Supporting Information for:

Ipomoeassin F Binds Sec61α to Inhibit Protein Translocation

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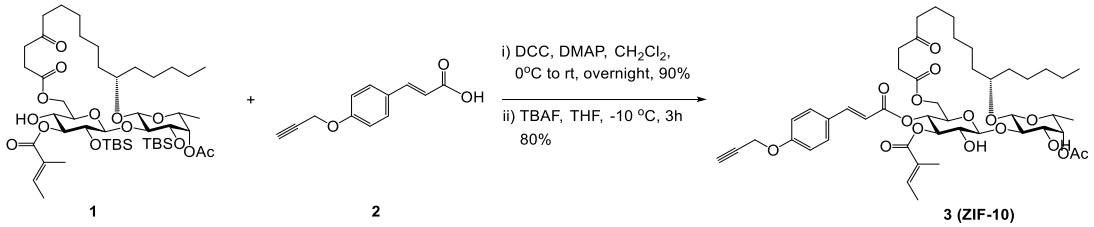
CHEMISTRY

General Methods

Reactions were carried out in oven-dried glassware. All reagents were purchased from commercial sources and were used without further purification unless noted. Unless stated otherwise, all reactions were carried out under a nitrogen atmosphere and monitored by thin layer chromatography (TLC) using Silica Gel GF_{254} plates (Agela) with detection by charring with 5% (v/v) H_2SO_4 in EtOH or by visualizing in UV light (254 nm or 365 nm). Column chromatography was performed on silica gel (230–450 mesh, Sorbent). The ratio between silica gel and crude product ranged from 100 to 50:1 (w/w). NMR data were collected on a Bruker 400 MHz NMR spectrometer and a Bruker 400 MHz system. ^{1}H NMR spectra were obtained in deuteriochloroform ($CDCl_3$) with chloroform ($CHCl_3$, $\delta = 7.27$ for ^{1}H) as an internal reference. ^{13}C NMR spectra were proton decoupled and were in $CDCl_3$ with $CHCl_3$ ($\delta = 77.0$ for ^{13}C) as an internal reference. Chemical shifts are reported in ppm (δ). Data are presented in the form: chemical shift (multiplicity, coupling constants, and integration). ^{1}H data are reported as though they were first order. The errors between the coupling constants for two coupled protons were less than 0.5 Hz, and the average number was reported. Proton assignments, when made, were done so with the aid of COSY NMR spectra. For some compounds, HSQC and HMBC NMR were also applied to assign the proton signals. Optical rotations were measured on an Autopol III Automatic Polarimeter at 25 ± 1 °C for solutions in a 1.0 dm cell. High resolution mass spectrum (HRMS) and were acquired in the ESI mode.

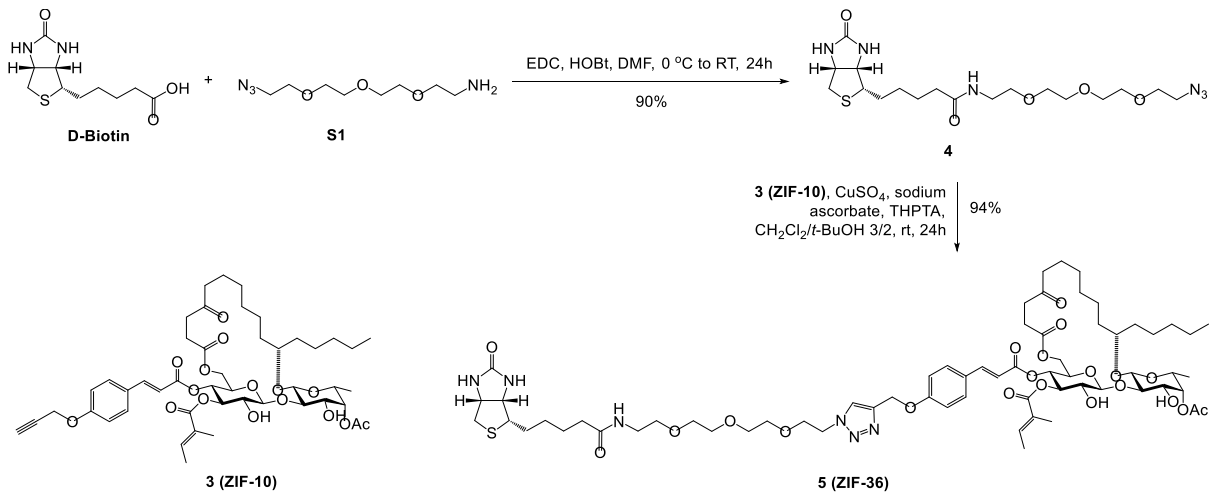
Synthetic Procedures and Analytical Data

Compound 3.



