Supporting information

A practical solution-phase synthesis of an antagonistic peptide of TNF-α based on hydrophobic tag strategy

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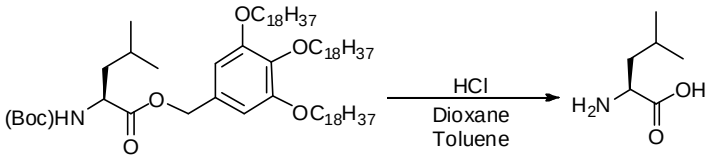
1. General information

^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ with TMS as the initial standard or CD_3OD on 600 MHz NMR spectrometers. The following abbreviations were used to explain multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; sext, sextet; sept, septet; m, multiple and coupling constants, *J*, are reported in Hz. MS spectra were recorded by TOF-ESI and MS-MALDI-TOF. TLC analysis was carried out with F254 plates, detection of compounds was achieved by UV absorption (254 nm) and by charring after spraying with 12 molybdo(VI) phosphoric acid n-hydrate in 95% ethanol.

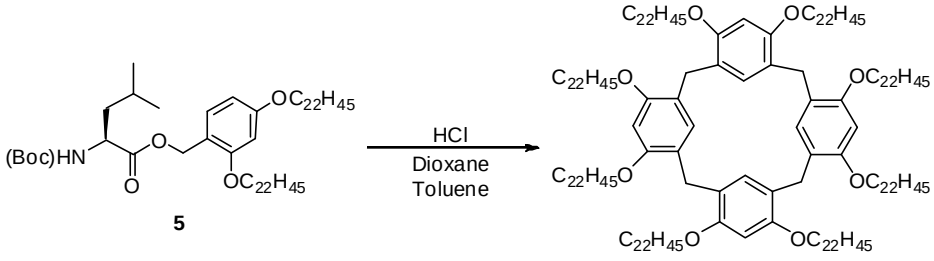
2. Additional schemes

Scheme S1 Investigation of hydrophobic tag HO-**TAGa** (**1**)



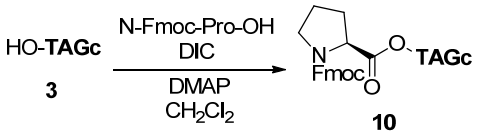
4 **S1**

Scheme S2 Investigation of hydrophobic tag HO-**TAGb** (**2**)



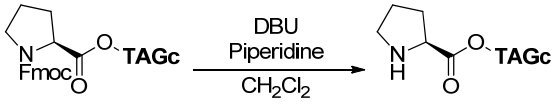
5 **S2**

Scheme S3 Introduction of N-Fmoc-proline to HO-**TAGc** (**3**)



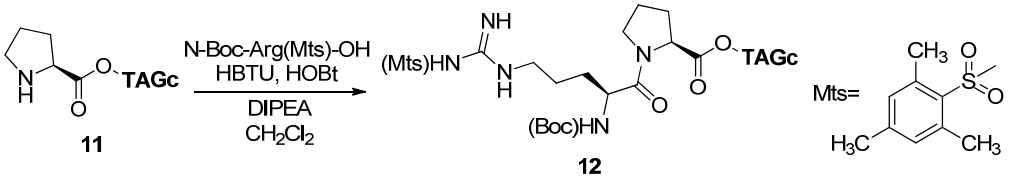
3 **10**

Scheme S4 N-Fmoc group deprotection of N-Fmoc-Pro-O-**TAGc** (**10**)



10 **11**

Scheme S5 Introduction of N-Boc-arginine(Mts) to H-Pro-O-**TAGc** (**11**)



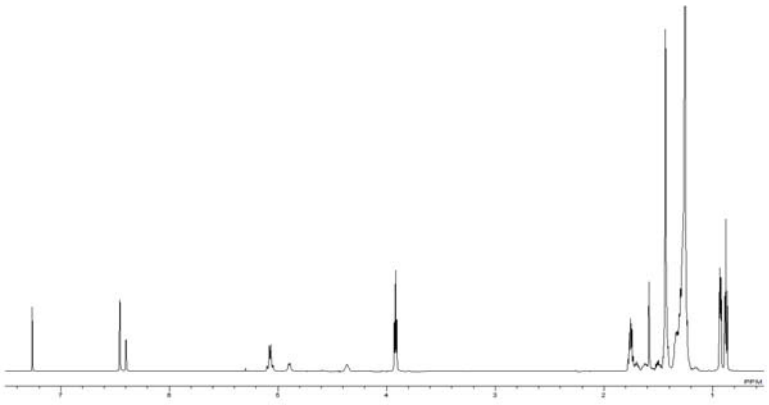


Figure S1 ^{1}H NMR spectrum of N-Boc-Leu-O-**TAGc** (**6**)

