

Supporting Information

for

Cell Impermeable Inhibitors Confirm that Intracellular Human Transglutaminase 2 is Responsible for the Transglutaminase-Associated Cancer Phenotype

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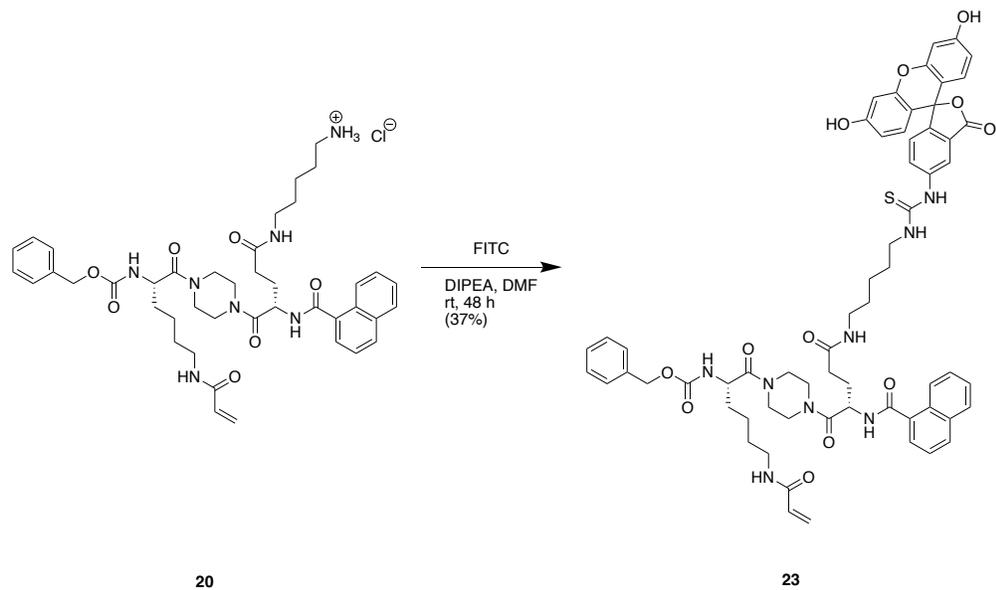
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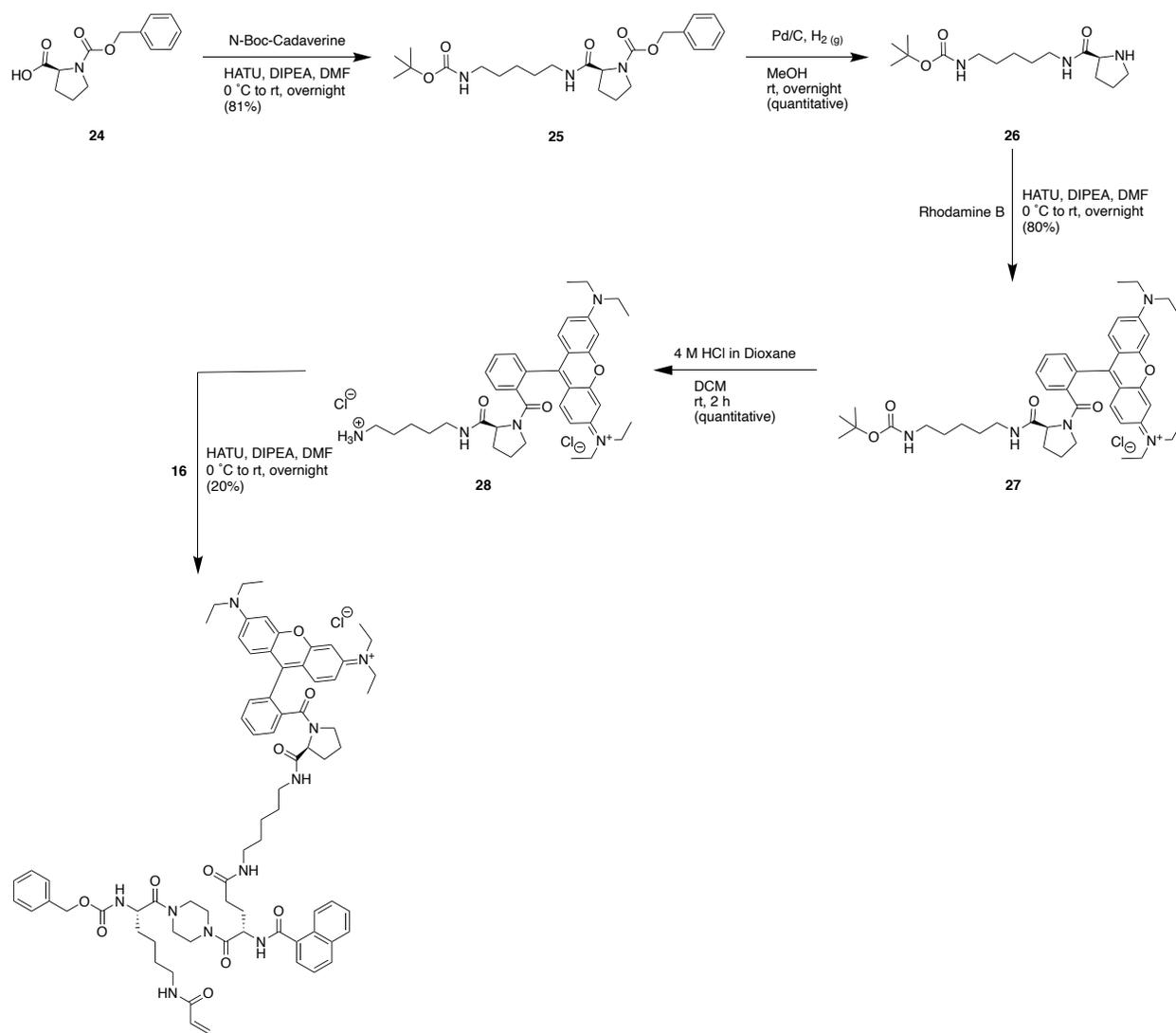
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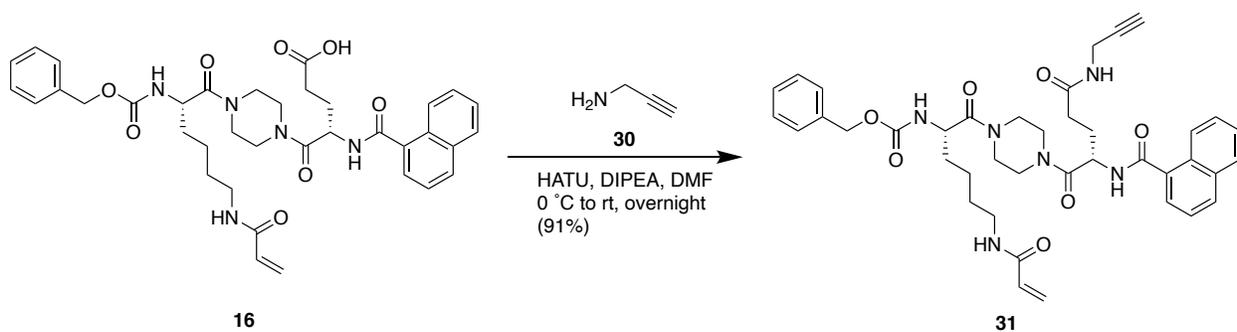
Supplementary synthetic schemes



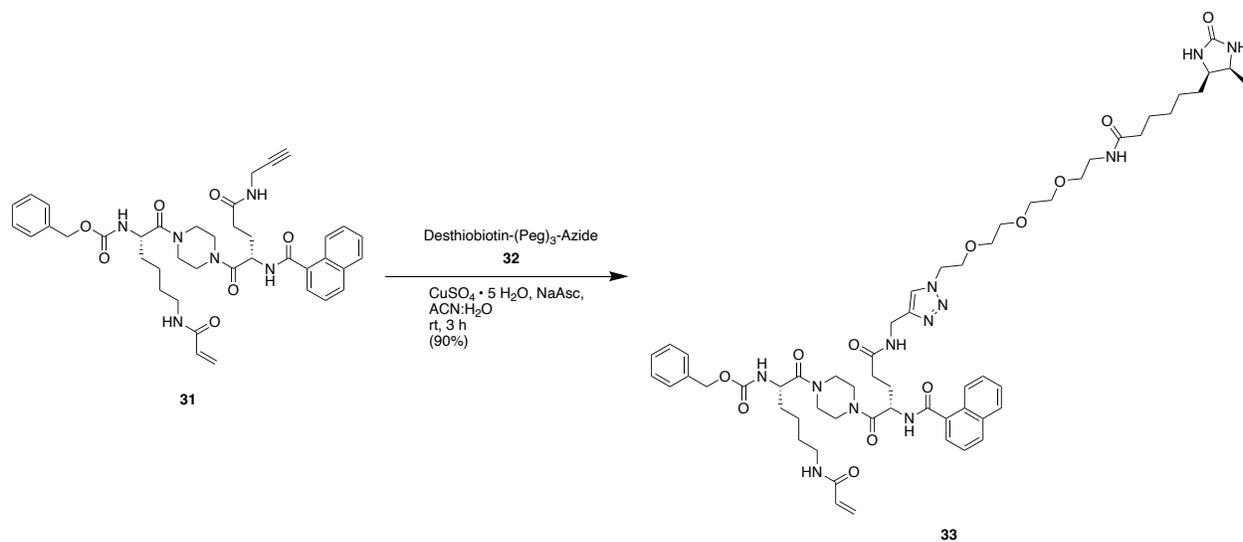
Scheme S1. Synthetic scheme from key intermediate to generate fluorescent FITC probe **23**.



Scheme S2. Synthetic scheme from key intermediate **16** to generate fluorescent rhodamine B probe **29**.



Scheme S3. Synthetic scheme from key intermediate **16** to generate propargyl derivative **31**, appropriate for subsequent ‘click’ modification.



Scheme S4. CuAAC ‘click’ reaction to generate the desthiobiotin derivative **33** used in the pull-down assay.

Supplementary kinetic traces and fitting

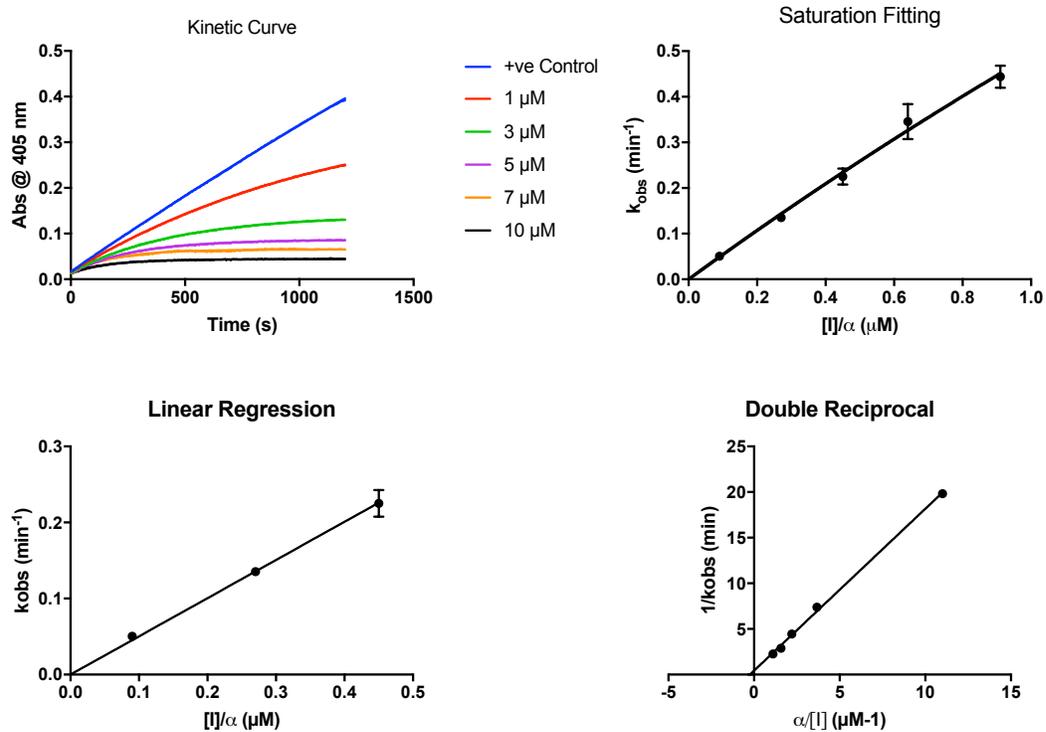


Figure S1. Kinetic traces and fitting of 18 (NCEG2).

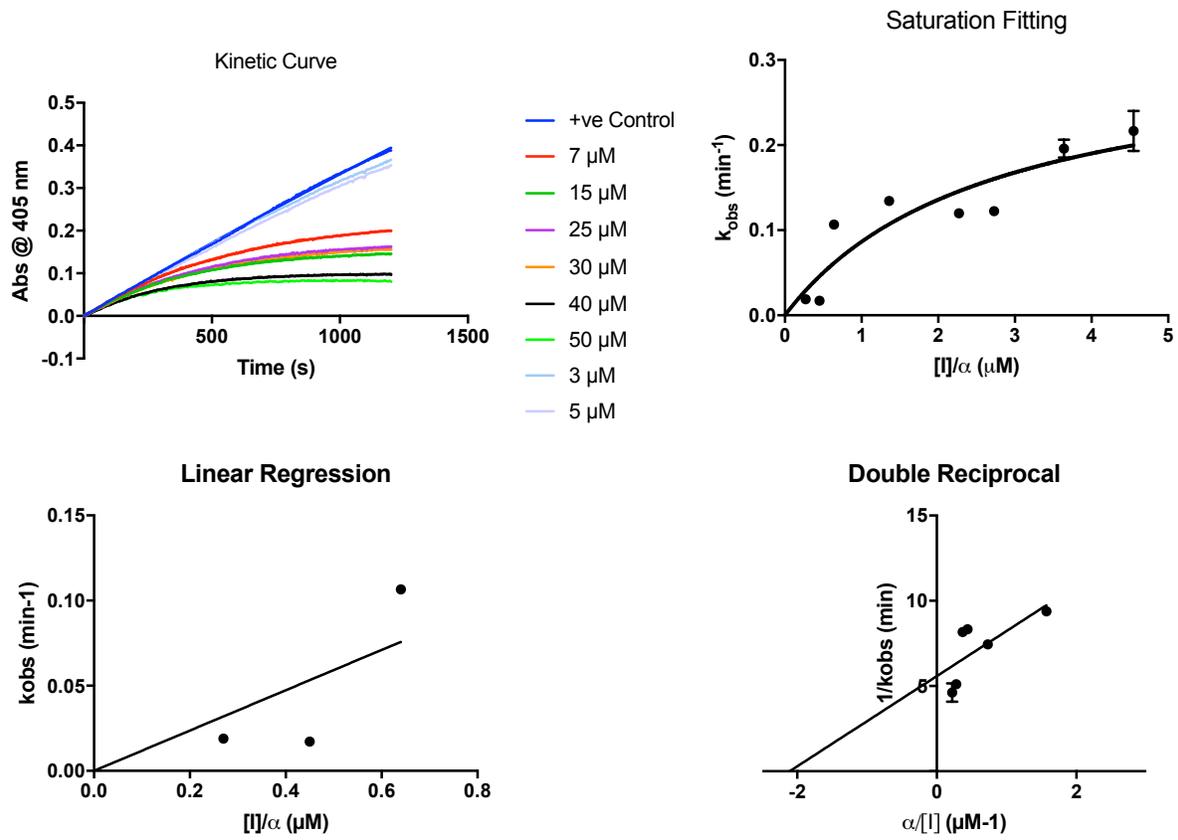


Figure S2. Kinetic traces and fitting for **22**.

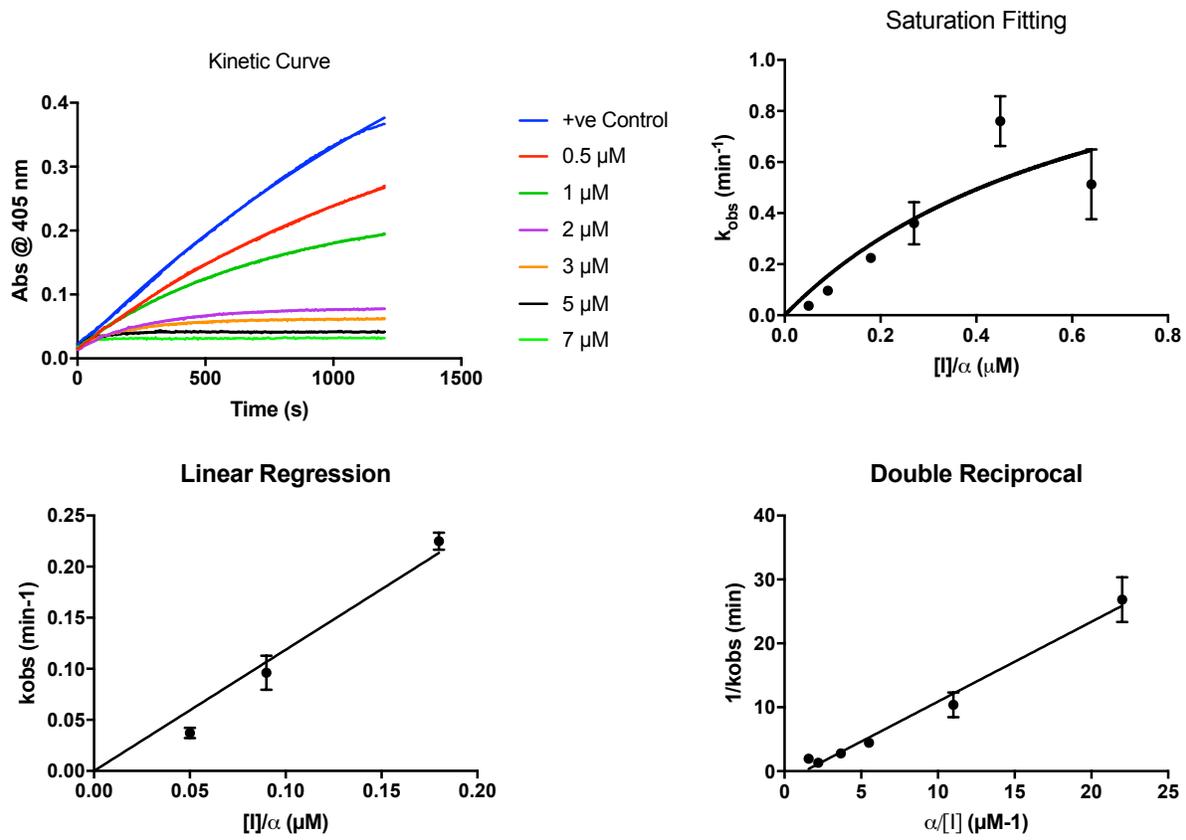


Figure S3. Kinetic traces and fitting for **23**.