4-Propargyloxy cinnamic acid (17.4 mg, 0.086 mmol) was added in one portion to a solution of **1** (40 mg, 0.043 mmol), DCC (17.8 mg, 0.086 mmol) and 4-dimethylaminopyridine (2.6 mg, 0.022 mmol) in CH_2Cl_2 (2 mL). The reaction was stirred at room temperature for 12 h. At this point, TLC (silica, 1:3 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was diluted with ether (2 mL) and hexanes (1 mL), stirred for 20 min then filtered thru a pad of celite using ether (3 mL) as the eluent and the filtrate concentrated *in vacuo*. The residue was purified by flash column chromatography (silica, EtOAc–hexanes, 1:3→ 1:1) gave the protected intermediate (43 mg, 90%) as a colorless syrup. To a solution of the intermediate (41 mg, 0.037 mmol) in THF (2 mL) was added TBAF (1M solution in THF,

0.22 mL, 0.22 mmol, 6 equiv) at –10 °C. The reaction mixture was stirred at the same temperature for 6 h at which point TLC (silica, 2:1 EtOAc–hexanes) showed it was complete. The reaction mixture was diluted with Et_2O (20 mL), washed with 1M HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), brine (10 mL). The aqueous layer was extracted with Et_2O (20 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–hexanes, 1:1→ 2:1) gave analogue **3** (26.0 mg, 80%) as a white solid. $[\alpha]_D^{25}$ –55.3° (*c* 1 $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, *J* = 16.0 Hz, 1H, Ph-C*H*=CH-), 7.50 – 7.43 (m, 2H, 2 × Ar*H*), 7.01 – 6.95 (m, 2H, 2 × Ar*H*), 6.92 – 6.85 (m, 1H, Me-C*H*-C(Me)-C=O), 6.22 (d, *J* = 16.0 Hz, 1H, Ph-CH=C*H*-), 5.30 (t, *J* = 10.0 Hz, 1H, H-4-Glu*p*), 5.18 – 5.10 (m, 2H, H-3-Glu*p*, H-4-Fuc*p*), 4.73 (d, *J* = 2.4 Hz, 2H, HC≡C-CH_2), 4.63 – 4.58 (m, 2H, H-1-Glu*p*, O*H*), 4.45 (dd, *J* = 12.4, 3.2 Hz, 1H, H-6-Glu*p*), 4.40 (d, *J* = 7.6 Hz, 1H, H-1-Fuc*p*), 4.14 (dd, *J* = 12.4, 2.4 Hz, 1H, H-6-Glu*p*), 4.04 (br, 1H, O*H*), 3.91 (dd, *J* = 9.6, 3.2 Hz, 1H, H-3-Fuc*p*), 3.78 – 3.72 (m, 1H, H-5-Glu*p*), 3.71 – 3.59 (m, 4H, H-2-Glu*p*, H-2-Fuc*p*, H-5-Fuc*p*, -CH_2-C*H*-CH_2-), 2.83 – 2.39 (m, 7H), 2.18 (s, 3H, CH_3-C=O), 1.79 – 1.72 (m, 6H, C*H*$_3$-CH-C(C*H*$_3$)-C=O), 1.70 – 1.20 (m, 18H), 1.18 (d, *J* = 6.4 Hz, 3H, H-6-Fuc*p*), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.04, 171.76, 171.70, 168.82, 165.61, 159.43, 145.55, 139.74, 129.89(2), 127.56, 127.55, 115.21(2), 114.62, 105.66, 100.19, 82.74, 79.73, 77.91, 75.98, 75.85, 73.95, 72.68, 72.59, 72.42, 68.79, 67.32, 61.78, 55.79, 41.82, 37.56, 34.32, 33.08, 31.89, 29.10, 29.02, 28.28, 24.66, 24.48, 23.45, 22.63, 20.92, 16.32, 14.57, 14.08, 11.95. HRMS (ESI) *m*/*z* calcd for $C_{47}H_{64}NaO_{16}$ $[M+Na]^+$ 907.4092. Found: 907.4089.