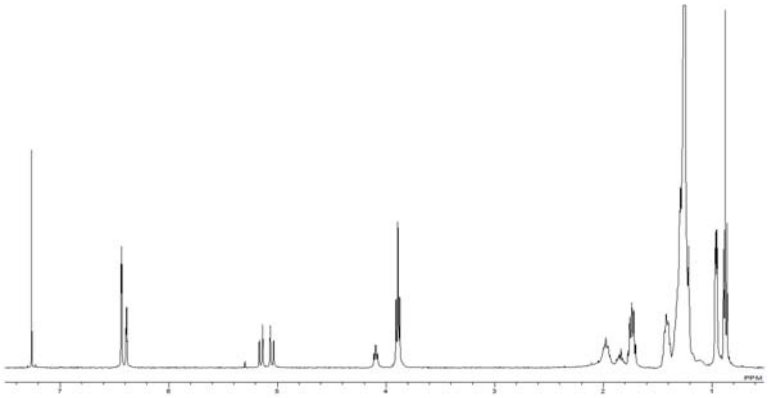


Figure S2 ^{1}H NMR spectrum of H-Leu-O-**TAGc** (**7**)

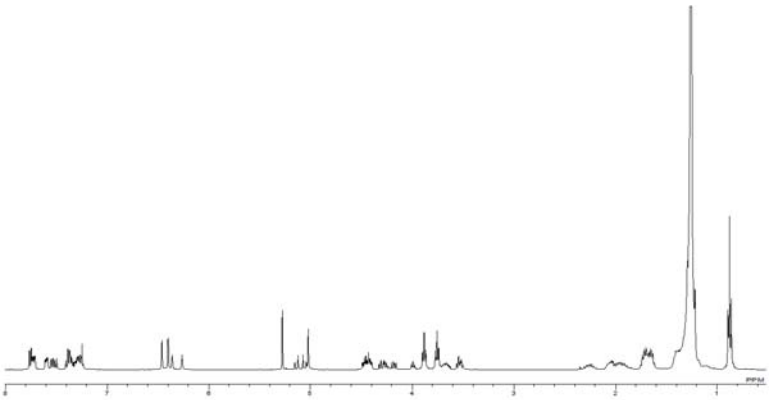


Figure S3 ^{1}H NMR spectrum of N-Fmoc-Pro-O-**TAGc** (**10**)

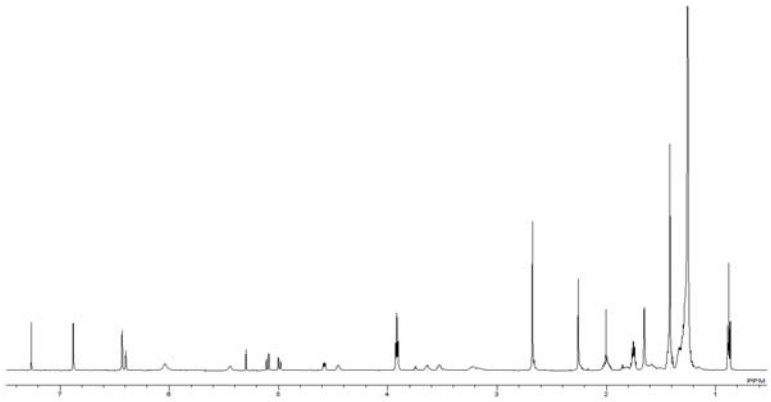


Figure S4 ^{1}H NMR spectrum of N-Boc-Arg(Mts)-Pro-O-**TAGc** (**12**)

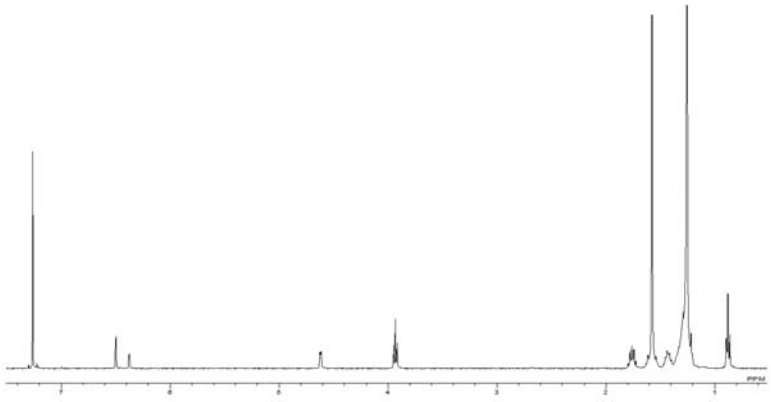


Figure S5 ^{1}H NMR spectrum of a precipitate obtained through N-terminal Fmoc group deprotection of N-Fmoc-Arg(Mts)-Pro-O-**TAGc** (**14**)