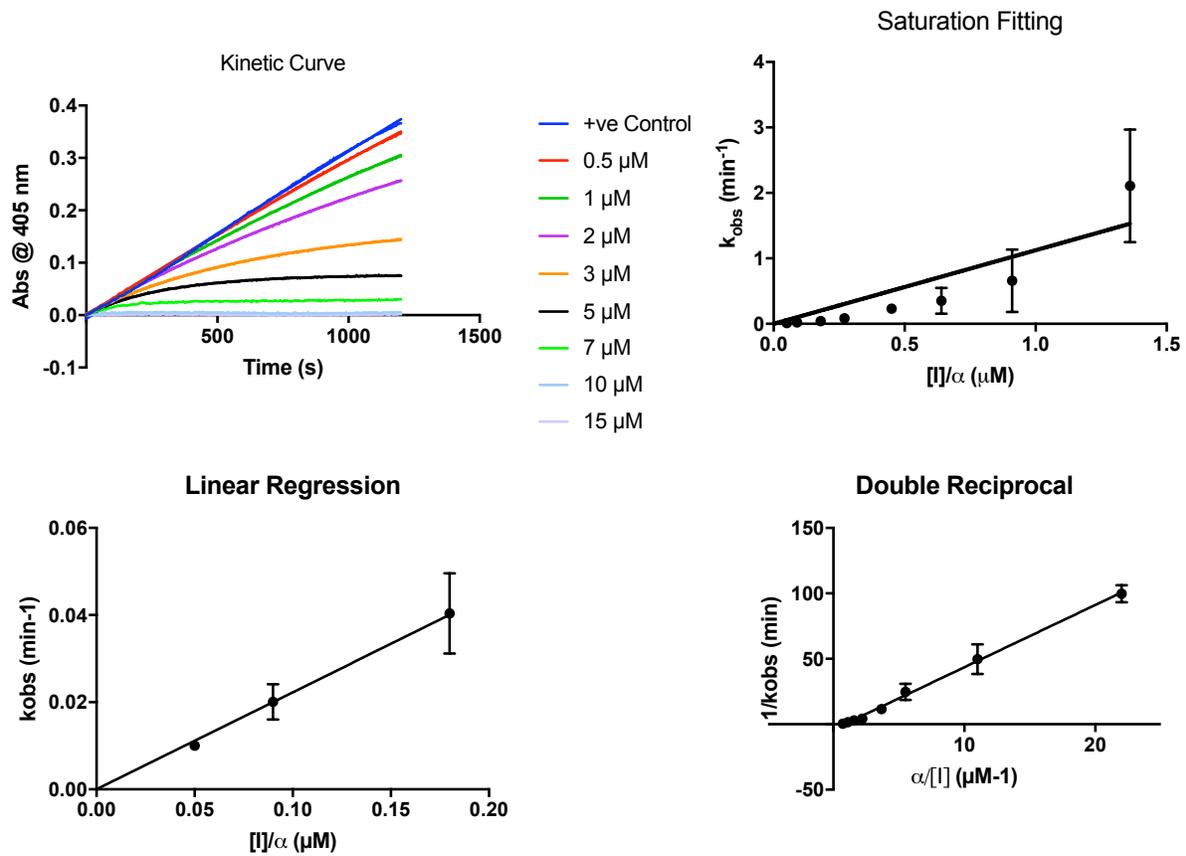


Figure S4. Kinetic traces and fitting for **29** (NCEG-RHB).

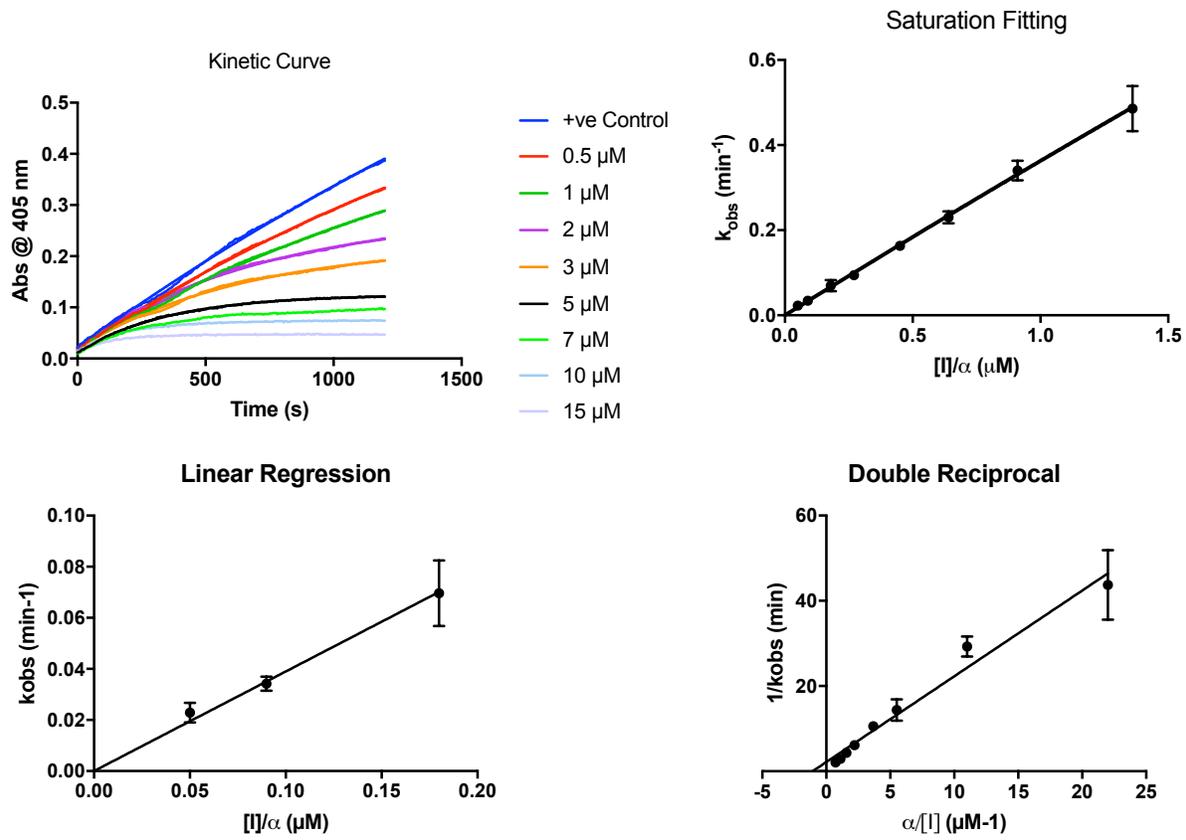


Figure S5. Kinetic traces and fitting for 31.

Molecular docking of compounds in TG2 active site

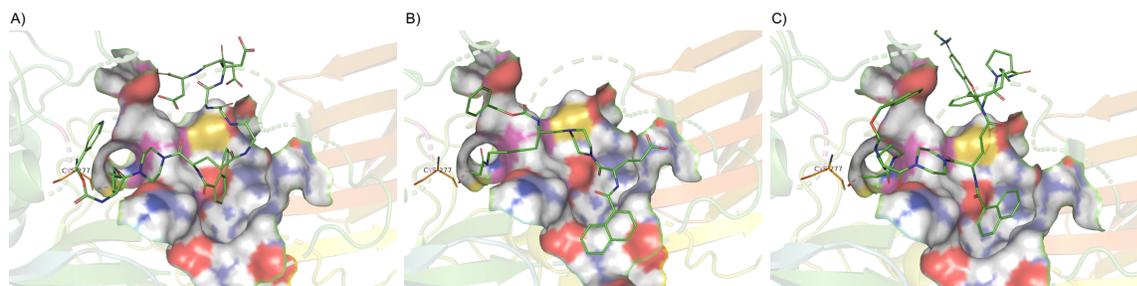


Figure S6. Structural representation of inhibitors **16**, **18** (NCEG2), and **29** (NCEG-RHB) covalently bound to the catalytic pocket of TG2 (PDB: 2Q3Z) via CYS277 (orange residue). A) inhibitor **18**, S score =9.63. B) inhibitor **16**, S score -9.92. C) inhibitor **29**, S score -10.93.

Isozyme selectivity

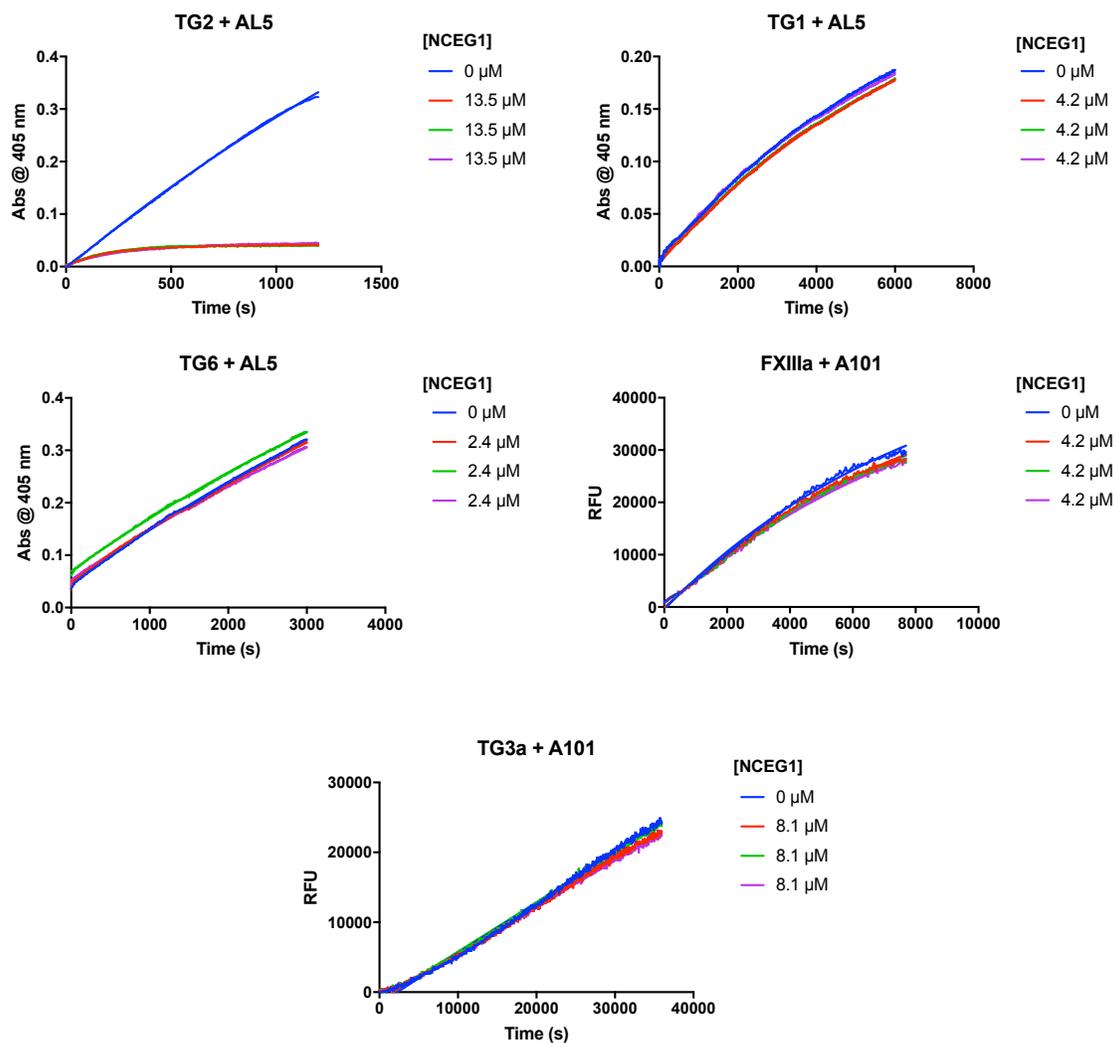


Figure S7. Isozyme selectivity of **17** with respect to therapeutically relevant human transglutaminases TG2, TG1, TG6, FXIIIa, and TG3a, using either chromogenic substrate AL5 or fluorescently quenched isopeptidase substrate A101.

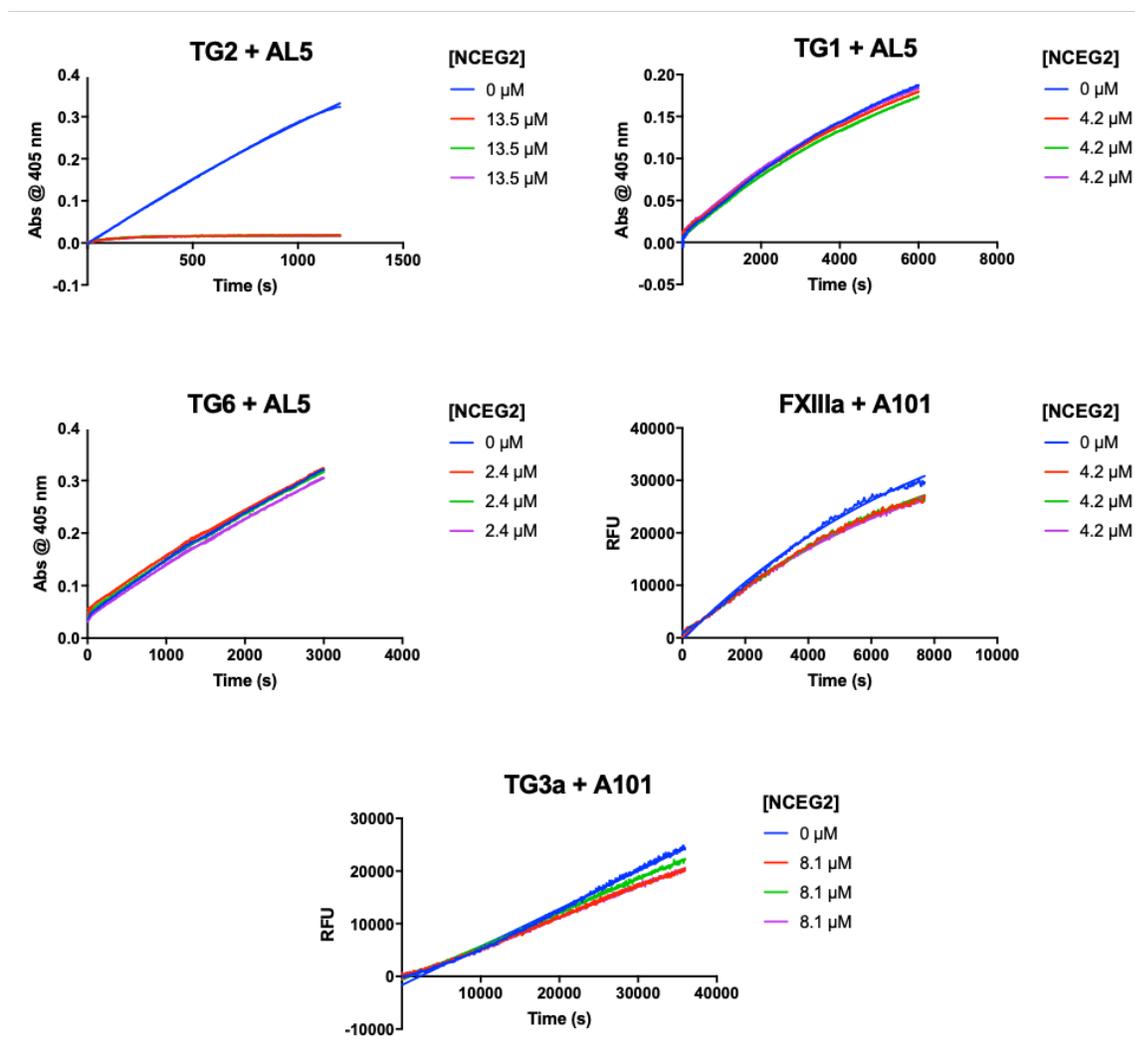


Figure S8. Isozyme selectivity of **18** (NCEG2) with respect to therapeutically relevant human transglutaminases TG2, TG1, TG6, FXIIIa, and TG3a, using either chromogenic substrate AL5 or fluorescently quenched isopeptidase substrate A101.

Supplementary fluorescent labelling images

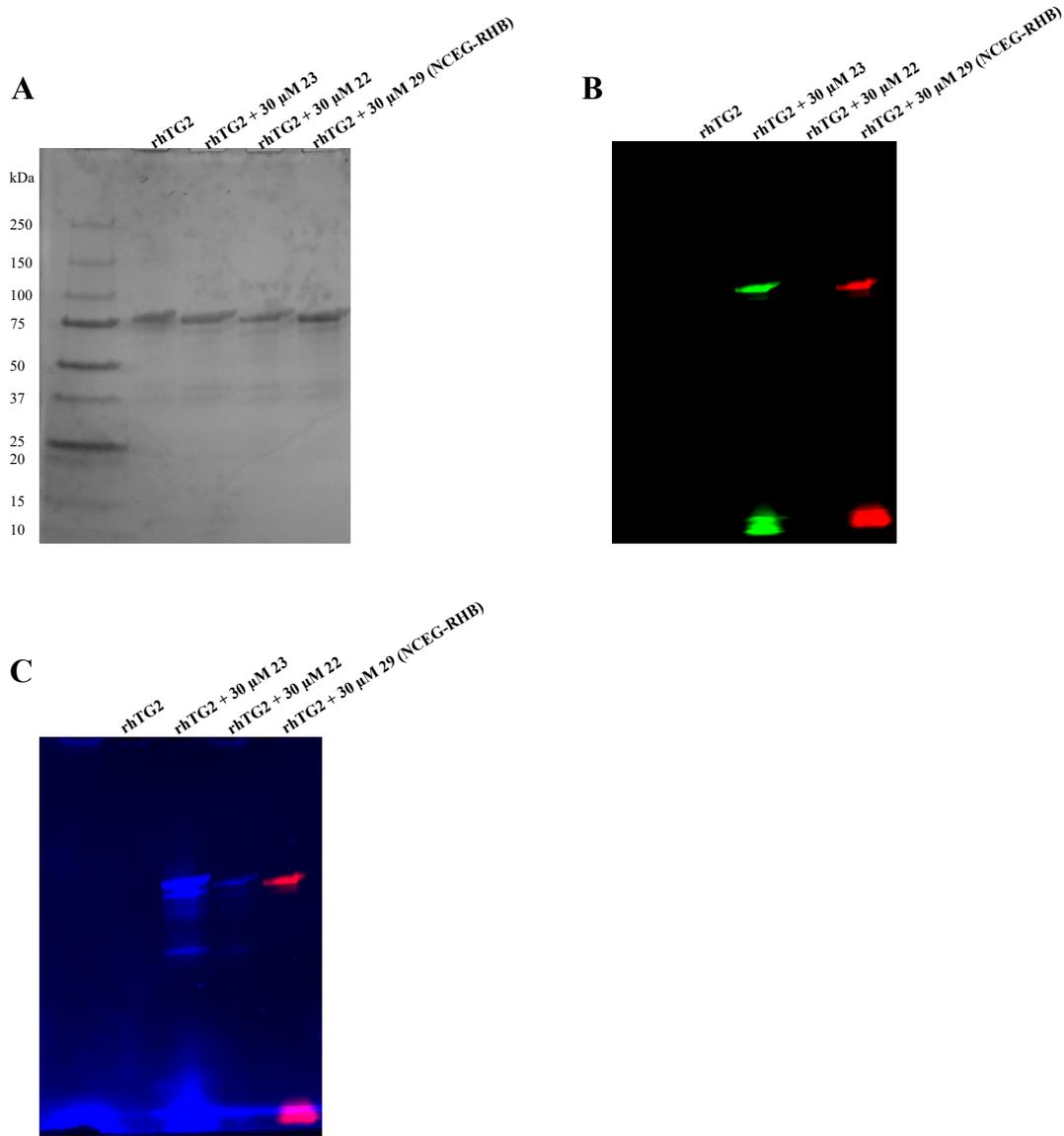


Figure S9. **A)** Coomassie stain of full SDS-PAGE used for fluorescent labelling of purified rhTG2. **B)** Multi-channel blue epi illumination with 530/28 nm filter (green band colour) and green epi illumination with 605/50 nm filter (red band colour) of full SDS-PAGE used for fluorescent labelling of purified rhTG2. **C)** Multi-channel UV trans illumination with 530/28 nm filter (blue band colour) and green epi illumination with 605/50 nm filter (red band colour) of full SDS-PAGE used for fluorescent labelling of purified rhTG2.