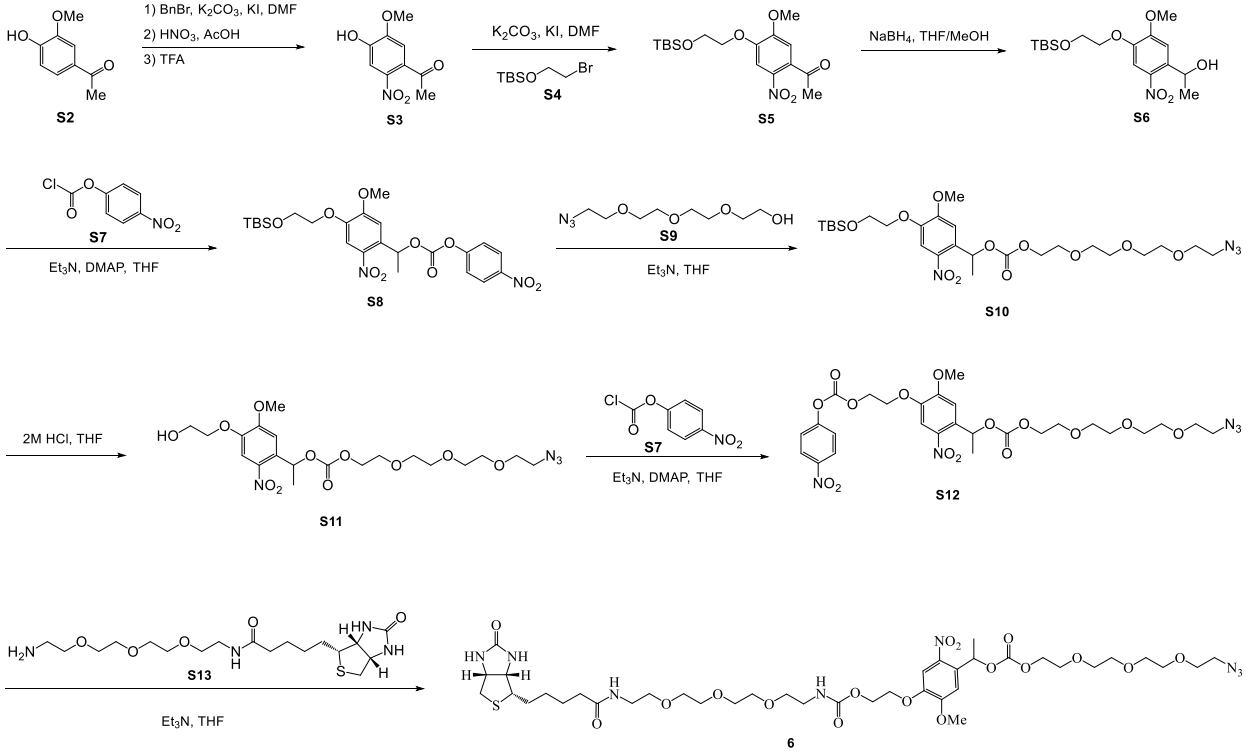


Compound 5.

Biotin linker **4** was prepared from D-biotin and 1-amino11-azido-3,6,9-trioxaundecane (**S1**) following the literature reported procedure[1] with a yield of 90%. Compound **3** (24.8 mg, 0.028 mmol) and biotin linker **4** (6.23 mg, 0.014 mmol) were dissolved in a mixture of CH_2Cl_2/*t*-BuOH (3:2, w/w, 1 mL). To the mixture were added an aqueous solution of $CuSO_4$ (0.3 M, 1 eq, 93 μL) and a aqueous solution of sodium

