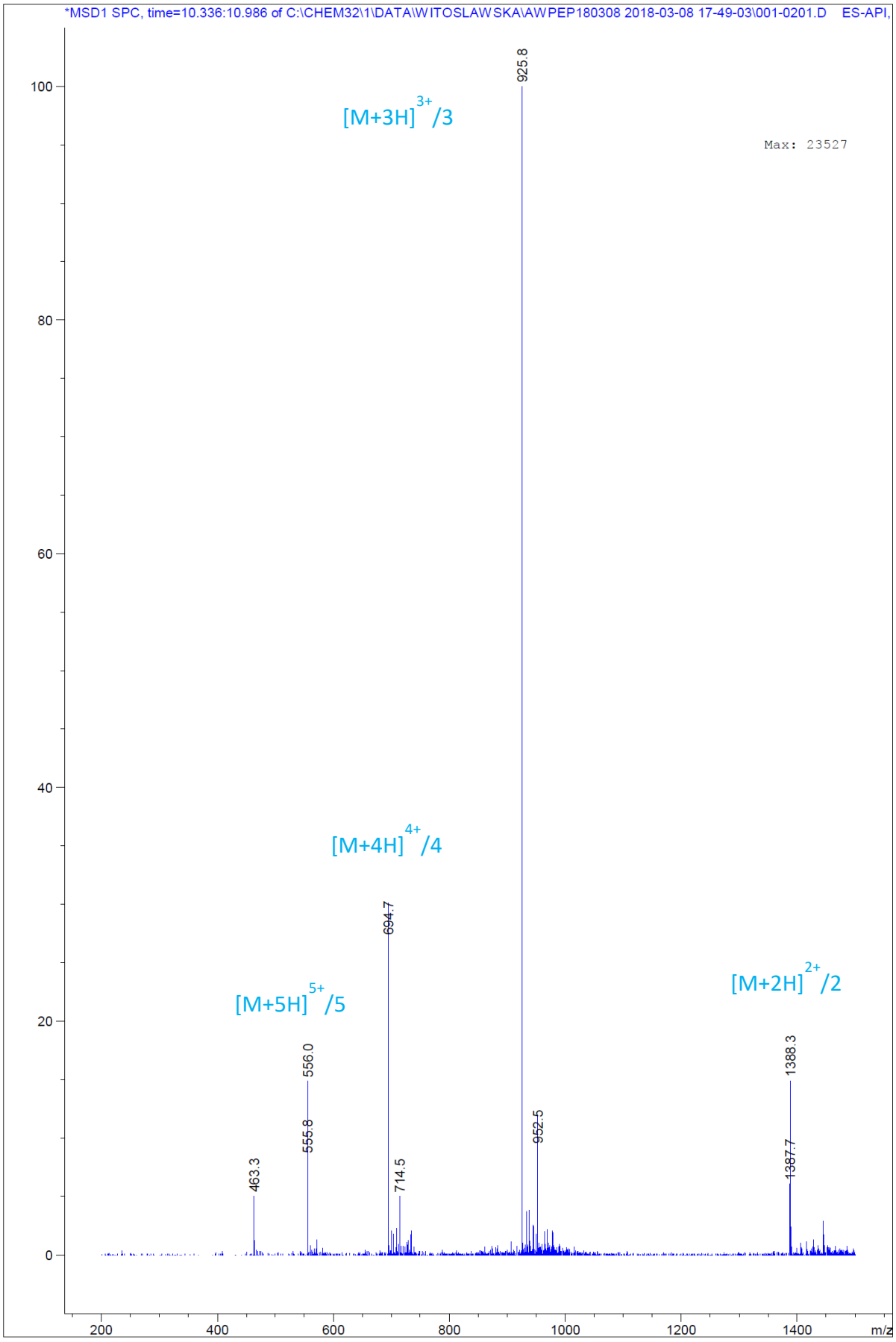


TAT-SP012 **14**

Purity was evaluated by LC-MS using a 0% B to 25% B in 20 min gradient (eluent A Water+0.1%TFA, Eluent B ACN+0.1% TFA).



MS Spectrum


*MSD1 SPC, time=10.336:10.986 of C:\CHEM32\1\DATA\WITOSLAWSKA\AWPEP180308 2018-03-08 17-49-03\001-0201.D ES-API,
Max: 23527
[M+3H]3+/3
[M+4H]4+/4
[M+5H]5+/5
[M+2H]2+/2
925.8
694.7
556.0
555.8
463.3
714.5
952.5
1388.3
1387.7
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
m/z