Supplementary cellular evaluation figures

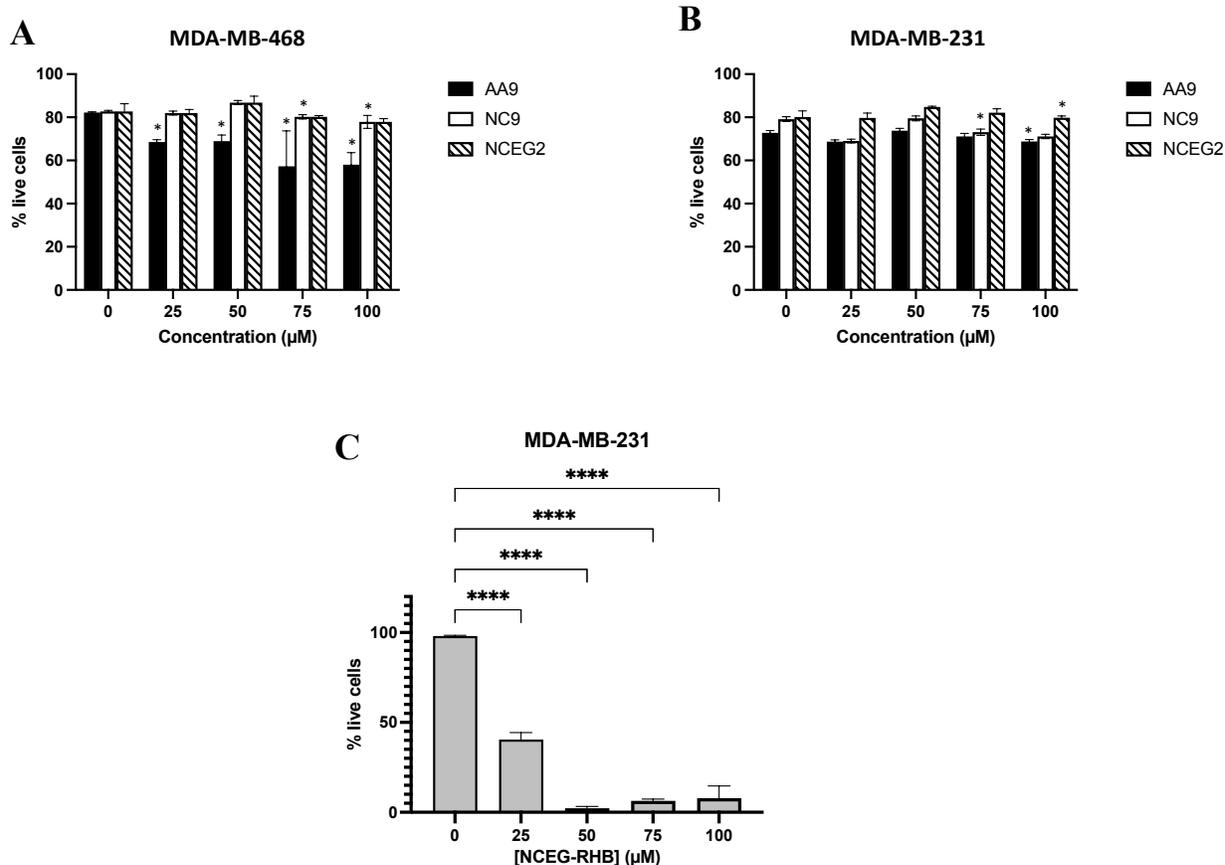


Figure S10. Viability assay: Treatment of (A) MDA-MB-468 breast cancer cell line with cell impermeable inhibitor **18** (NCEG2) and cell permeable inhibitors NC9 and AA9, employed at the indicated concentrations and (B) MDA-MB-231 breast cancer cell line with cell impermeable inhibitor **18** (NCEG2) and cell permeable inhibitors NC9, AA9, and (C) NCEG-RHB employed at the indicated concentrations. Experiments were carried out in triplicate and are represented as average \pm SD of the percentage of living cells; (*) P value < 0.05 ; (****) P value < 0.0001 .

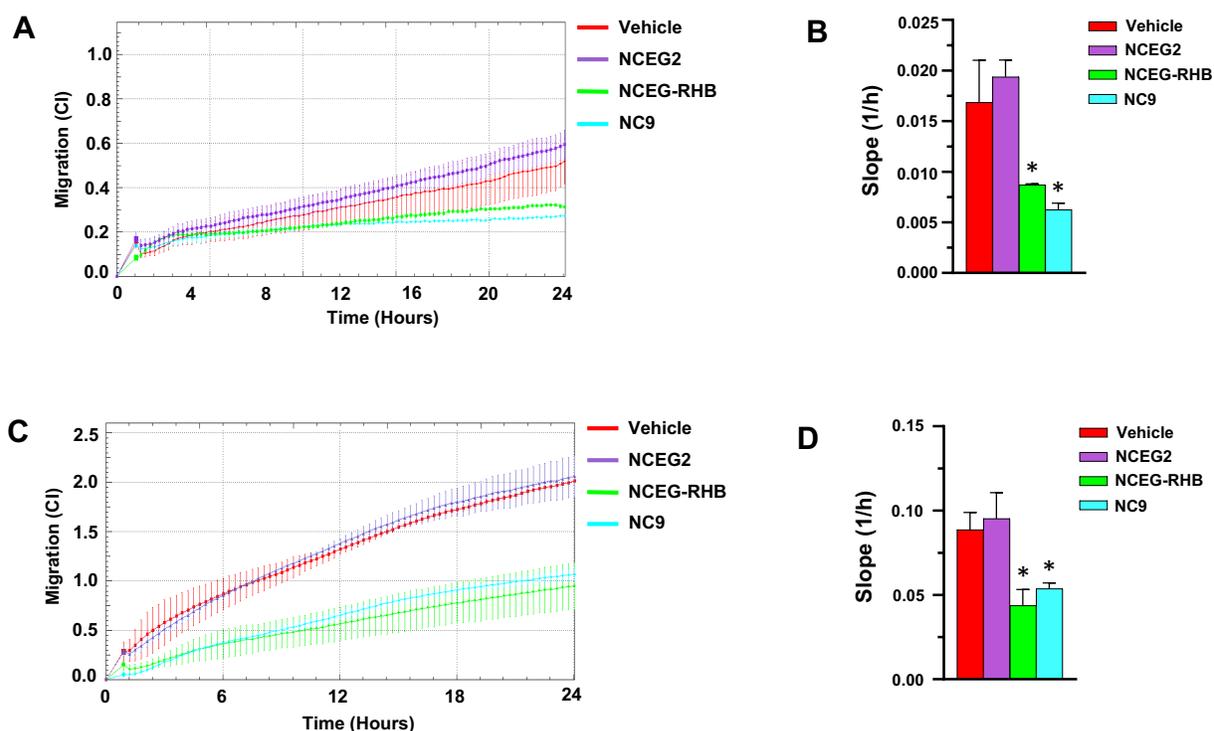
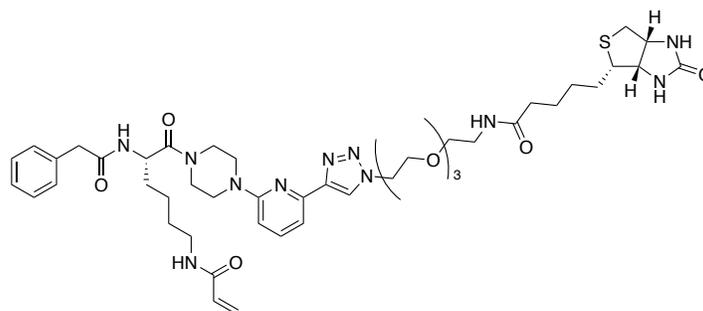


Figure S11. Migration assay: **A)** Dynamic monitoring of the migration of MDA-MB-436 cells. We recorded for 24 hours the migration of the cells in the presence only of the vehicle or of the indicated TG2 inhibitors, used at 25 μM concentrations. Data are reported as Cell Index (CI) values \pm SD. **B)** Slope analysis shown at 24 h comprising the steepness, inclination, gradient, and changing rate of the CI curves of MDA-MB-436 cells over the time. * $P < 0.05$ versus vehicle. **C)** Dynamic monitoring of the migration of MDA-MB-231 cells. We recorded for 24 hours the migration of the cells in the presence only of the vehicle or of the indicated TG2 inhibitors, used at 25 μM concentrations. Data are reported as Cell Index (CI) values \pm SD. **D)** Slope analysis shown at 24 h comprising the steepness, inclination, gradient, and changing rate of the CI curves of MDA-MB-231 cells over the time. * $P < 0.05$ versus vehicle.

Biotinylated inhibitor published by Hauser *et al.*



Hauser's Biotinylated Inhibitor

Figure S12. Structure of biotinylated TG2 inhibitor recently published by Hauser *et al.* [53].

SDS-PAGE of pull-down from *E. coli*

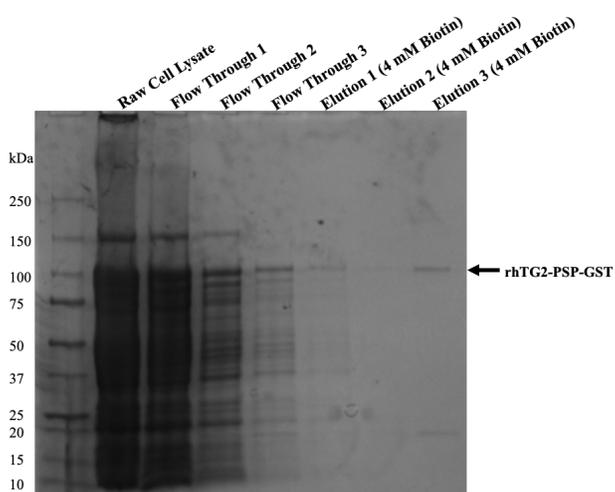


Figure S13. SDS-PAGE of pull-down experiment of recombinant human TG2 from *E. coli* cell lysate visualized with Coomassie staining.

Synthesis and characterization of intermediates and final compounds

Experimental Section

Where available, commercially available reagents and solvents were purchased from suppliers including Sigma-Aldrich, Oakwood Products, Combi-Blocks, and Fisher Scientific and used without further purification. Solid phase peptide synthesis resin was purchased from MilliporeSigma. Thin layer chromatography (TLC) was performed using SiliCycle aluminium backed TLC plates 200- μm thickness with F-254 indicator and visualized using short wave UV light. Preparatory-TLC was executed on SiliCycle SiliaPlate glass backed TLC with extra hard layer 60 \AA 250 μm thickness plates and F-254 indicator. Flash chromatography purification was performed using 230-400 mesh silica gel. All ^1H - and ^{13}C -NMR spectra were referenced to the indicated deuterated solvent and acquired using a Bruker 400-MHz or 600-MHz instrument to report the peaks in ppm. The high-resolution mass spectra were obtained using an electrospray ionization source (ESI) and quadrupole time-of-flight (QTOF) analyser. All final compounds were further evaluated for purity by HPLC where indicated.

General synthetic procedure A

To a round bottom flask equipped with magnetic stir bar was added the acid (1 eq) solubilized in a 1:1 mixture of THF:H₂O (0.1 M). To the flask was added finely ground potassium hydroxide (4 eq). The reaction mixture was then stirred for 2 h. Upon completion, the reaction mixture was diluted with H₂O and washed once with ether. The aqueous phase was then acidified to pH 2 with 1 M HCl. The aqueous layer was then extracted three times with ethyl acetate. The organic layers were combined, washed once with brine, dried over MgSO₄, filtered, and concentrated under reduced pressure to yield the product without further purification.

General synthetic procedure B

To a round bottom flask equipped with magnetic stir bar was added the acid (1.5 eq), HATU (1.5 eq), and DIPEA (3 eq) solubilized in DMF (0.5 M) at 0 °C. After 0.5 h, the amine (1 eq) was added dropwise as a solution in DMF (0.5 M) with DIPEA (1 eq). The ice bath was removed, and the reaction was allowed to stir overnight at room temperature. Upon completion, the reaction mixture was quenched with H₂O and extracted once with ethyl acetate. The organic layer was then washed with 1 M HCl, H₂O, sat. NaHCO₃ solution, and brine. The organic phase was then dried over MgSO₄, filtered, and concentrated under reduced pressure. The resulting crude oil was then purified *via* flash chromatography to obtain the product.

SPPS Wang resin loading procedure

To pre-load the first residue onto Wang resin, a procedure using a symmetrical anhydride was adapted from Atherton *et al.* [55]. To a dry SPPS reaction vessel was added 1 gram of Wang resin. The resin was suspended in 15 mL dry DMF under inert atmosphere and allowed to swell for 30 min. To form the corresponding anhydride to load onto the resin, 4.937 g Fmoc-Asp(OtBu)-OH (12 mmol, 10 eq) was solubilized in 120 mL dry DCM in a flame-dried round-bottom flask equipped with magnetic stir bar under inert atmosphere. To the flask was added 0.929 mL DIC (6 mmol, 5 eq) and the solution was stirred for 20 min at 0 °C. The volatiles were then removed from the round bottom flask under reduced pressure the residue was re-solubilized in a minimum amount of DMF. The solution was then added along with 0.015 g DMAP (1.2 mmol, 0.1 eq) to the SPPS reaction vessel, which was subsequently sealed and occasionally stirred for 1 h. Upon

completion, the resin was washed with DMF and DCM. The resin was then dried under reduced pressure and the loading was quantified according to the procedure published by Gude *et al.* [56]. The resin was finally capped using 0.319 mL acetic anhydride (6 mmol, 5 eq), 1.176 mL DIPEA (12 mmol, 10 eq) in 15 mL DCM for 15 min. The resin was once again washed and dried under reduced pressure.

General synthetic procedure C

Wang resin was preloaded with Fmoc-Asp(OtBu)-OH offline using the resin loading protocol described above. Resin was loaded into a CEM Liberty Blue Peptide Synthesizer, and the cell impermeable inhibitors were generated using 2 min couplings at 90 °C (0.2 M amino acid (5 eq) in DMF, 1.0 M DIC (10 eq) in DMF, and 1.0 M Oxyma Pure (5 eq) in DMF and 1 min Fmoc deprotections at 90°C. The deprotection solution contained 20% Piperidine in DMF with 0.1 M Oxyma Pure to prevent aspartimide formation. Upon completion, the peptidic inhibitors were cleaved from resin using a cleavage cocktail of 95% TFA, 2.5% H₂O, and 2.5% TIPS for 4 h. The crude peptide was precipitated using cold ether, collected by centrifugation, and purified by semi-preparative HPLC.

Synthesis of compound 2

To a round bottom flask equipped with magnetic stir bar was added 5.000 g of commercially available Z-Glu(OtBu)-OH **1** (14.821 mmol, 1 eq) solubilized in 30 mL DMF with 2.656 g cesium carbonate (8.151 mmol, 0.55 eq) and stirred for 1 h. After which 0.923 mL iodomethane (14.821 mmol, 1 eq) was added and the reaction mixture was stirred overnight. Upon completion, the reaction mixture was diluted with ethyl acetate and washed sequentially with 1 M HCl, H₂O, sat. NaHCO₃ solution, and brine. The organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure to yield 5.04 g (97%) of the product as a white solid.

¹H NMR (400 MHz, CDCl₃) δ 7.39 – 7.28 (m, 5H), 5.46 (d, *J* = 8.2 Hz, 1H), 5.09 (s, 2H), 4.38 (td, *J* = 8.3, 5.0 Hz, 1H), 3.73 (s, 3H), 2.40 – 2.21 (m, 2H), 2.20 – 2.07 (m, 1H), 2.00 – 1.86 (m, 1H), 1.42 (s, 9H).

¹³C NMR (101 MHz, CDCl₃) δ 172.55, 172.02, 156.02, 136.33, 128.59, 128.24, 128.17, 80.87, 67.08, 53.54, 52.53, 31.47, 28.13, 27.67.

HRMS (ESI-QTOF) *m/z* [M + Na]⁺ calcd for C₁₈H₂₅NO₆Na 374.1580; found 374.1583.

Synthesis of compound 3

To a round bottom flask under inert atmosphere was added 5.04 g compound **2** (14.451 mmol, 1 eq) solubilized in 143 mL methanol. Palladium on carbon (0.305 g, 2.869 mmol, 20 mol%) was then added to the flask which was subsequently evacuated and backfilled three times with N₂ (g). The flask was then evacuated and backfilled three times with H₂ (g) and stirred overnight. Upon completion, the mixture was evacuated and backfilled three times with N₂ (g) and filtered over a pad a celite. The filtrate was then concentrated under reduced pressure to yield the product as a clear oil in quantitative yield (3.057 g) and carried forward without further purification.

^1H NMR (400 MHz, MeOD) δ 3.79 (s, 3H), 2.50 – 2.30 (m, 2H), 2.13 – 1.90 (m, 2H), 1.45 (s, 9H).

^{13}C NMR (101 MHz, MeOD) δ 173.71, 173.44, 81.97, 53.85, 53.12, 31.98, 28.77, 28.32.

HRMS (ESI-QTOF) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{10}\text{H}_{19}\text{NO}_4\text{Na}$ 240.1212; found 240.1216.

Synthesis of compound 5

To a round bottom flask equipped with magnetic stir bar was added 3.057 g compound **3** (14.343 mmol, 1 eq) solubilized in 143 mL DCM with 2.199 mL triethylamine (15.778 mmol, 1.2 eq). To the flask was added 2.734 g 1-naphthoyl chloride **4** (14.343 mmol, 1.1 eq) dropwise and the reaction was stirred. After five minutes 0.175 g DMAP (1.434 mmol, 10 mol%) was added and the reaction mixture was stirred for 2 h. Upon completion the reaction mixture was washed sequentially with 1 M HCl, H_2O , sat. NaHCO_3 solution, and brine. The organic layer was then dried over MgSO_4 , filtered, and concentrated under reduced pressure. The resulting crude oil was purified *via* flash chromatography to yield 4.47 g (84%) of the product as a white solid.