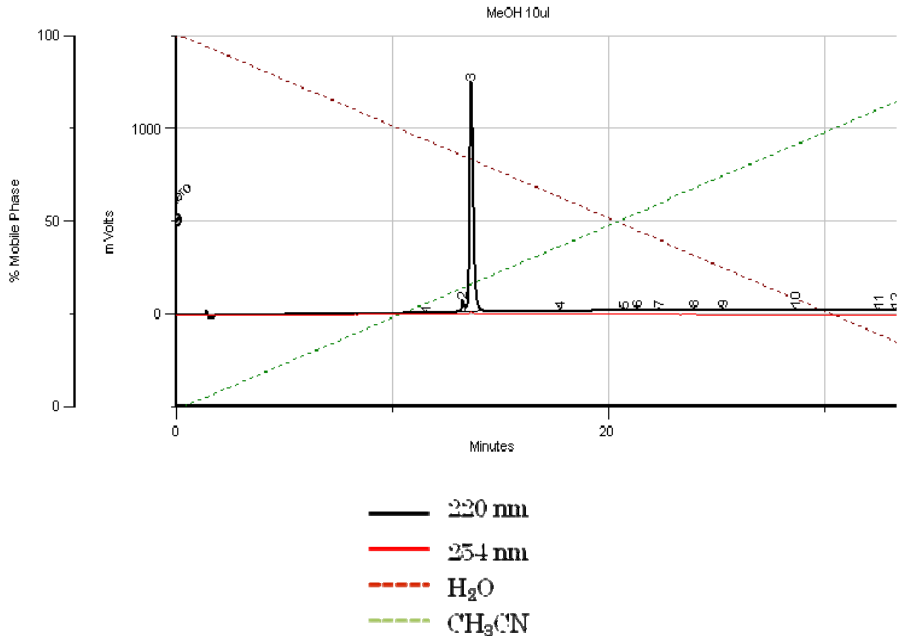
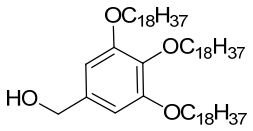


Figure S6 HPLC spectrum of A-TNF-α (**17**)

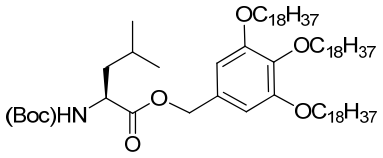
3. Experimental information

3.1. Investigation of hydrophobic tag HO-TAGa (1)



1

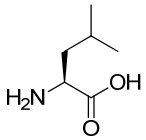
3.1.1. Preparation of N-Boc-Leu-O-TAGa (4)



4

The hydrophobic tag **1** (458 mg, 0.500 mmol) was dissolved in CH_2Cl_2 (10 mL). N-Boc-Leu-OH (173 mg, 0.750 mmol), DMAP (12.2 mg, 0.100 mmol), and DIC (94.7 mg, 0.750 mmol) were then added to the solution. The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to the reaction mixture to give **4** quantitatively as a precipitate.

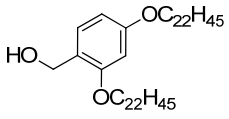
3.1.2. Acidic deprotection of N-Boc-Leu-O-TAGa (4)



S1

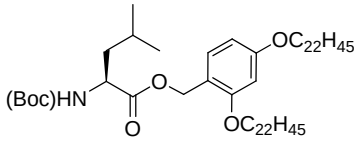
Compound **4** (113 mg, 0.100 mmol) was dissolved in 50% TFA in CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to give complex precipitate, including decomposed **1**, and hydrolyzed leucine **S1** was obtained in 86% yield from the filtrate by addition of IPE.

3.2. Investigation of hydrophobic tag HO-TAGb (2)



2

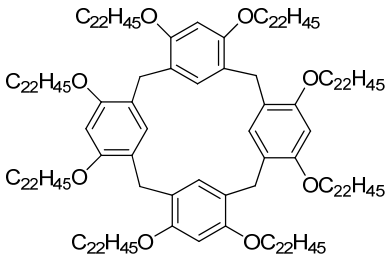
3.2.1. Preparation of N-Boc-Leu-O-TAGb (5)



5

The hydrophobic tag **2** (379 mg, 0.500 mmol) was dissolved in CH_2Cl_2 (10 mL). N-Boc-Leu-OH (173 mg, 0.750 mmol), DMAP (12.2 mg, 0.100 mmol), and DIC (94.7 mg, 0.750 mmol) were then added to the solution. The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to the reaction mixture to give **5** quantitatively as a precipitate.

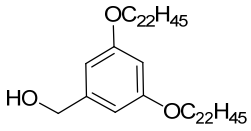
3.2.2. Acidic deprotection of N-Boc-Leu-O-TAGb (5)



S2

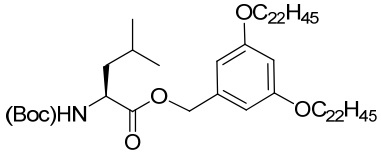
Compound **5** (97.1 mg, 0.100 mmol) was dissolved in 50% TFA in CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to give resorcinarene **S2**, cleaved and cyclized product of **5**, quantitatively as a precipitate.