^1H NMR (400 MHz, CDCl_3) δ 8.41 – 8.33 (m, 1H), 7.92 (dt, $J = 8.3, 1.2$ Hz, 1H), 7.88 – 7.84 (m, 1H), 7.67 (dd, $J = 7.1, 1.2$ Hz, 1H), 7.59 – 7.49 (m, 2H), 7.45 (dd, $J = 8.3, 7.0$ Hz, 1H), 6.78 (d, $J = 7.9$ Hz, 1H), 3.81 (s, 3H), 2.54 – 2.24 (m, 3H), 2.18 – 2.01 (m, 1H), 1.41 (s, 9H).

^{13}C NMR (101 MHz, CDCl_3) δ 172.56, 172.28, 169.46, 133.80, 133.71, 131.09, 130.31, 128.39, 127.36, 126.57, 125.55, 125.39, 124.76, 81.13, 52.74, 52.36, 31.71, 28.14, 27.39.

HRMS (ESI-QTOF) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{21}\text{H}_{25}\text{NO}_5\text{Na}$ 394.1630; found 394.1641.

Synthesis of compound 6

Compound **6** was synthesized according to general synthetic procedure A. The product was obtained as a white solid (4.30 g, 99%).

^1H NMR (400 MHz, CDCl_3) δ 8.39 – 8.29 (m, 1H), 7.89 (dt, $J = 8.2, 1.0$ Hz, 1H), 7.86 – 7.80 (m, 1H), 7.63 (dd, $J = 7.1, 1.3$ Hz, 1H), 7.57 – 7.46 (m, 2H), 7.40 (dd, $J = 8.3, 7.0$ Hz, 1H), 7.09 (d, $J = 7.4$ Hz, 1H), 4.85 (td, $J = 7.8, 5.0$ Hz, 1H), 2.57 – 2.25 (m, 3H), 2.18 – 2.06 (m, 1H), 1.40 (s, 9H).

^{13}C NMR (101 MHz, CDCl_3) δ 175.21, 173.05, 170.30, 133.74, 133.16, 131.26, 130.24, 128.39, 127.42, 126.59, 125.62, 125.46, 124.71, 81.56, 52.64, 31.89, 28.10, 26.93.

HRMS (ESI-QTOF) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{20}\text{H}_{23}\text{NO}_5\text{Na}$ 380.1474; found 380.1468.

Synthesis of compound 8

To a round bottom flask equipped with magnetic stir bar was added 2.000 g of commercially available Z-Lys-OH **7** (7.13 mmol, 1 eq) suspended in 50 mL MeOH under nitrogen atmosphere. The suspension was cooled to 0 °C and 0.78 mL thionyl chloride (10.7 mmol, 1.5 eq) was added

dropwise. The reaction mixture was allowed to warm to room temperature and was stirred overnight. Upon completion, the volatiles were removed under reduced pressure to yield the product in quantitative yield (2.35 g) as a white solid and was carried forward without further purification or characterization.

Synthesis of compound 10

To a round bottom flask equipped with magnetic stir bar was added 4.348 g compound **8** (13.144 mmol, 1 eq) solubilized in 40 mL ACN with 6.868 mL DIPEA (39.431 mmol, 3 eq) and 0.161 g DMAP (1.314 mmol, 10 mol%) at 0 °C. Acryloyl chloride **9** (1.267 mL, 15.772 mmol, 1.2 eq) was solubilized in 12 mL ACN and added dropwise to the reaction mixture *via* dropping funnel. The reaction was stirred for 1 h after which the reaction mixture was concentrated under reduced pressure and the resulting oil was taken up in ethyl acetate. The organic mixture was washed once with 1 M HCl, H₂O, sat. NaHCO₃ solution, and brine. The organic layer was then dried over MgSO₄, filtered, and concentrated under reduced pressure. The resulting crude oil was purified *via* flash chromatography to yield 2.65 g of the product (58%) as a white solid.

¹H NMR (400 MHz, CDCl₃) δ 7.29 (d, *J* = 5.0 Hz, 5H), 6.58 – 6.47 (m, 1H), 6.20 (dd, *J* = 17.0, 1.8 Hz, 1H), 6.07 (dd, *J* = 17.0, 10.1 Hz, 1H), 5.75 (d, *J* = 8.1 Hz, 1H), 5.51 (dd, *J* = 10.0, 1.8 Hz, 1H), 5.04 (s, 2H), 4.26 (td, *J* = 8.2, 4.7 Hz, 1H), 3.66 (s, 3H), 3.23 (qt, *J* = 10.6, 5.6 Hz, 2H), 1.84 – 1.58 (m, 2H), 1.57 – 1.40 (m, 2H), 1.40 – 1.25 (m, 2H).

¹³C NMR (101 MHz, CDCl₃) δ 172.91, 165.85, 156.18, 136.18, 130.89, 128.44, 128.10, 127.95, 126.08, 66.84, 53.66, 52.30, 38.84, 31.79, 28.73, 22.44.

Synthesis of compound 11

Compound **11** was synthesized according to general synthetic procedure A. The product was obtained as a white solid (7.807 g, quantitative yield).

¹H NMR (400 MHz, CDCl₃) δ 7.36 – 7.27 (m, 5H), 6.30 – 6.19 (m, 2H), 6.08 (dd, *J* = 17.0, 10.3 Hz, 1H), 5.75 (d, *J* = 8.0 Hz, 1H), 5.60 (dd, *J* = 10.3, 1.4 Hz, 1H), 5.08 (d, *J* = 1.8 Hz, 2H), 4.35 (q, *J* = 7.3 Hz, 1H), 3.29 (d, *J* = 2.9 Hz, 2H), 1.95 – 1.67 (m, 2H), 1.60 – 1.30 (m, 4H).

¹³C NMR (101 MHz, CDCl₃) δ 174.91, 166.49, 156.40, 136.18, 130.43, 128.54, 128.21, 128.06, 127.11, 67.06, 53.52, 39.22, 31.79, 28.73, 22.22.

HRMS (ESI-QTOF) *m/z* [M + Na]⁺ calcd for C₁₇H₂₂N₂O₅Na 357.1426; found 357.1446.

Synthesis of compound 13

Compound **13** was synthesized according to general synthetic procedure B. The product was obtained after flash chromatography as a white solid (1.816 g, 98%).

¹H NMR (400 MHz, CDCl₃) δ 7.34 – 7.27 (m, 5H), 6.29 (t, *J* = 5.7 Hz, 1H), 6.21 (dd, *J* = 17.0, 1.7 Hz, 1H), 6.06 (dd, *J* = 17.0, 10.2 Hz, 1H), 5.95 (d, *J* = 8.3 Hz, 1H), 5.54 (dd, *J* = 10.1, 1.7 Hz,

¹H), 5.04 (s, 2H), 4.59 (td, $J = 8.2, 4.4$ Hz, 1H), 3.66 – 3.21 (m, 10H), 1.70 – 1.61 (m, 1H), 1.60 – 1.46 (m, 3H), 1.43 (s, 9H), 1.40 – 1.30 (m, 2H).

¹³C NMR (101 MHz, CDCl₃) δ 170.47, 165.76, 156.23, 154.42, 136.24, 130.93, 128.52, 128.18, 127.99, 126.15, 80.42, 66.91, 50.33, 45.36, 41.96, 38.96, 32.75, 28.80, 28.35, 22.26.

HRMS (ESI-QTOF) m/z [M + Na]⁺ calcd for C₂₆H₃₈N₄O₆Na 525.2689; found 525.2674.

Synthesis of compound 14

To a round bottom flask equipped with magnetic stir bar was added 1.816 g compound **13** (3.613 mmol, 1 eq) solubilized in 36 mL of a 20% v/v TFA in DCM solution. The reaction mixture was stirred for 2 h upon which the solvent was removed under reduced pressure. The resulting oil was co-evaporated 3 times with DCM under reduced pressure to yield the product in quantitative yield (1.866 g) as a brown oil. The product was carried forward as the TFA salt without further purification or characterization.

Synthesis of compound 15

Compound **15** was synthesized according to general synthetic procedure B. The product was obtained after flash chromatography as a white solid (1.778 g, 66%)

¹H NMR (400 MHz, CDCl₃) δ 8.32 (d, $J = 7.9$ Hz, 1H), 7.88 (d, $J = 8.3$ Hz, 1H), 7.83 (dd, $J = 7.6, 1.8$ Hz, 1H), 7.63 (ddd, $J = 7.1, 3.0, 1.2$ Hz, 1H), 7.55 – 7.46 (m, 2H), 7.41 (dd, $J = 8.3, 7.0$ Hz, 1H), 7.31 (d, $J = 3.4$ Hz, 5H), 7.25 – 7.18 (m, 1H), 6.31 – 6.27 (m, 1H), 6.26 – 6.15 (m, 1H), 6.12 – 5.98 (m, 2H), 5.57 – 5.50 (m, 1H), 5.32 – 5.26 (m, 1H), 5.05 (s, 2H), 4.64 – 4.57 (m, 1H), 3.94 – 3.11 (m, 11H), 2.54 – 2.30 (m, 2H), 2.18 – 2.06 (m, 1H), 1.87 – 1.76 (m, 1H), 1.66 – 1.28 (m, 15H).

¹³C NMR (101 MHz, CDCl₃) δ 172.08, 170.60, 170.24, 169.20, 165.63, 156.20, 136.26, 133.64, 130.90, 130.14, 128.51, 128.32, 128.18, 128.00, 127.17, 126.41, 126.08, 125.28, 124.65, 80.89, 66.89, 50.31, 48.40, 45.04, 42.10, 38.85, 32.54, 30.56, 28.84, 28.09, 27.87, 22.24. (Rotamers of piperazine observed at 45.04 ppm and 42.10 ppm)

HRMS (ESI-QTOF) m/z [M + Na]⁺ calcd for C₄₁H₅₁N₅O₈Na 764.3635; found 764.3645.

Synthesis of compound 16

To a round bottom flask equipped with magnetic stir bar was added 0.100 g compound **15** (0.135 mmol, 1 eq) solubilized in 1.5 mL of a 20% v/v TFA in DCM solution. The reaction mixture was stirred for 4 h upon which the solvent was removed under reduced pressure. The resulting brown oil was then precipitated using cold ether. The crude solid was further purified by flash chromatography to yield 0.039 g (42%) of product as a white solid.

¹H NMR (600 MHz, CDCl₃) δ 8.31 – 8.24 (m, 1H), 7.88 (t, $J = 8.1$ Hz, 1H), 7.85 – 7.80 (m, 1H), 7.66 – 7.61 (m, 1H), 7.57 – 7.45 (m, 2H), 7.43 – 7.27 (m, 7H), 6.34 – 6.19 (m, 2H), 6.13 – 5.96

(m, 2H), 5.61 – 5.51 (m, 1H), 5.40 – 5.30 (m, 1H), 5.10 – 5.02 (m, 2H), 4.71 – 4.60 (m, 1H), 3.93 – 3.15 (m, 11H), 2.66 – 2.55 (m, 1H), 2.53 – 2.43 (m, 1H), 2.22 – 2.08 (m, 1H), 2.00 – 1.80 (m, 1H), 1.72 – 1.42 (m, 4H), 1.40 – 1.28 (m, 2H).

¹³C NMR (151 MHz, CDCl₃) δ 175.18, 170.62, 169.64, 166.38, 166.11, 156.40, 136.30, 133.75, 133.34, 133.31, 131.22, 130.67, 130.18, 128.66, 128.48, 128.33, 128.14, 128.12, 127.40, 126.60, 125.64, 125.34, 124.78, 67.16, 67.15, 67.12, 67.10, 50.50, 48.82, 48.77, 48.60, 45.57, 42.15, 39.13, 32.64, 29.51, 28.83, 22.33.

HRMS (ESI-QTOF) m/z [M + Na]⁺ calcd for C₃₇H₄₃N₅O₈Na 708.3009; found 708.2991.

Synthesis of compound 17

Compound **17** was synthesized according to general synthetic procedure C using Fmoc-Aminohexanoic acid-OH as linker on 0.1 mmol scale (3.4 mg isolated).

HRMS (ESI-QTOF) m/z [M – H][–] calcd for C₅₅H₆₈N₉O₁₈ 1142.4682; found 1442.4698.

Purity: 95.5%

Synthesis of compound 18 (NCEG2)

Compound **18** was synthesized according to general synthetic procedure C using three glycine residues (iterative couplings and deprotections) as linker on 0.1 mmol scale (7.4 mg isolated).

HRMS (ESI-QTOF) m/z [M – H][–] calcd for C₅₅H₆₆N₁₁O₂₀ 1200.4486; found 1200.4473.

Purity: 96.4%

Synthesis of compound 19

Compound **19** was synthesized according to general synthetic procedure B. The product was obtained after flash chromatography as a white solid (0.098 g, 63%).

HRMS (ESI-QTOF) m/z [M + Na]⁺ calcd for C₄₇H₆₃N₇O₉Na 892.4585; found 892.4579.

Synthesis of compound 20

To a round bottom flask equipped with magnetic stir bar was added 0.319 g **19** (0.367 mmol, 1 eq) solubilized in 2 mL of a 1:1 mixture of 4 M HCl in dioxane and DCM. The reaction was stirred for 2 h after which the solvents were removed under reduced pressure. The product was carried forward as the HCl salt in quantitative yield (0.296 g) and without further purification or characterization.

Synthesis of compound 22

Compound **22** was synthesized according to general synthetic procedure B. The product was obtained after flash chromatography as a vibrant yellow solid (0.073 g, 59%).

HRMS (ESI-QTOF) m/z $[M + Na]^+$ calcd for $C_{56}H_{68}N_8O_{10}Na$ 1035.4956; found 1035.4944.

Purity: 85.3%

Synthesis of compound 23

To a round bottom flask equipped with magnetic stir bar was added 0.099 g compound **20** (0.123 mmol, 1 eq) solubilized in 2 mL DMF with 0.021 mL DIPEA (0.123 mmol, 1 eq). To the flask was added 0.053 g fluorescein isothiocyanate isomer I (0.135 mmol, 1.1 eq) and 0.043 mL DIPEA (0.246 mmol, 2 eq). The reaction mixture was stirred for 48 h at room temperature. The volatiles were then evaporated by a stream of compressed air overnight. The resulting crude oil was purified *via* flash chromatography twice to yield 0.052 g (37%) of the product as a yellow solid.

HRMS (ESI-QTOF) m/z $[M + Na]^+$ calcd for $C_{63}H_{66}N_8O_{12}SNa$ 1181.4419; found 1181.4425.

Purity: 89.9%

Synthesis of compound 25

Compound **25** was synthesized according to general synthetic procedure B. The product was obtained after flash chromatography as a colourless oil (0.470 g, 81%).

1H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.21 (m, 5H), 5.14 – 4.94 (m, 2H), 4.26 – 4.19 (m, 1H), 3.56 – 3.31 (m, 2H), 3.19 – 2.90 (m, 4H), 2.25 – 2.01 (m, 1H), 1.94 – 1.72 (m, 3H), 1.45 – 1.16 (m, 15H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.05, 155.98, 136.29, 128.39, 127.98, 127.69, 78.74, 67.08, 60.50, 46.92, 40.20, 39.02, 29.36, 28.98, 28.32, 23.73.

HRMS (ESI-QTOF) m/z $[M + Na]^+$ calcd for $C_{23}H_{35}N_3O_5Na$ 456.2474; found 256.2462.

Synthesis of compound 26

To a round bottom flask under inert atmosphere was added 0.470 g compound **25** (1.084 mmol, 1 eq) solubilized in 11 mL methanol. Palladium on carbon (0.023 g, 0.217 mmol, 20 mol%) was then added to the flask which was subsequently evacuated and backfilled three times with N_2 (g). The flask was then evacuated and backfilled three times with H_2 (g) and stirred overnight. Upon completion, the mixture was evacuated and backfilled three times with N_2 (g) and filtered over a pad a celite. The filtrate was then concentrated under reduced pressure to yield the product as a clear oil in quantitative yield (0.325 g) and carried forward without further purification or characterization.

Synthesis of compound 27

Compound **27** was synthesized according to general synthetic procedure B excluding the 1 M HCl washes. The product was obtained after flash chromatography as a purple solid (0.663 g, 80%).

^1H NMR (400 MHz, CDCl_3) δ 7.77 – 7.48 (m, 3H), 7.35 – 7.27 (m, 2H), 7.24 – 7.14 (m, 1H), 6.93 – 6.85 (m, 1H), 6.82 – 6.74 (m, 2H), 6.67 (dd, $J = 3.5, 2.4$ Hz, 1H), 4.19 (dd, $J = 7.7, 5.3$ Hz, 1H), 3.65 – 3.46 (m, 10H), 3.32 – 2.87 (m, 5H), 2.11 – 1.14 (m, 31H).

^{13}C NMR (101 MHz, CDCl_3) δ 172.43, 170.79, 167.88, 167.48, 157.94, 155.64, 136.96, 136.48, 133.35, 132.37, 131.44, 130.61, 130.49, 130.29, 130.23, 130.16, 129.87, 129.80, 129.41, 127.92, 127.34, 115.78, 114.61, 114.13, 114.06, 113.89, 113.61, 113.35, 62.54, 60.17, 50.49, 46.16, 39.25, 32.51, 29.57, 29.05, 28.97, 28.55, 28.53, 28.28, 25.45, 24.02, 23.96, 22.19, 12.66.

HRMS (ESI-QTOF) m/z $[\text{M}]^+$ calcd for $\text{C}_{43}\text{H}_{58}\text{N}_5\text{O}_5$ 724.4438; found 724.4414.

Synthesis of compound 28

To a round bottom flask equipped with magnetic stir bar was added 0.103 g **27** (0.135 mmol, 1 eq) solubilized in 1 mL of a 1:1 mixture of 4 M HCl in dioxane and DCM. The reaction was stirred for 2.5 h after which the solvents were removed under reduced pressure. The product was co-evaporated twice with DCM and then carried forward as the HCl salt in quantitative yield (0.094 g) and without further purification or characterization.

Synthesis of compound 29

Compound **29** was synthesized according to general synthetic procedure B excluding the 1 M HCl washes. The product was obtained after flash chromatography as a purple solid (0.035 g, 20%).

HRMS (ESI-QTOF) m/z $[\text{M}]^+$ calcd for $\text{C}_{75}\text{H}_{91}\text{N}_{10}\text{O}_{10}$ 1291.6920; found 1291.6892.

Purity: 91.9%

Synthesis of compound 31

Compound **31** was synthesized according to general synthetic procedure B. The product was obtained after flash chromatography as a white solid (0.103 g, 91%).

^1H NMR (600 MHz, CDCl_3) δ 8.35 – 8.28 (m, 1H), 7.95 – 7.90 (m, 1H), 7.86 (d, $J = 8.1$ Hz, 1H), 7.68 – 7.63 (m, 1H), 7.58 – 7.49 (m, 2H), 7.45 – 7.30 (m, 6H), 6.27 – 6.17 (m, 1H), 6.16 – 6.01 (m, 2H), 5.95 – 5.81 (m, 1H), 5.61 – 5.53 (m, 1H), 5.23 – 5.13 (m, 1H), 5.09 – 5.02 (m, 2H), 4.66 – 4.57 (m, 1H), 4.17 – 4.06 (m, 1H), 4.06 – 3.94 (m, 1H), 3.87 – 3.12 (m, 10H), 2.54 – 2.43 (m, 1H), 2.42 – 2.34 (m, 1H), 2.25 – 2.14 (m, 2H), 1.97 – 1.86 (m, 1H), 1.72 – 1.44 (m, 4H), 1.42 – 1.31 (m, 2H).

^{13}C NMR (151 MHz, CDCl_3) δ 172.00, 170.67, 170.00, 169.90, 165.78, 156.25, 136.31, 136.29, 133.80, 133.79, 131.38, 130.94, 130.15, 130.13, 128.74, 128.63, 128.55, 128.31, 128.29, 128.14, 128.11, 128.09, 127.46, 127.44, 127.41, 126.62, 126.41, 126.40, 125.68, 125.65, 125.22, 125.20, 124.76, 80.05, 71.30, 67.06, 50.36, 50.34, 50.25, 49.03, 49.01, 48.99, 48.94, 45.60, 45.54, 45.16, 45.11, 42.05, 41.79, 41.73, 39.04, 39.00, 38.97, 32.74, 32.72, 32.68, 32.65, 32.03, 32.01, 29.29, 29.27, 29.24, 29.19, 29.18, 29.00, 28.93, 28.90, 22.38, 22.29, 22.27.

HRMS (ESI-QTOF) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{40}\text{H}_{46}\text{N}_6\text{O}_7\text{Na}$ 745.3326; found 745.3307.

Purity: 94.7%

Synthesis of compound 32

Compound **32** was synthesized according to general synthetic procedure B using commercially available desthiobiotin and amine-peg3-azide. The product was obtained after flash chromatography as an orange oil (0.320 g, >99%).

^1H NMR (400 MHz, CDCl_3) δ 6.62 (t, $J = 5.6$ Hz, 1H), 5.91 (s, 1H), 5.16 (s, 1H), 3.86 – 3.75 (m, 1H), 3.73 – 3.57 (m, 11H), 3.54 (dd, $J = 5.6, 4.6$ Hz, 2H), 3.45 – 3.33 (m, 4H), 2.17 (t, $J = 7.4$ Hz, 2H), 1.63 (p, $J = 7.1$ Hz, 2H), 1.53 – 1.19 (m, 8H), 1.09 (d, $J = 6.5$ Hz, 3H).

^{13}C NMR (101 MHz, CDCl_3) δ 173.33, 164.08, 70.73, 70.58, 70.22, 70.11, 70.01, 56.19, 51.54, 50.73, 39.23, 35.97, 29.51, 28.63, 25.92, 25.26, 15.80.

HRMS (ESI-QTOF) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{18}\text{H}_{34}\text{N}_6\text{O}_5\text{Na}$ 437.2488; found 437.2462.

Synthesis of compound 33

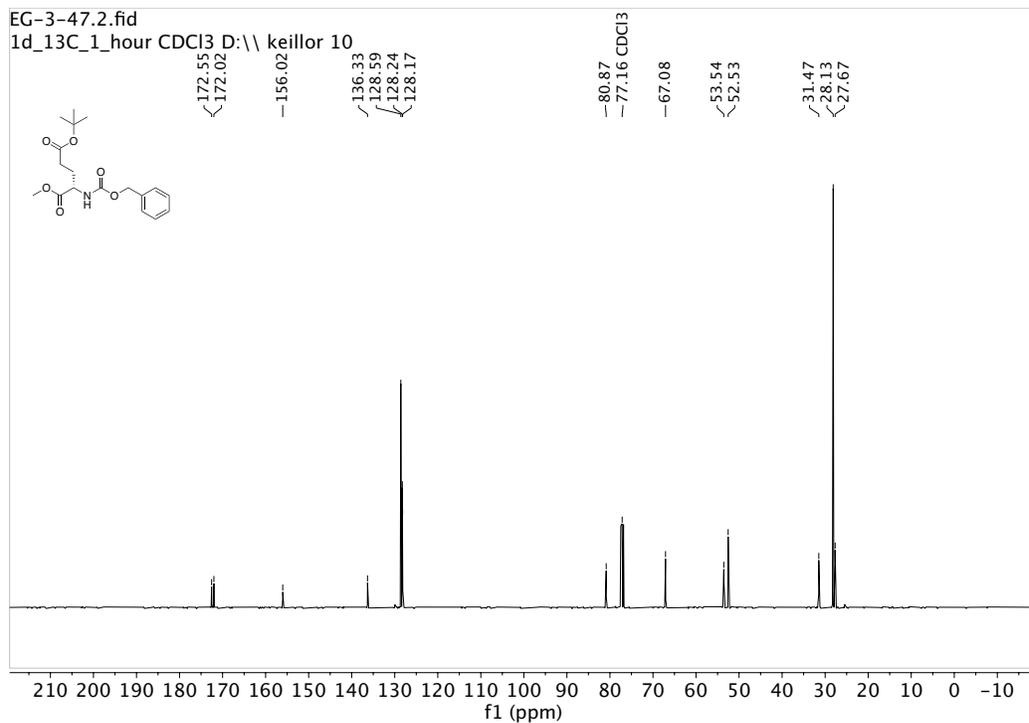
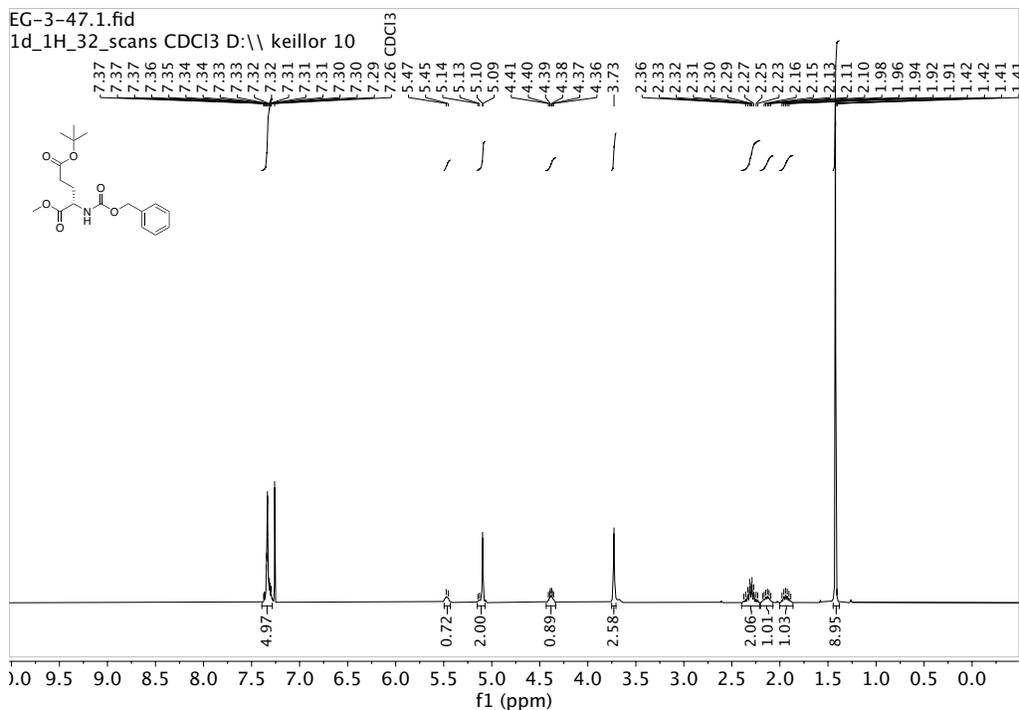
To a round bottom flask equipped with magnetic stir bar was added a slurry of 0.038 g alkyne **31** (0.053 mmol, 1 eq), desthiobiotin-peg3-azide **32** (0.053 mmol, 1 eq), and copper (II) sulfate pentahydrate (0.053 mmol, 1 eq) in 1 mL 1:1 ACN: H_2O . To the flask was then added 0.010 g sodium ascorbate (0.053 mmol, 1 eq) and the reaction was stirred at room temperature for 3 h. Upon completion the reaction mixture was filtered and subsequently concentrated under reduced pressure. The resulting crude oil was purified *via* preparatory-TLC to obtain the product as a clear oil (0.054 g, 90%)

HRMS (ESI-QTOF) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{58}\text{H}_{80}\text{N}_{12}\text{O}_{12}\text{Na}$ 1159.5916; found 1159.5928.

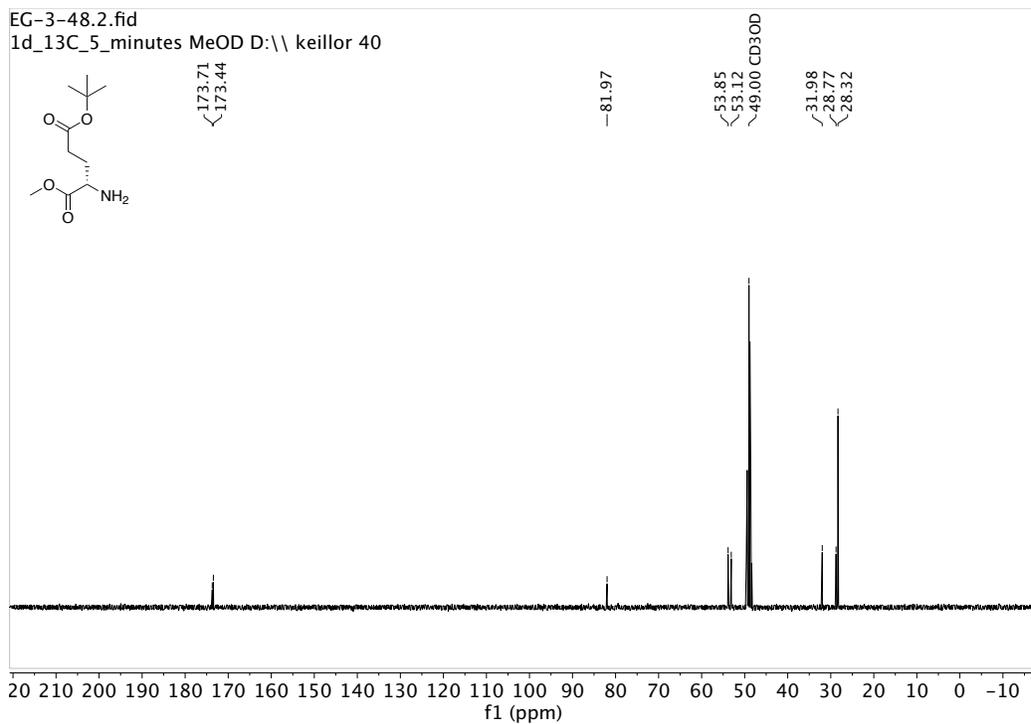
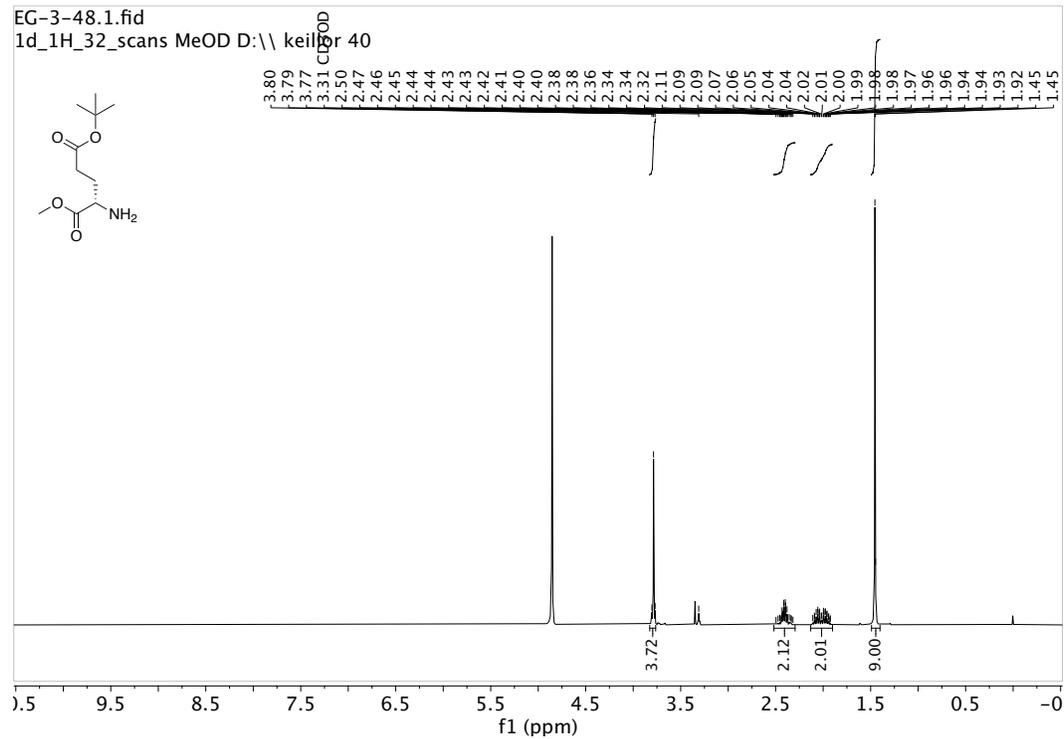
Purity: 92.4%

NMR spectra of intermediates and final compounds

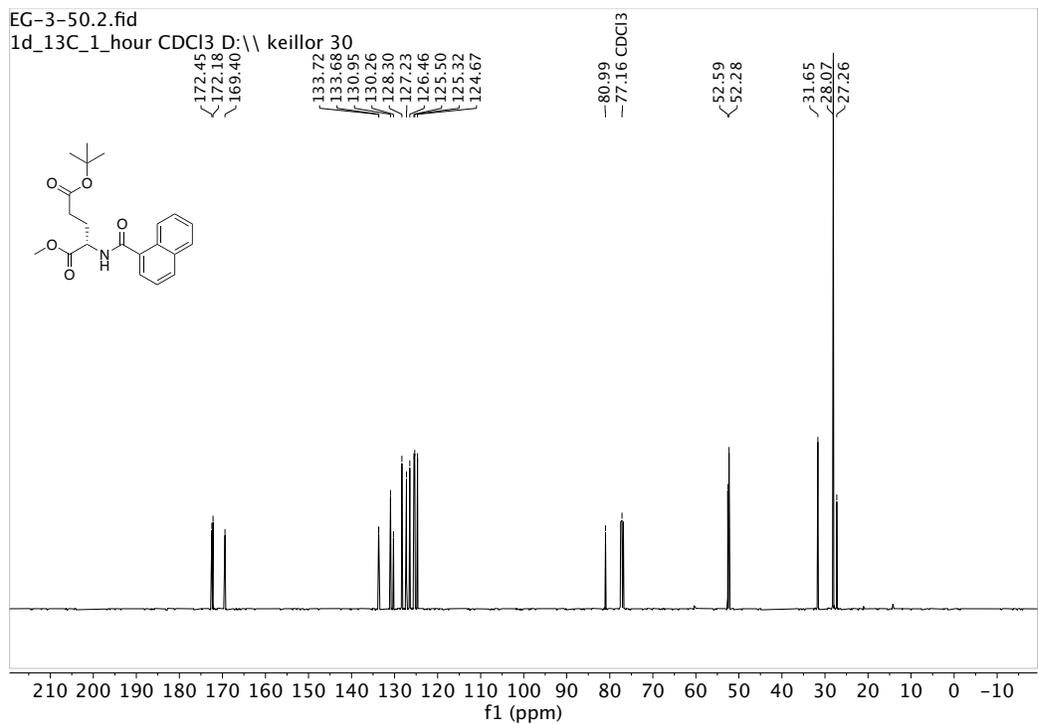
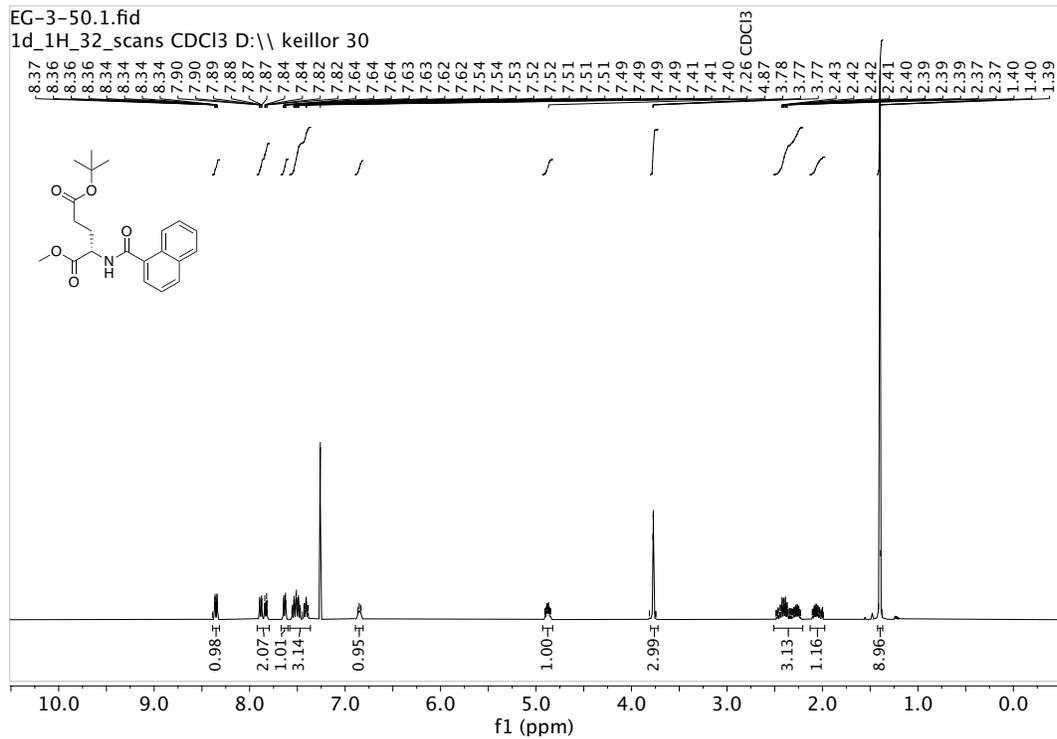
Compound 2