3.3. Investigation of hydrophobic tag HO-TAGc (3)



3

3.3.1. Preparation of N-Boc-Leu-O-TAGc (6)

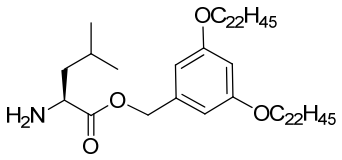


6

The hydrophobic tag **3** (379 mg, 0.500 mmol) was dissolved in CH_2Cl_2 (10 mL). N-Boc-Leu-OH (173 mg, 0.750 mmol), DMAP (12.2 mg, 0.100 mmol), and DIC (94.7 mg, 0.750 mmol) were then added to the solution. The reaction mixture was

stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to the reaction mixture to give **6** quantitatively as a precipitate.

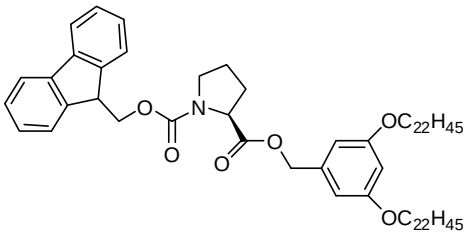
3.3.2. Acidic deprotection of N-Boc-Leu-O-TAGc (6)



9

Compound **6** (97.1 mg, 0.100 mmol) was dissolved in 50% TFA in CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to give H-Leu-O-**TAGc** (**9**) quantitatively as a precipitate.

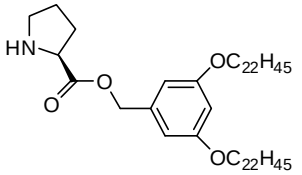
3.4. Preparation of N-Fmoc-Pro-O-TAGc (10)



10

The hydrophobic tag **3** (757 mg, 1.00 mmol) was dissolved in CH_2Cl_2 (10 mL). N-Fmoc-Pro-OH (506 mg, 1.50 mmol), DMAP (24.4 mg, 0.200 mmol), and DIC (189 mg, 1.50 mmol) were then added to the solution. The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to the reaction mixture to give **10** quantitatively as a precipitate.

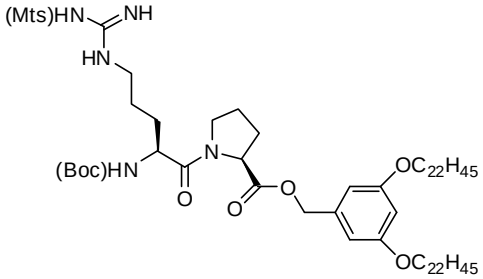
3.5. N-Fmoc group deprotection of N-Fmoc-Pro-O-TAGc (10)



11

Compound **10** (1.08 g, 1.00 mmol) was dissolved in 1% DBU and 1% piperidine in CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature until the reaction completed. After the completion, 6 M HCl was added to the solution to neutralize (pH 7.0), and then CH_3CN was added to give H-Pro-O-**TAGc** (**11**) quantitatively as a precipitate.

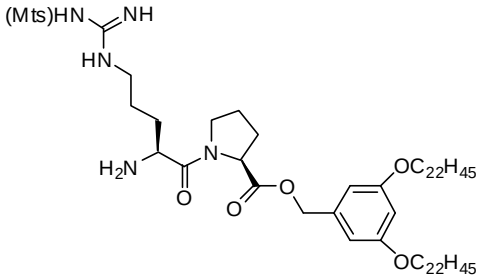
3.6. Preparation of N-Boc-Arg(Mts)-Pro-O-TAGc (12)



12

Compound **11** (854 mg, 1.00 mmol) was dissolved in CH_2Cl_2 (10 mL). N-Boc-Arg(Mts)-OH (548 mg, 1.20 mmol), HBTU (455 mg, 1.20 mmol), HOBT (162 mg, 1.20 mmol), and DIPEA (310 mg, 2.40 mmol) were then added to the solution. The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to the reaction mixture to give **12** quantitatively as a precipitate.

3.7. N-Boc group deprotection of N-Boc-Arg(Mts)-Pro-O-TAGc (12)



13

Compound **12** (1.29 g, 1.00 mmol) was dissolved in toluene (10 mL), and then 4 M HCl/dioxane (5 mL) was added. The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to give H-Arg(Mts)-Pro-O-TAGc (**13**) quantitatively as a precipitate.

3.8. General method for the elongation of H-Peptide-O-TAGc

H-Peptide-O-**TAGc** was dissolved in THF (10 mL). Fmoc-AAs (1.2 mol equiv.), HBTU (1.2 mol equiv.), HOBT (1.2 mol equiv.), and DIPEA (2.4 mol equiv.) were then added to the solution. The reaction mixture was stirred at room temperature until the reaction completed. After the completion, CH_3CN was added to the reaction mixture to give N-Fmoc-AA-Peptide-O-**TAGc** quantitatively as a precipitate.

3.9. General method for N-Fmoc group deprotection of N-Fmoc-Peptide-O-TAGc

N-Fmoc-Peptide-O-**TAGc** was dissolved in 1% DBU and 1% piperidine in CH_2Cl_2

(20 mL). The reaction mixture was stirred at room temperature until the reaction completed. After the completion, 6 M HCl was added to the solution to neutralize (pH 7.0), and then CH_3CN was added to give H-Peptide-O-**TAGc** quantitatively as a precipitate.