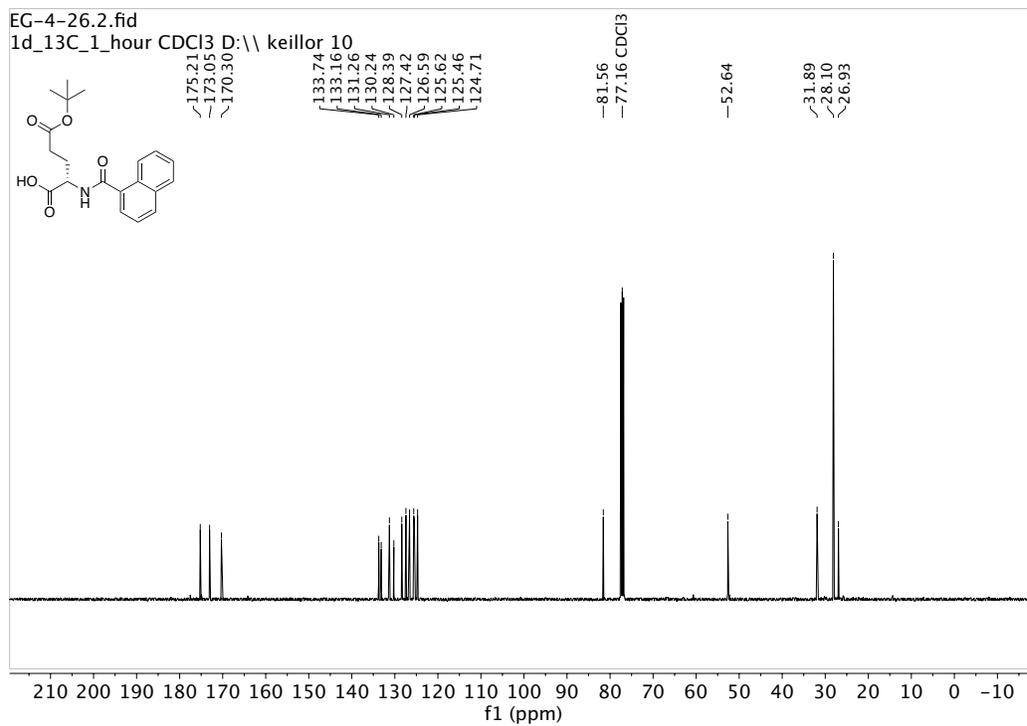
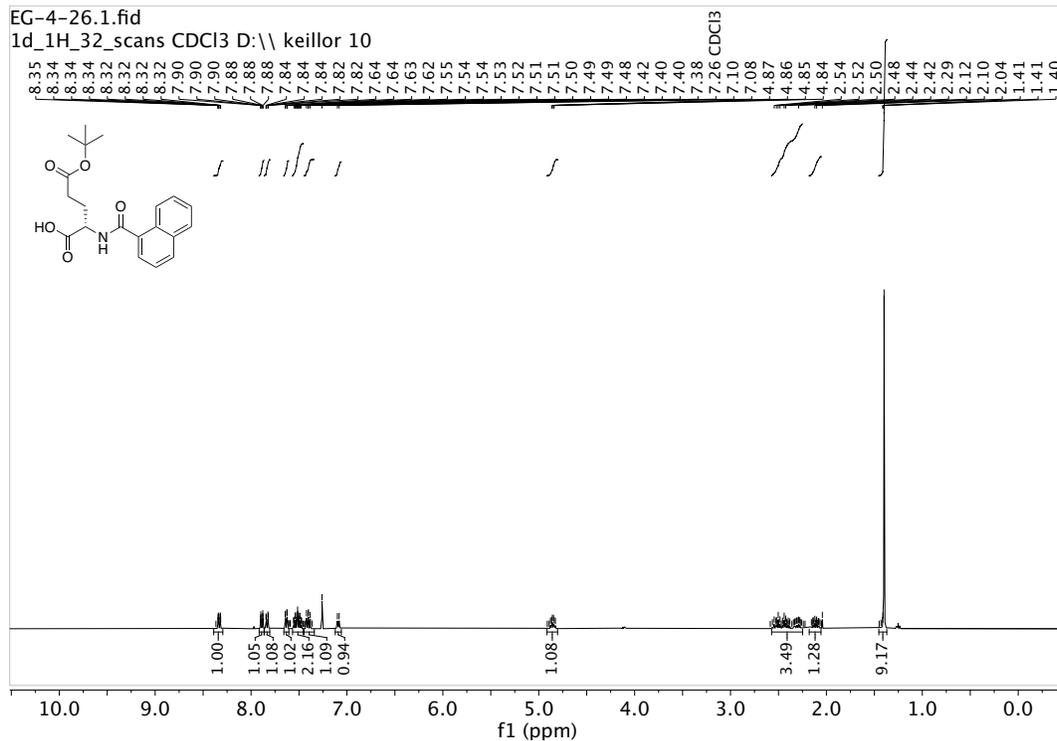
Compound 3



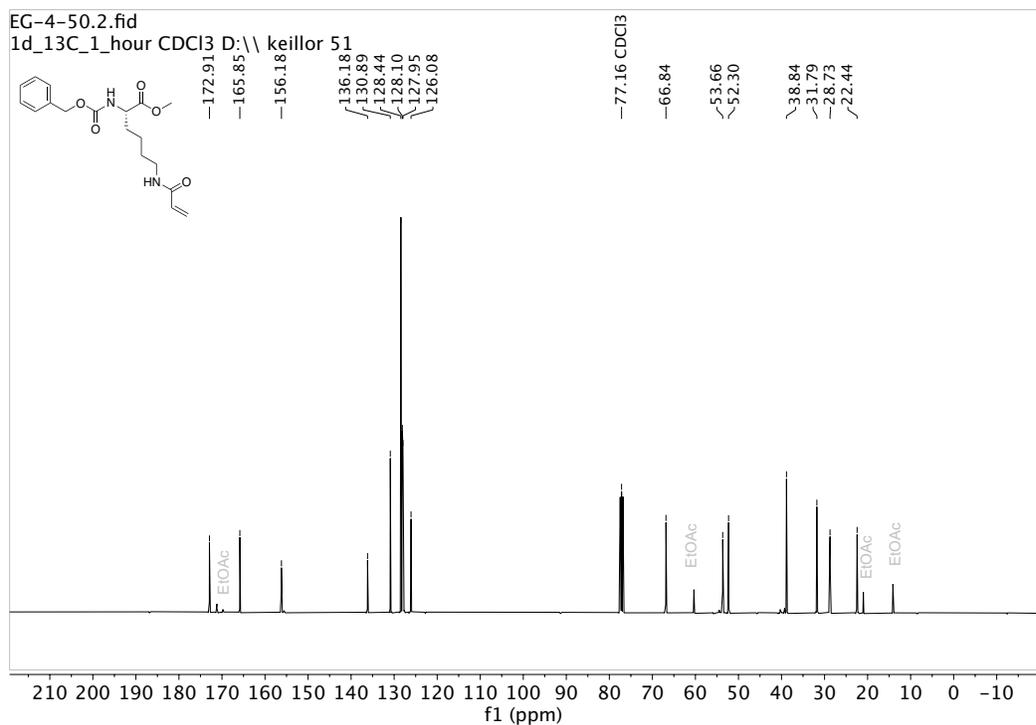
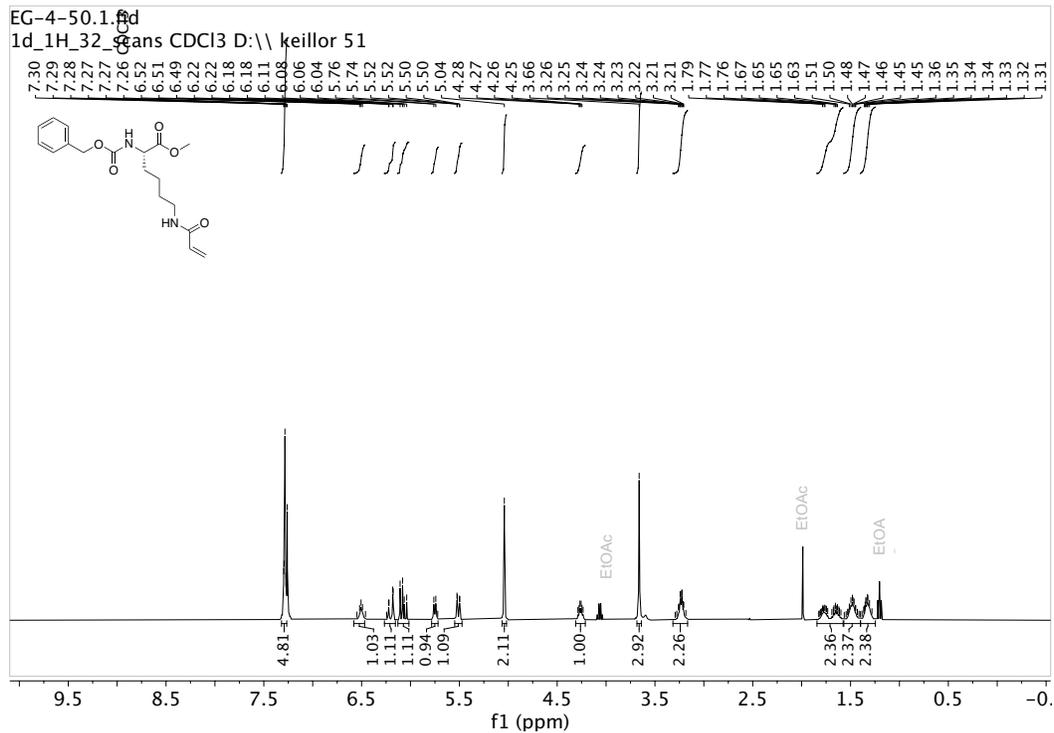
Compound 5



Compound 6



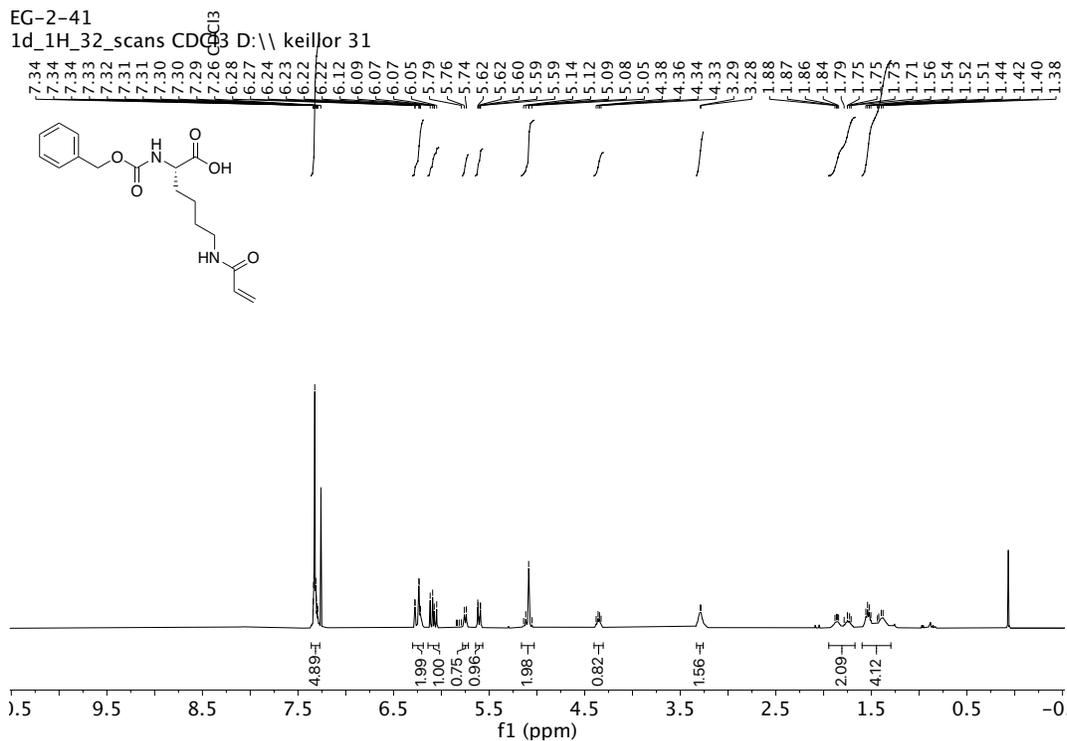
Compound 10



Compound 11

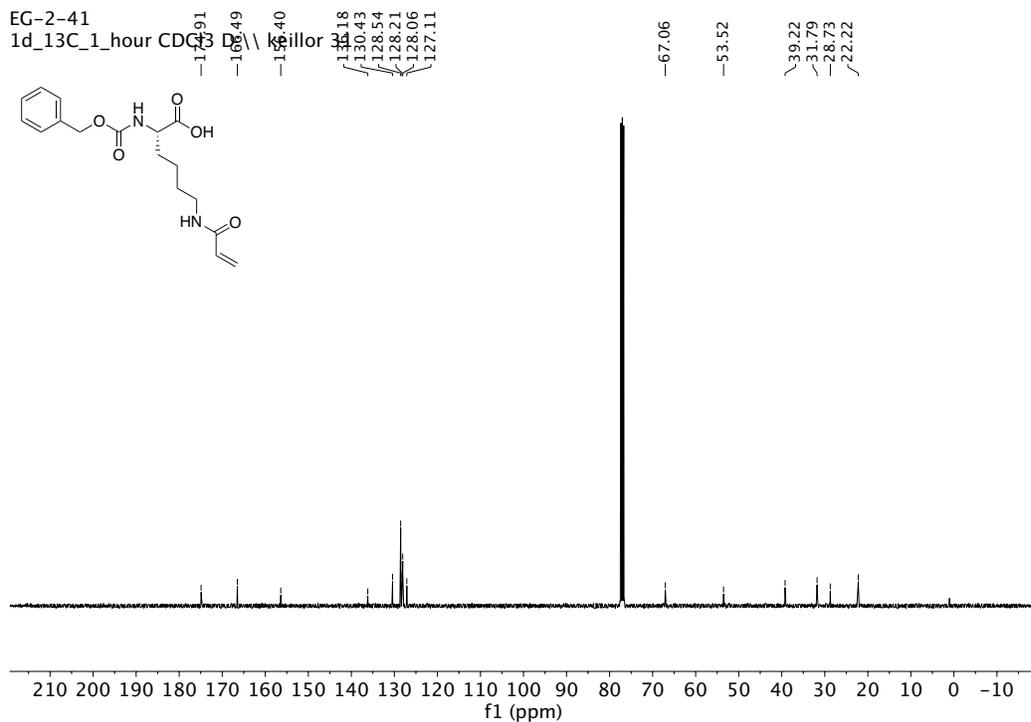
EG-2-41

1d_1H_32_scans CDCl₃ D:\\ keillor 31

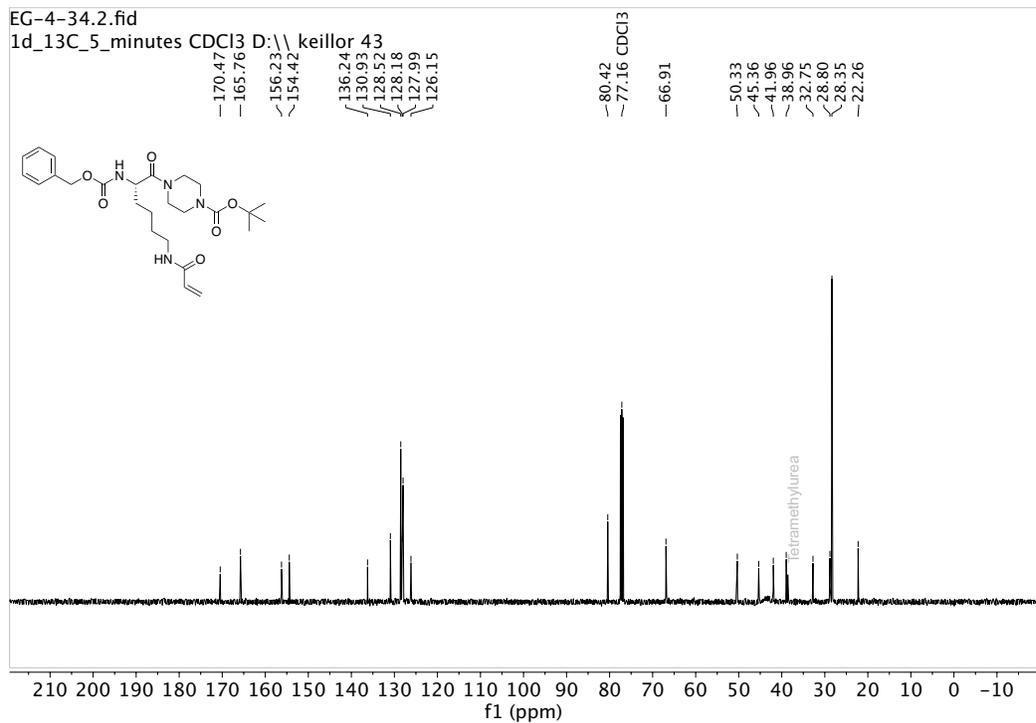
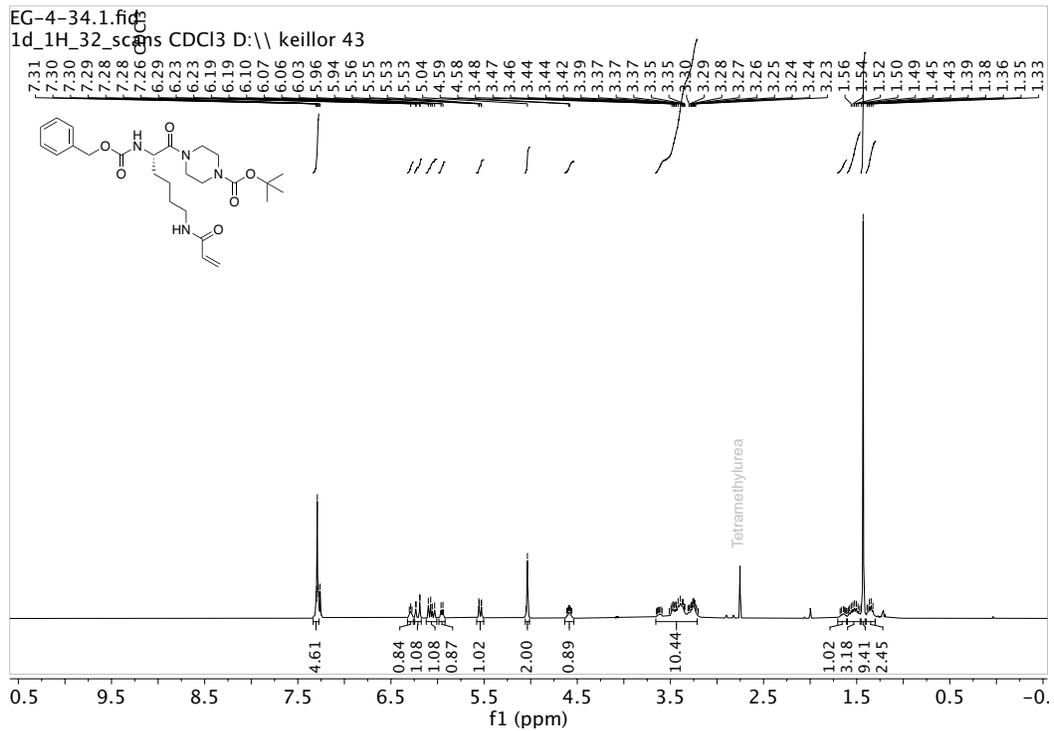


EG-2-41

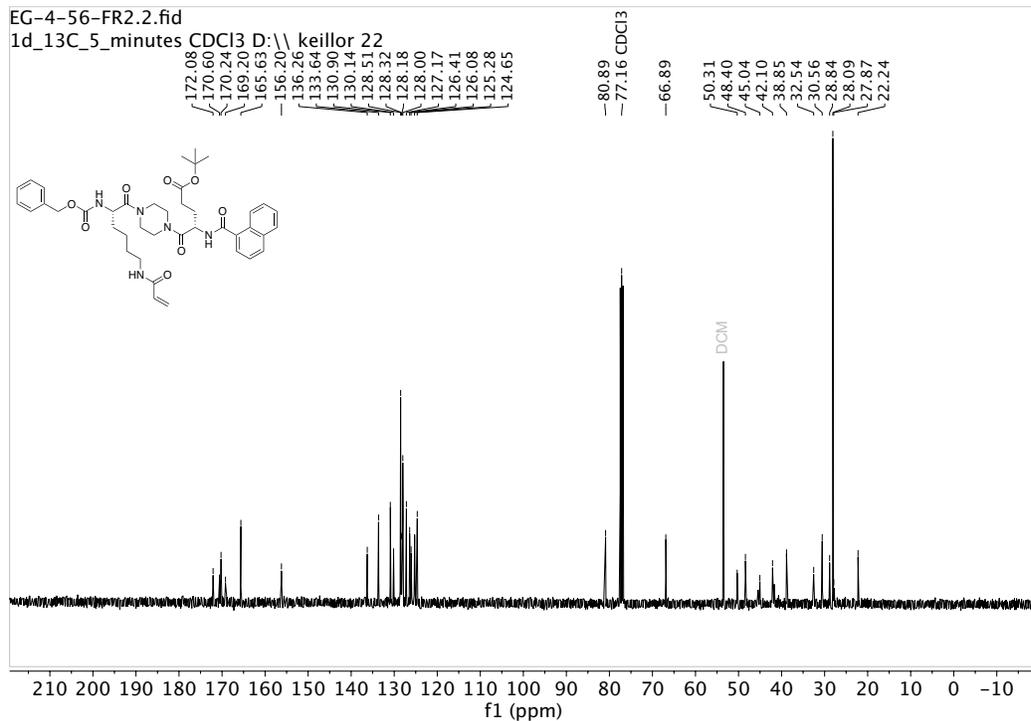
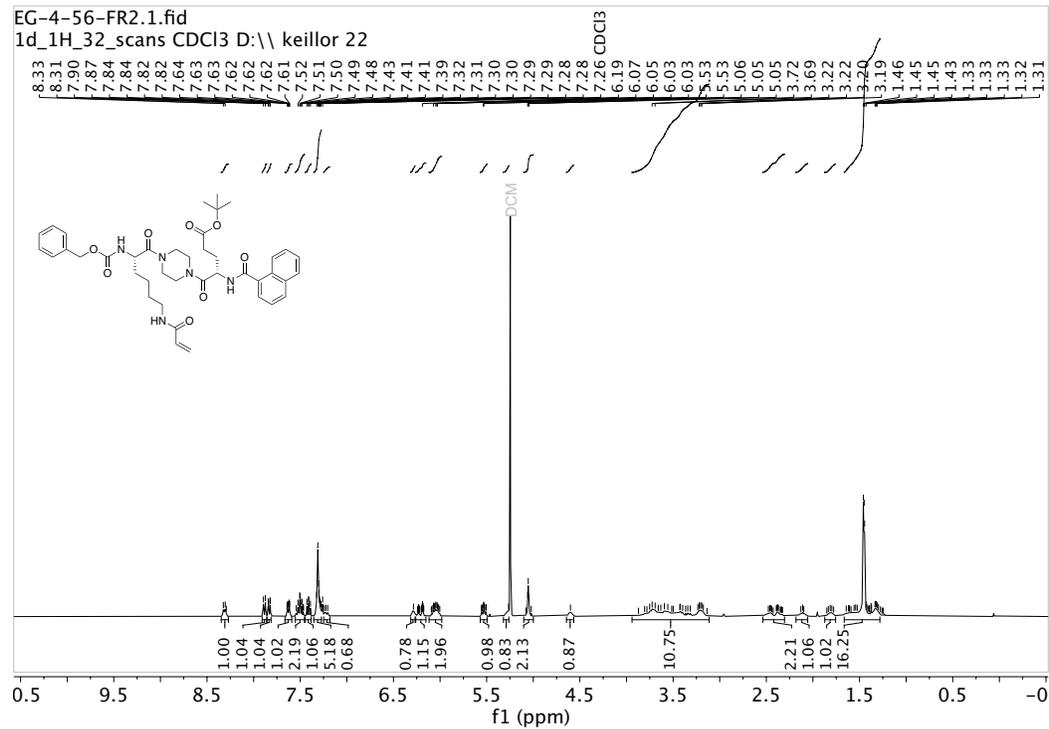
1d_13C_1_hour CDCl₃ D:\\ keillor 31



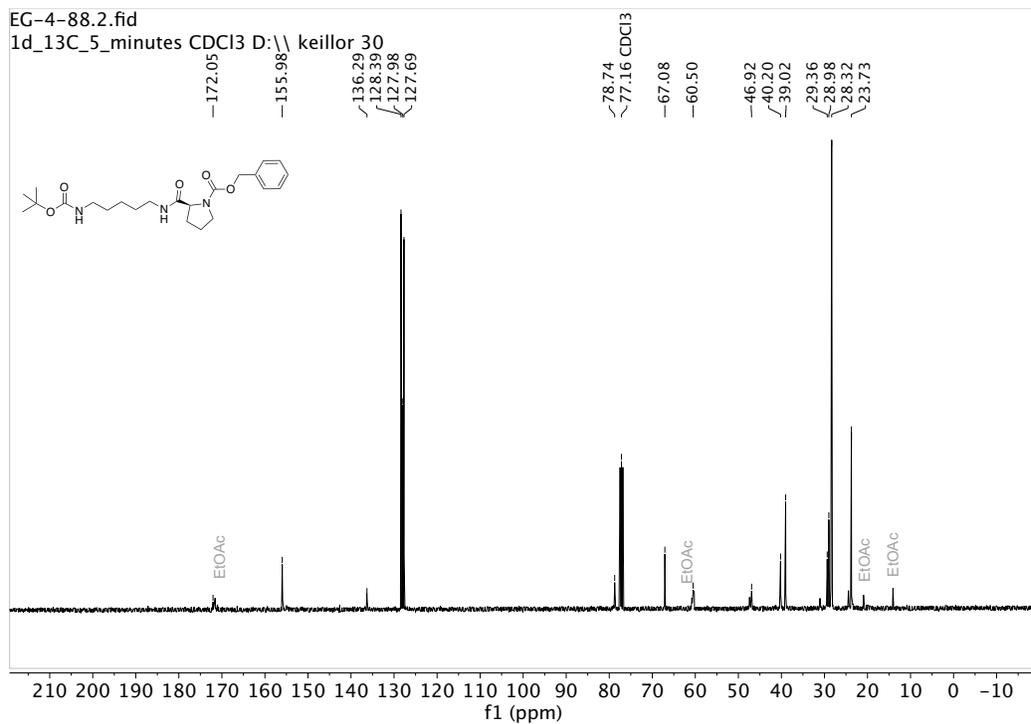
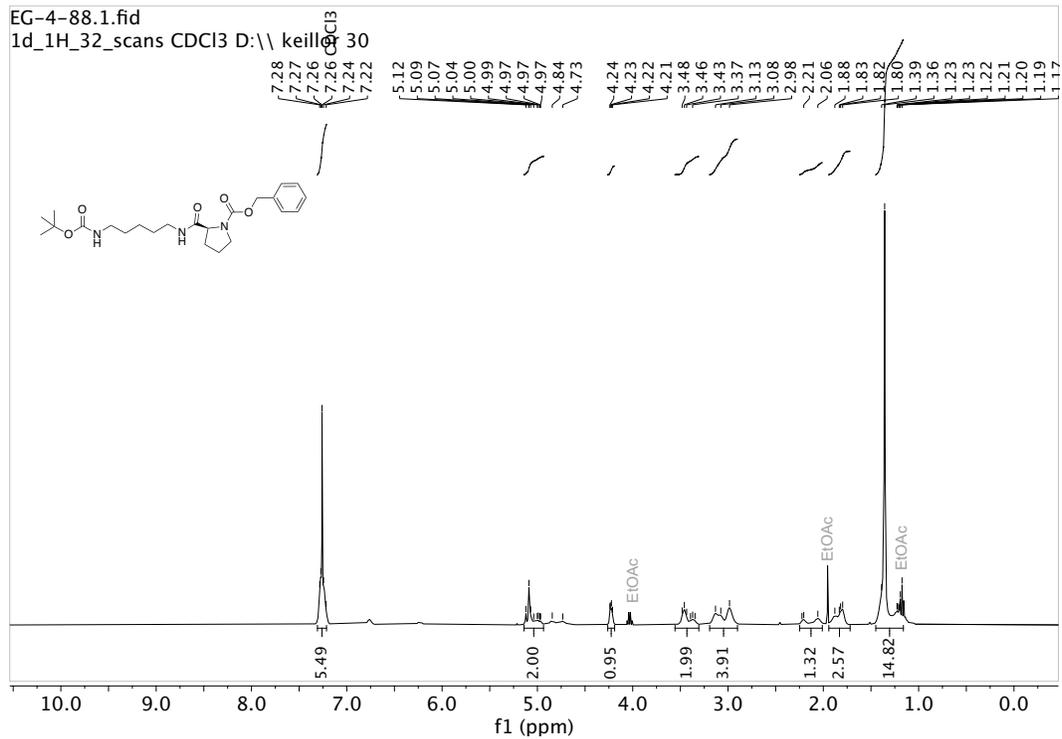
Compound 13



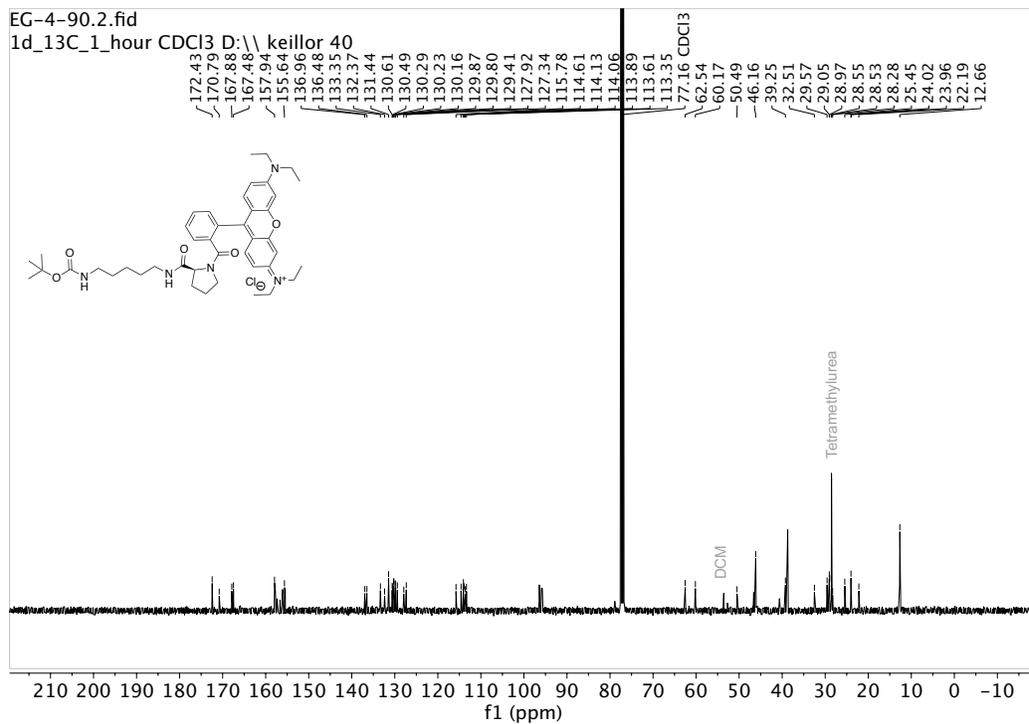
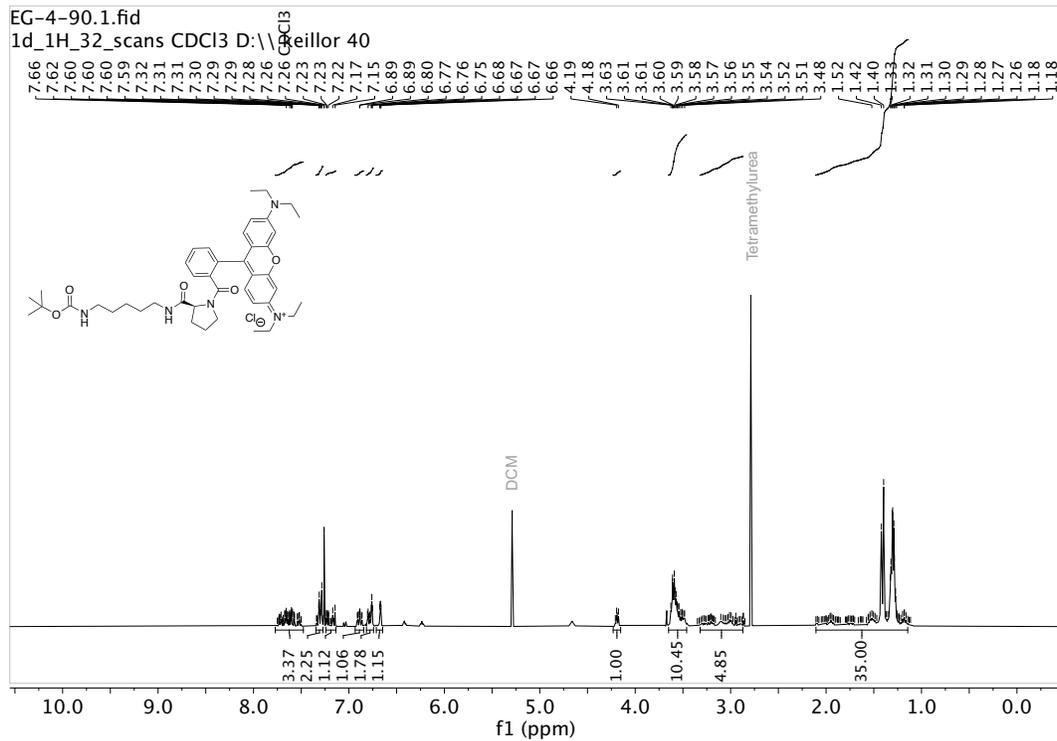
Compound 15



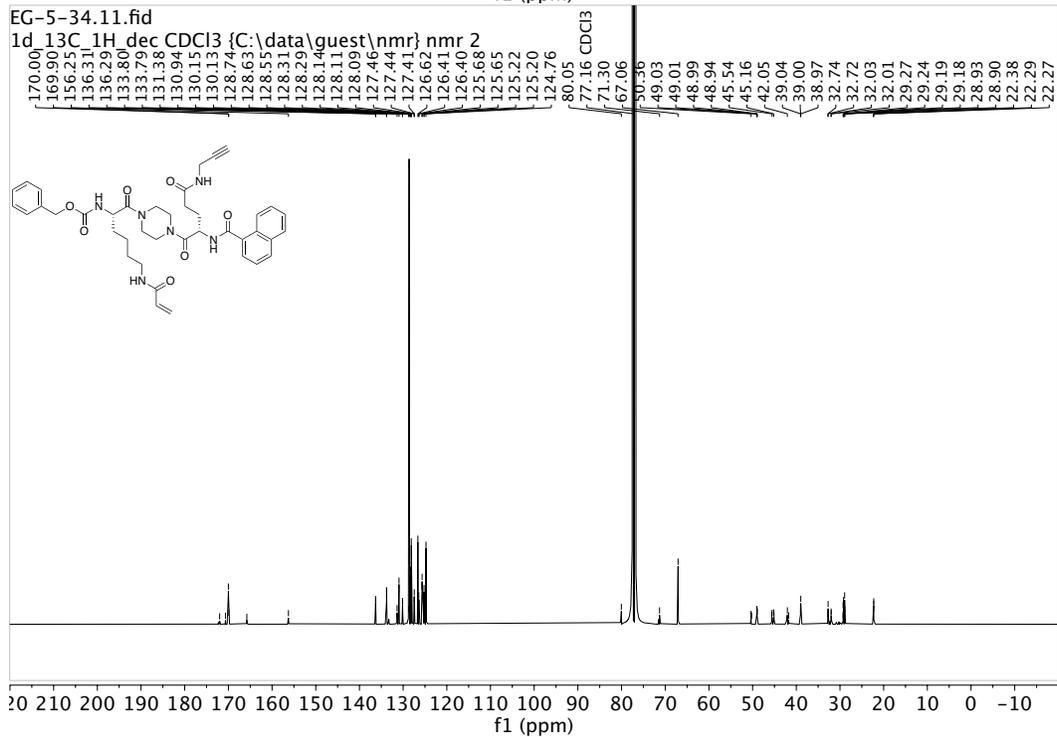
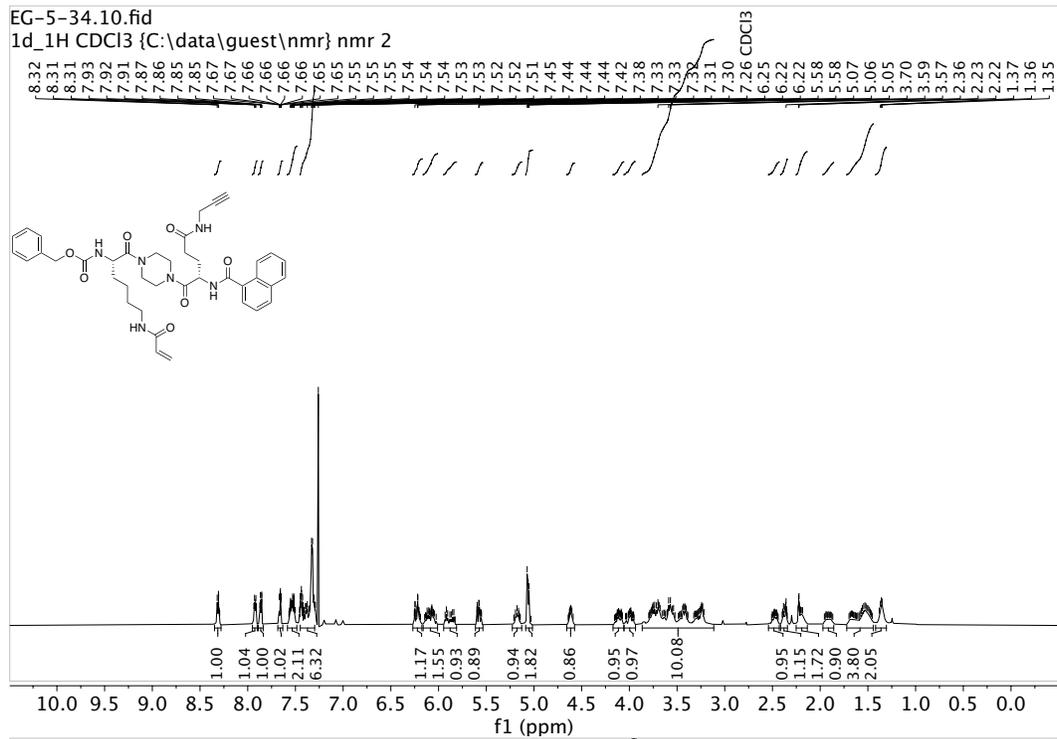
Compound 25