3.10. Acidic deprotection of N-Boc-A-TNF-α(Mts)-O-TAGc (15)

Boc-Asp(*t*Bu)-Phe-Leu-Pro-His(Trt)-Tyr(*t*Bu)-Lys(Boc)-Asn(Trt)-Thr(*t*Bu)
-Ser(*t*Bu)-Leu-Gly-His(Trt)-Arg(Mts)-Pro-O-**TAGc**

15

H-Asp-Phe-Leu-Pro-His-Tyr-Lys-Asn-Thr-Ser-Leu-Gly-His-Arg(Mts)-Pro-OH

S3

Compound **16** (38.6 mg, 0.0100 mmol) was dissolved in 2.5% TIS and 2.5% H_2O in TFA (5 mL). The reaction mixture was stirred at room temperature until the reaction completed. After the completion, the solution was filtrated by hydrophilic PTFE filter, and then IPE was added to the filtrate to give H-A-TNF-α(Mts)-OH (**S3**) in 94% yield as a precipitate.

3.11. Mts group deprotection of H-A-TNF-α(Mts)-OH (S3)

H-Asp-Phe-Leu-Pro-His-Tyr-Lys-Asn-Thr-Ser-Leu-Gly-His-Arg-Pro-OH

16

Compound **S3** (19.6 mg, 0.100 mmol) was dissolved in 1 M TFMSA/TFA (5 mL) in the presence of thioanisole. The reaction mixture was stirred at 0 °C until the reaction completed. After the completion, IPE was added to the reaction mixture to give H-A-TNF-α-OH (**16**) in 91% yield, >98% purity as a precipitate. Purity was determined by HPLC.

4. Spectra information

N-Boc-Leu-O-**TAGc** (**6**)

^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.45 (2H, s), 6.40 (1H, s), 5.07 (2H, dd, *J*=19.8, 12.5 Hz), 4.90 (1H, d, *J*=8.1 Hz), 4.41-4.30 (1H, m), 3.92 (4H, t, *J*=6.6 Hz), 1.76 (4H, quint, *J*=7.3 Hz), 1.70 (1H, sext, *J*=6.6 Hz), 1.66-1.60 (1H, m), 1.53-1.47 (1H, m), 1.43 (9H, s), 1.38-1.12 (76H, m), 0.93 (3H, d, *J*=6.6 Hz), 0.92 (3H, d, *J*=6.6 Hz), 0.88 (6H, t, *J*=6.6 Hz) ^{13}C NMR ($CDCl_3$, 150 MHz) δ 173.3, 160.4, 155.4, 106.3, 101.2, 68.1, 66.9, 52.2, 41.8, 31.9, 29.7, 29.7, 29.7, 29.6, 29.6, 29.4, 29.4, 29.3, 28.3, 26.1, 24.8, 22.8, 22.7, 21.9, 14.1 HRMS calc. for $C_{62}H_{115}NO_6$ 992.8622 (M+Na), found 992.8658

H-Leu-O-**TAGc** (**9**)

^{1}H NMR ($CDCl_3$, 600 MHz) δ 9.30-8.53 (2H, bs), 6.44 (2H, d, *J*=1.8 Hz), 6.39 (1H, t, *J*=1.8 Hz), 5.15 (1H, d, *J*=12.4 Hz), 5.05 (1H, d, *J*=12.4 Hz), 4.10 (1H, t, *J*=6.9 Hz), 3.89 (4H, t, *J*=6.9 Hz), 2.11-1.79 (3H, m), 1.74 (4H, quint, *J*=7.4 Hz), 1.48-1.15 (76H, m), 0.96 (6H, dd, *J*=6.4, 2.3 Hz), 0.88 (6H, t, *J*=6.9 Hz) ^{13}C NMR ($CDCl_3$, 150 MHz) δ 169.7, 160.4, 136.5, 106.5, 101.5, 68.1, 51.7, 39.5, 31.9, 29.7, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 26.1, 24.4, 22.7, 22.4, 21.9, 14.1 HRMS calc. for $C_{57}H_{107}NO_4$ 892.8098 (M+Na), found 892.8115

N-Fmoc-Pro-O-**TAGc** (**10**) **dr 1:1**

^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.76 (1H, d, *J*=7.8 Hz), 7.72 (1H, dd, *J*=7.8, 3.2 Hz), 7.60 (1H, dd, *J*=7.3, 3.2 Hz), 7.52 (1H, dd, *J*=15.1, 7.3 Hz), 7.43-7.33 (2H, m), 7.32-7.22 (2H, m), 6.46 (1H, d, *J*=1.8 Hz), 6.40 (1H, d, *J*=1.8 Hz), 6.36, 6.26 (1H, t, *J*=1.8 Hz), 5.14, 5.06 (1H, d, *J*=12.4 Hz), 5.02 (1H, m), 4.52-4.36 (2H, m), 4.34-4.23 (1H, m), 4.18, 3.99 (1H, dd, t, *J*=10.5, 7.8 Hz, 6.9 Hz), 3.88 (2H, t, *J*=6.9 Hz), 3.76 (2H, t, *J*=6.9 Hz), 3.73-3.61 (1H, m), 3.58-3.48 (1H, m), 2.36-2.15 (1H, m), 2.14-1.81 (3H, m), 1.80-1.56 (4H, m), 1.48-1.04 (76H, m), 0.88 (6H, t, *J*=6.9 Hz) ^{13}C NMR ($CDCl_3$, 150 MHz) δ 172.4, 172.4, 160.4, 160.3, 154.8, 154.4, 144.3, 144.1, 143.8, 143.6, 141.3, 141.2, 141.1, 137.6, 137.4, 127.6, 127.6, 127.5, 127.0, 127.0, 126.9, 125.2, 125.1, 125.0, 124.9, 119.9, 119.8, 106.5, 106.2, 101.0, 100.9, 68.0, 67.9, 67.5, 67.4, 66.8, 66.8, 59.3, 58.9, 53.4, 47.2, 47.1, 47.0, 46.4, 31.9, 31.0, 29.9, 29.8, 29.7, 29.7, 29.6, 29.6, 29.6, 29.4, 29.3, 29.2, 29.1, 26.0, 26.0, 24.3, 23.4, 22.7, 14.1 HRMS calc. for $C_{71}H_{113}NO_6$ 1098.8466 (M+Na), found 1098.8464

H-Pro-O-**TAGc** (**11**)

HRMS calc. for $C_{56}H_{103}NO_4$ 854.7965 (M+H), found 854.7898

N-Boc-Arg(Mts)-Pro-O-**TAGc** (**12**)

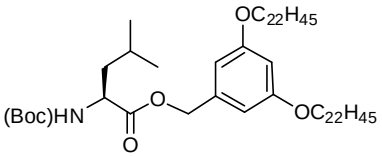
^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.88 (2H, s), 6.43 (2H, d, *J*=2.2 Hz), 6.40 (1H, t, *J*=2.2 Hz), 5.10 (1H, d, *J*=12.5 Hz), 4.99 (1H, d, *J*=12.5 Hz), 4.58 (1H, dd, J=8.1, 5.1 Hz), 4.49-4.39 (1H, m), 3.92 (4H, t, *J*=6.6 Hz), 3.69-3.59 (1H, m), 3.57-3.48 (1H, m), 3.33-3.02 (2H, m), 2.68 (6H, s), 2.30-2.20 (4H, m), 2.06-1.94 (3H, m), 1.75 (4H, quint, *J*=7.3 Hz), 1.63-1.53 (2H, m), 1.42 (11H, s), 1.36-1.19 (76H, m), 0.88 (6H, t, *J*=7.3 Hz) ^{13}C NMR ($CDCl_3$, 150 MHz) δ 160.4, 160.4, 156.4, 155.7, 140.3, 138.2, 137.8, 137.7, 137.3, 131.3, 128.9, 125.1, 106.1, 104.9, 101.0, 100.3, 79.8, 68.0, 68.0, 67.9, 67.8, 65.1, 58.9, 53.3, 46.9, 40.6, 31.8, 29.6, 29.6, 29.6, 29.5, 29.3, 29.3, 29.2, 29.2, 28.8, 28.2, 26.0, 24.9, 22.8, 22.6, 21.3, 20.7, 14.0 HRMS calc. for $C_{76}H_{133}N_5O_9S$ 1291.9824 (M+H), found 1292.9849

H-Arg(Mts)-Pro-O-**TAGc** (**13**)
HRMS calc. for $C_{71}H_{125}N_5O_7S$ 1192.9378 (M+H), found 1192.9269

N-Boc-A-TNF-α(Mts)-O-**TAGc** (**15**)
HRMS calc. for $C_{224}H_{314}N_{24}O_{30}S$ 3875.3401 (M+Na), found 3875.1307

H-A-TNF-α(Mts)-OH (**S3**)
HRMS calc. for $C_{90}H_{130}N_{24}O_{24}S$ 982.9798 $(M+H)^{2+}$, found 982.9800

H-A-TNF-α-OH (**16**)
HRMS calc. for $C_{81}H_{120}N_{24}O_{22}$ 891.4583 $(M+H)^{2+}$, found 891.4601



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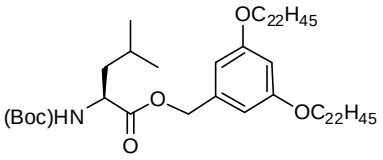