Compound 27



Compound 31



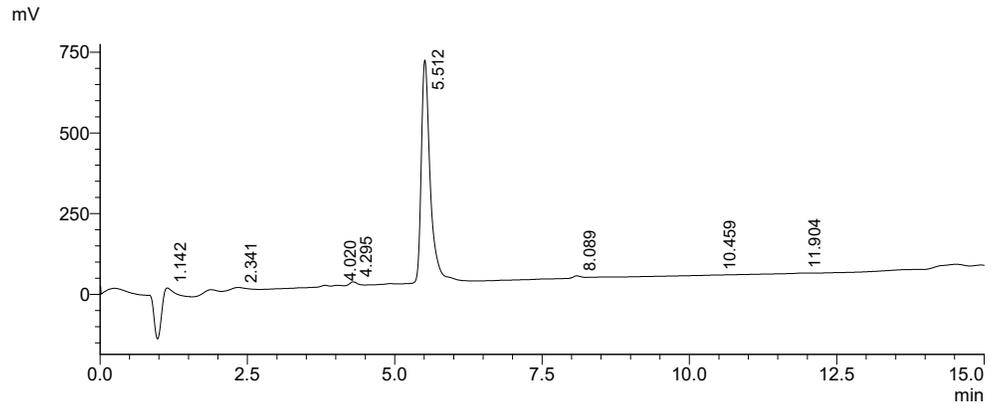
HPLC traces of final compounds

Compound 17:

<Sample Information>

Sample Name : EG-4-29
Sample ID : EG-4-29
Data Filename : EG-4-29
Method Filename : 5-95% Method.lcm
Batch Filename : EG-4-29
Vial # : 1-19
Injection Volume : 30 uL
Date Acquired : 12/15/2021 11:59:11 AM
Date Processed : 12/15/2021 12:14:15 PM
Sample Type : Unknown
Acquired by : Eric Gates
Processed by : Eric Gates

<Chromatogram>



Peak Table

Peak#	Ret. Time	Area%
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2	2.341	1.503
3	4.020	0.498
4	4.295	1.070
5	5.512	95.488
6	8.089	0.572
7	10.459	0.023
8	11.904	0.041
Total		100.000

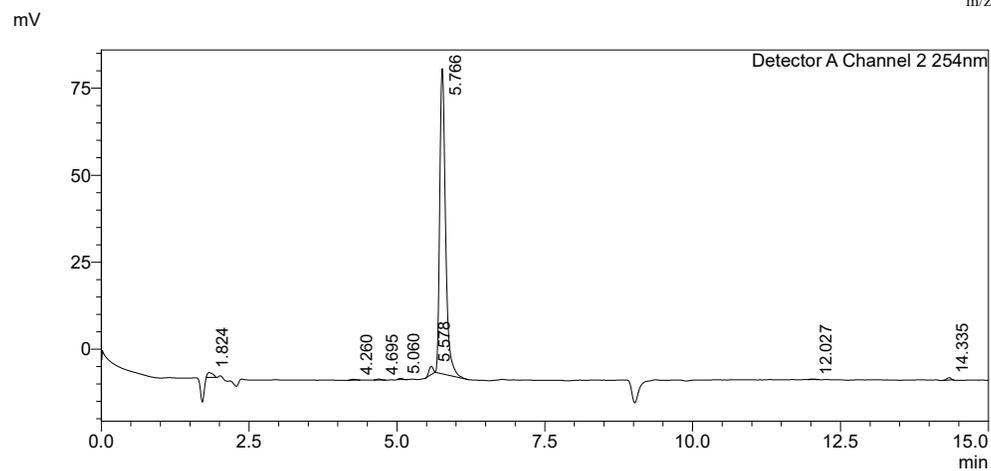
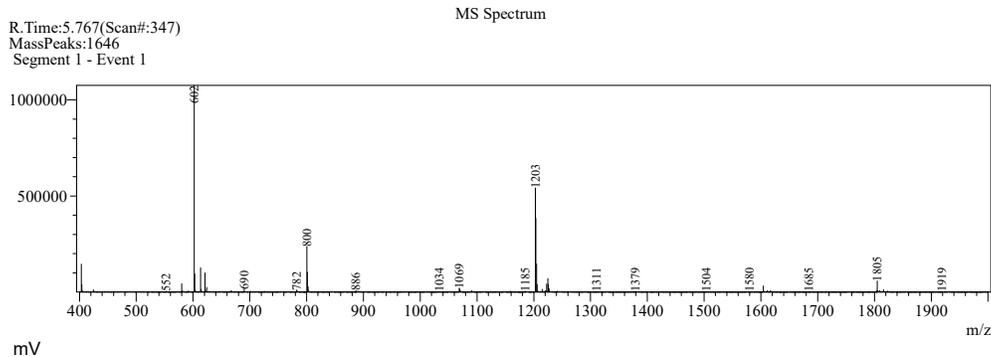
Detector A Channel 1 220nm

Compound 18 (NCEG2):

<Sample Information>

Sample Name : EG-5-11-Purity
Sample ID : EG-5-11-Purity
Data Filename : EG-5-11-Purity.lcd
Method Filename : 5-95% Method.lcm
Batch Filename :
Vial # : 0-2
Injection Volume : 10 uL
Date Acquired : 9/1/2022 11:01:55 AM
Date Processed : 9/1/2022 11:21:26 AM
Sample Type : Unknown
Acquired by : Boddy lab
Processed by : Boddy lab

<Chromatogram>



Peak Table
Detector A Channel 2 254nm

Peak#	Ret. Time	Area%
1	1.824	1.438
2	4.260	0.222
3	4.695	0.219
4	5.060	0.272
5	5.578	1.827
6	5.766	95.387
7	12.027	0.144
8	14.335	0.492
Total		100.000

Compound 22:

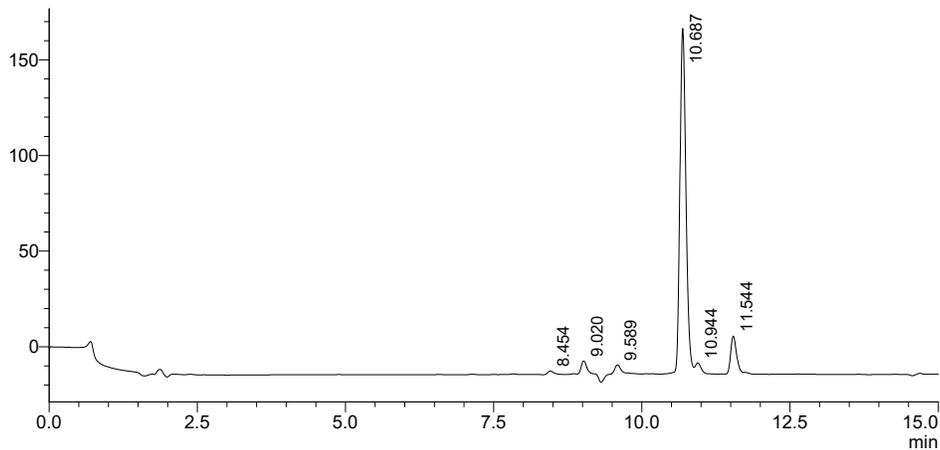
<Sample Information>

Sample Name : EG-4-65
Sample ID : EG-4-65
Data Filename : EG-4-65_002.lcd
Method Filename : 5-95% Method.lcm
Batch Filename : EG-4-64-65-87-Purity.lcb
Vial # : 0-5
Injection Volume : 10 uL
Date Acquired : 4/29/2022 10:20:06 AM
Date Processed : 4/29/2022 10:35:07 AM

Sample Type : Unknown
Acquired by : Boddy lab
Processed by : Boddy lab

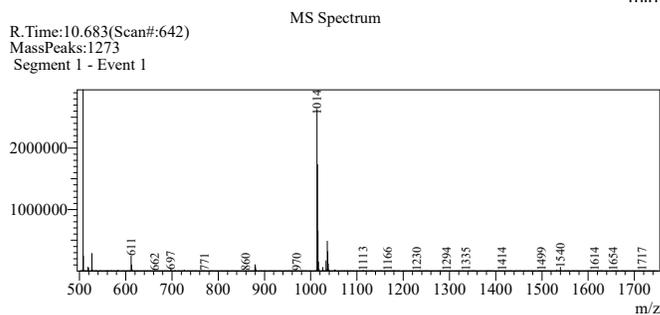
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mV



Peak Table	
Detector A Channel 2 254nm	
Ret. Time	Area%
8.454	0.580
9.020	2.489
9.589	1.625
10.687	85.300
10.944	1.605
11.544	8.401
	100.000

R. Time: 10.683 (Scan#: 642)
MassPeaks: 1273
Segment 1 - Event 1



Compound 23:

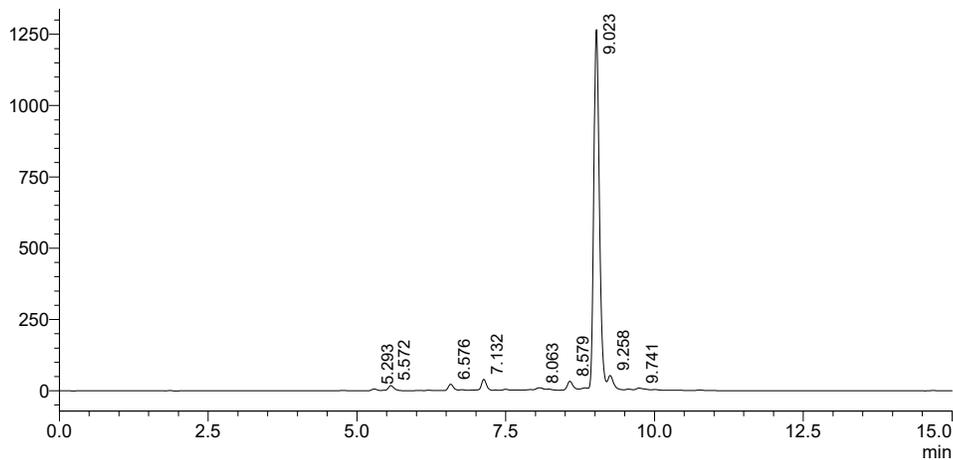
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Sample Name : EG-4-64
 Sample ID : EG-4-64
 Data Filename : EG-4-64_001.lcd
 Method Filename : 5-95% Method.lcm
 Batch Filename : EG-4-64-65-87-Purity.lcb
 Vial # : 0-4
 Injection Volume : 10 uL
 Date Acquired : 4/29/2022 10:04:34 AM
 Date Processed : 4/29/2022 10:19:36 AM

Sample Type : Unknown
 Acquired by : Eric Gates
 Processed by : Eric Gates

<Chromatogram>

mV

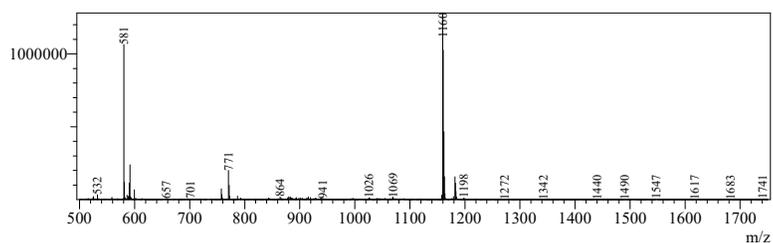


Peak Table
Detector A Channel 2 254nm

Ret. Time	Area%
5.293	0.255
5.572	0.943
6.576	1.268
7.132	2.143
8.063	0.425
8.579	1.827
9.023	89.915
9.258	3.059
9.741	0.166
	100.000

R.Time:9.017(Scan#:542)
MassPeaks:1293
Segment 1 - Event 1

MS Spectrum



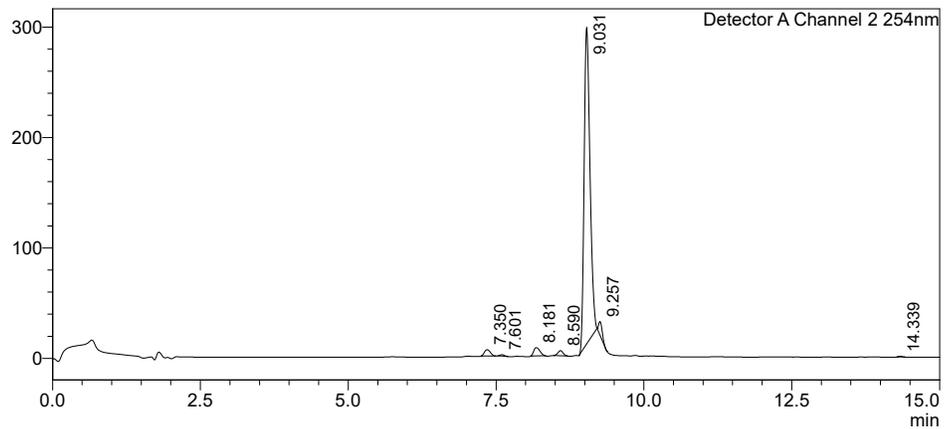
Compound 29 (NCEG-RHB):

<Sample Information>

Sample Name : EG-5-2-Purity
Sample ID : EG-5-2-Purity
Data Filename : EG-5-2-Purity_002.lcd
Method Filename : 5-95% Method.lcm
Batch Filename : EG-4-77-EG-5-2-6-Purity.lcb
Vial # : 0-3
Injection Volume : 10 uL
Date Acquired : 9/19/2022 11:35:22 AM
Date Processed : 9/19/2022 11:50:24 AM
Sample Type : Unknown
Acquired by : Boddy lab
Processed by : Boddy lab

<Chromatogram>

mV



Peak Table

Peak#	Ret. Time	Area%
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4	8.590	1.270
5	9.031	91.881
6	9.257	2.184
7	14.339	0.137
Total		100.000

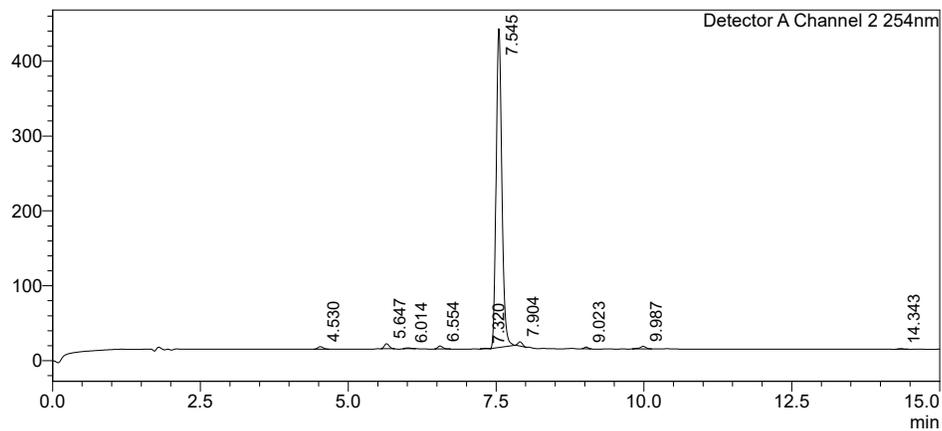
Compound 31:

<Sample Information>

Sample Name : EG-4-77-Purity
Sample ID : EG-4-77-Purity
Data Filename : EG-4-77-Purity_001.lcd
Method Filename : 5-95% Method.Tcm
Batch Filename : EG-4-77-EG-5-2-6-Purity.lcb
Vial # : 0-2
Injection Volume : 10 uL
Date Acquired : 9/19/2022 11:19:51 AM
Date Processed : 9/19/2022 11:49:31 AM
Sample Type : Unknown
Acquired by : Boddy lab
Processed by : Boddy lab

<Chromatogram>

mV



Peak Table
Detector A Channel 2 254nm

Peak#	Ret. Time	Area%
1	4.530	0.673
2	5.647	1.273
3	6.014	0.224
4	6.554	0.787
5	7.320	0.115
6	7.545	94.734
7	7.904	0.937
8	9.023	0.317
9	9.987	0.825
10	14.343	0.116
Total		100.000

Compound 33:

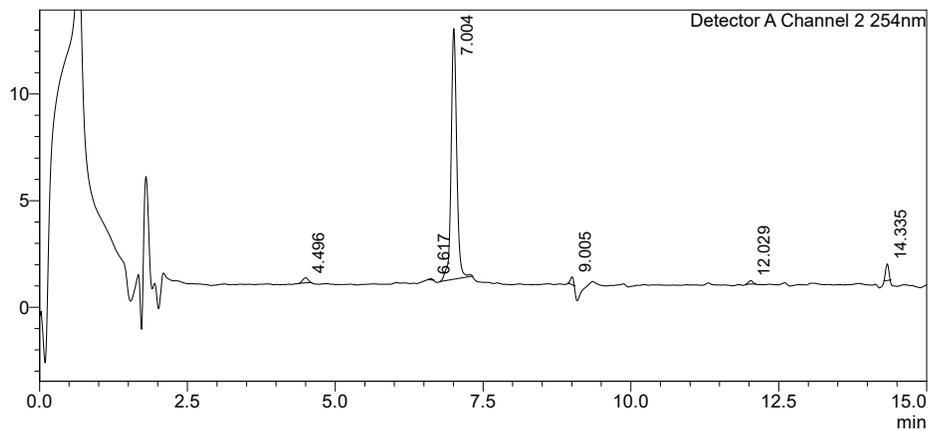
<Sample Information>

Sample Name : EG-5-6-Purity
Sample ID : EG-5-6-Purity
Data Filename : EG-5-6-Purity_003.lcd
Method Filename : 5-95% Method.lcm
Batch Filename : EG-4-77-EG-5-2-6-Purity.lcb
Vial # : 0-4
Injection Volume : 10 uL
Date Acquired : 9/19/2022 11:50:54 AM
Date Processed : 9/19/2022 12:05:56 PM

Sample Type : Unknown
Acquired by : Boddy lab
Processed by : Boddy lab

<Chromatogram>

mV



Peak Table
Detector A Channel 2 254nm

Peak#	Ret. Time	Area%
1	4.496	1.831
2	6.617	0.238
3	7.004	92.413
4	9.005	1.459
5	12.029	0.916
6	14.335	3.142
Total		100.000

References

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