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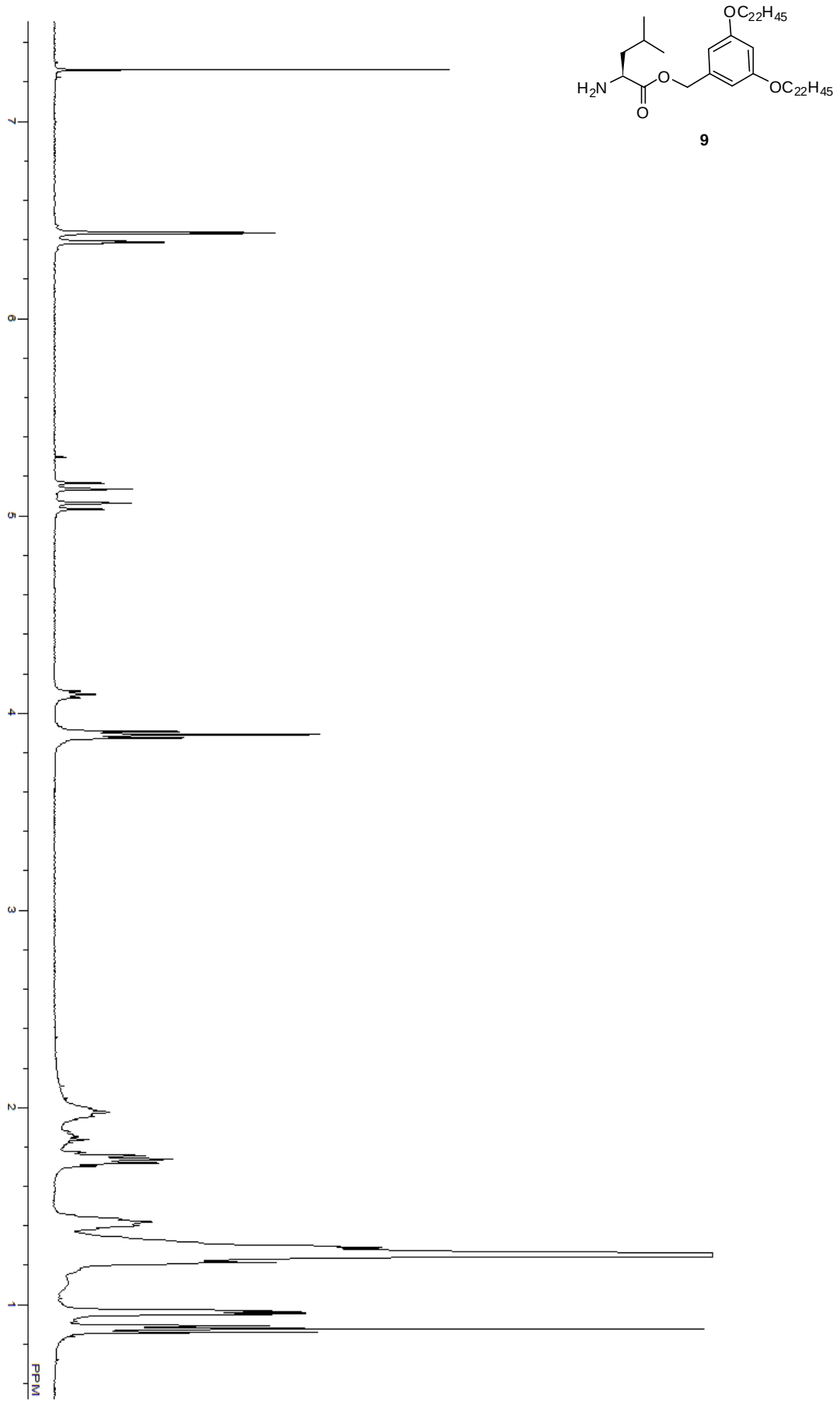

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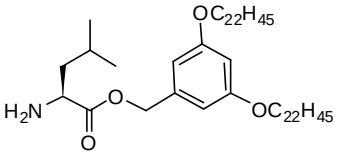

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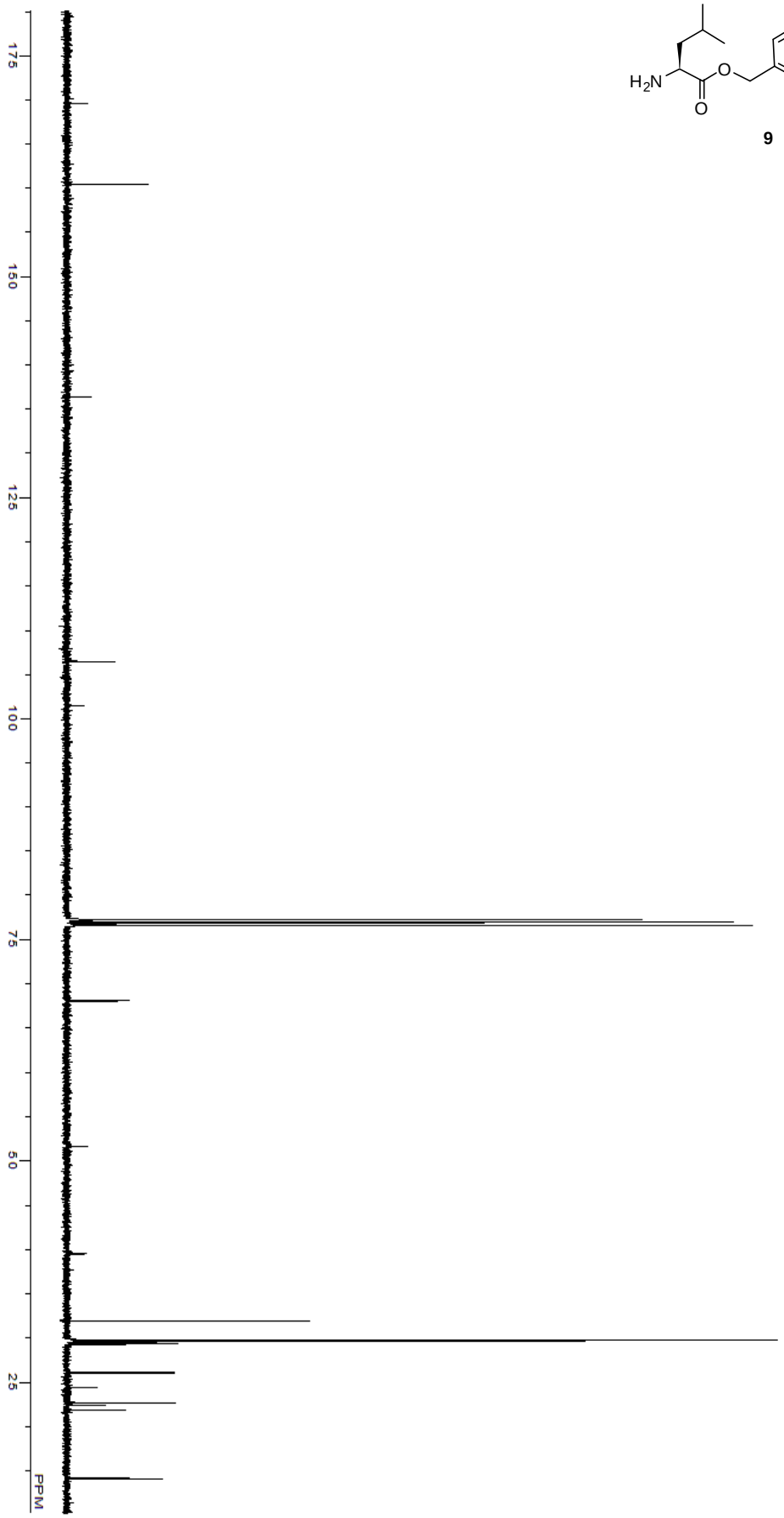

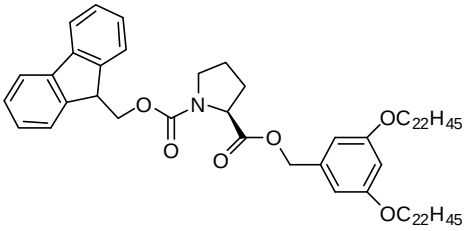

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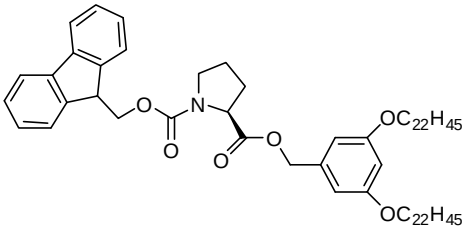

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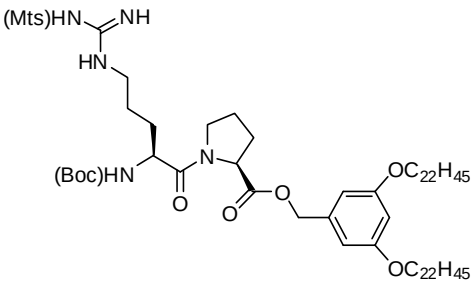

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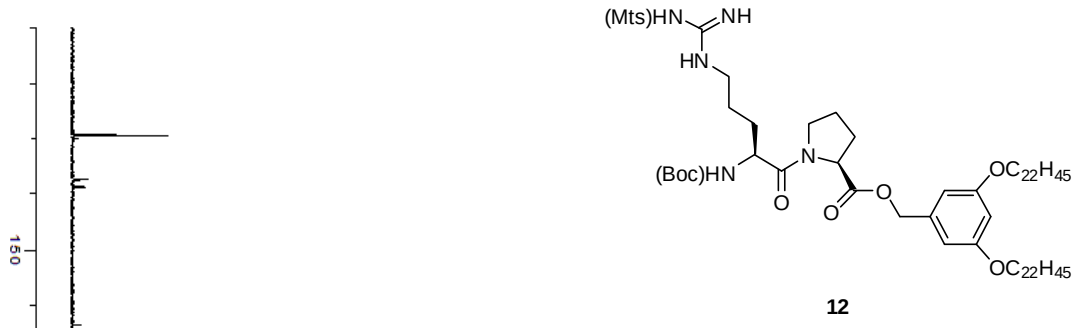
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NH
HN
(Boc)HN
N
O
O
O
OC22H45
OC22H45

12

150
125
100
75
50
25
PPM