ascorbate (1 M, 2 eq, 56 μL) and the mixture was vigorously stirred without light for 24 h. TLC analysis (silica, 1:10 MeOH–CH_2Cl_2) showed the reaction was done. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (silica, MeOH–CH_2Cl_2, 1:30 → 1:20 → 1:10) gave compound **5** (17.4 mg, 94%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (s, 1H, triazole-*H*), 7.57 (d, *J* = 16.0 Hz, 1H, Ph-C*H*=C-), 7.45 (d, *J* = 8.4 Hz, 2H, Ar*H*), 7.00 (d, *J* = 8.4 Hz, 2H, Ar*H*), 6.93 – 6.83 (m, 1H, Me-C*H*-C(Me)-C=O), 6.61 (br, 1H, N*H*), 6.31 – 6.18 (m, 2H, Ph-CH=C*H*-, N*H*), 5.35 – 5.21 (m, 3H, H-4-Glu*p*, , triazole-CH_2O-), 5.18 – 5.11 (m, 2H, H-3-Glu*p*, H-4-Fuc*p*), 4.63 (d, *J* = 8.0 Hz, 1H, H-1-Glu*p*), 4.57 (t, *J* = 5.2 Hz, 2H, PEG-CH_2), 4.54 – 4.44 (m, 2H, biotin NC*H*, H-6-Glu*p*), 4.40 (d, *J* = 7.6 Hz, 1H, H-1-Fuc*p*), 4.36 – 4.29 (m, 1H, biotin NC*H*), 4.16 – 4.08 (m, 1H, H-6-Glu*p*), 3.96 – 3.87 (m, 3H, H-3-Fuc*p*, PEG CH_2), 3.78 – 3.35 (m, 20H, H-2-Glu*p*, H-5-Glu*p*, H-2-Fuc*p*, H-5-Fuc*p*, -CH_2-C*H*-CH_2-, PEG CH_2), 3.20 – 3.08 (m, 2H), 2.95 – 2.38 (m, 8H), 2.28 – 2.10 (m, 5H), 1.79 – 1.20 (m, 28H), 1.17 (d, *J* = 6.4 Hz, 3H, H-6-Fuc*p*), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 210.1, 173.3, 171.7, 168.7, 165.6, 163.6, 160.2, 145.5, 143.3, 139.5, 130.0(×2), 127.6, 127.3, 124.3, 115.1(×2), 114.6, 105. 7, 100.4, 82.7, 79.9, 77.4, 75.8, 73.9, 72.8, 72.6, 72.4, 70.7, 70.5, 70.4, 70.1, 69.8, 69.4, 68.8, 67.4, 62.0, 60.3, 55.4, 50.7, 50.4, 41.8, 40.4, 39.2, 37.6, 35.7, 34.4, 33.2, 31.9, 29.1, 29.1, 28.3, 28.1, 28.0, 25.4, 24.7, 24.5, 23.5, 22.6, 21.0, 16.4, 14.6, 14.1, 12.0. HRMS (ESI) *m*/*z* calcd for $C_{65}H_{96}N_6NaO_{21}S$ $[M+Na]^+$ 1351.6247, found: 1351.6238.

Biotin Linker with photo cleavage moiety



Compound S3.

BnBr (1.54 g, 9.0 mmol) was added in one portion to a solution of compound **S2** (1.0 g, 6.0 mmol), K_2CO_3 (1.66 g, 12.0 mmol) and KI (200 mg, 1.2 mmol) in DMF (20 mL). The reaction mixture was stirred at 90 ^oC overnight. At this point, TLC (silica, 1:3 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was cooled to room temperature, and then filtered through a pad of Celite using Ethyl Acetate (50 mL) as the eluent and the filtrate concentrated. The residue was purified by column chromatography (silica, EtOAc–hexanes, 1: 4) gave desired compound (1.4 g, 91%) as a white solid. To a solution of this (1.3 g, 5.1 mmol) in AcOH (20 mL) was added HNO_3 (5 mL) at 0 ^oC. The reaction mixture was stirred at the same temperature for 12 h at which point TLC (silica, 1:3 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was diluted with Ice-Water (60 mL), and then filtered, washed with Ice-Water (20 mL). The precipitated product was recrystallized from ethanol to yield product (1.1g, 75%) as a yellow solid. The yellow solid (1.1 g, 4.3 mol) was slowly added to TFA (15 mL) at 0 ^oC for 1 h, and then the result mixture was stirred at room temperature overnight at which point TLC (silica, 1:3 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was diluted with Ice-Water (60 mL), and then filtered, washed with Ice-Water (20 mL). The precipitated product was purified by column chromatography (silica, EtOAc–hexanes, 1:4 → 1:1) gave compound **S3** (590 mg, 69%) as a white solid. ^1H NMR (400 MHz, $CDCl_3$) δ 7.67 (s, 1H), 6.81 (s, 1H), 4.02 (s, 3H), 2.49 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.83, 150.98, 146.61, 131.86, 110.70, 108.58, 56.74, 30.27.

Compound S5.

Compound **S4** (425 mg, 1.8 mmol) was added in one portion to a solution of compound **S3** (250 mg, 1.2 mmol), K_2CO_3 (327 mg, 2.4 mmol) and KI (39 mg, 0.24 mmol) in DMF (10 mL). The reaction mixture was stirred at 90 ^oC overnight. At this point, TLC (silica, 1:3 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was cooled to room temperature, and then filtered through a pad of Celite using Ethyl Acetate (50 mL) as the eluent and the filtrate concentrated. The residue was purified by column chromatography (silica, EtOAc–hexanes, 1: 4) gave desired compound **S5** (390 mg, 89%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.69 (s, 1H), 6.75 (s, 1H), 4.20 (t, J = 5.2 Hz, 2H), 4.03 (t, J = 5.2 Hz, 2H), 3.94 (s, 3H), 2.49 (s, 3H), 0.89 (s, 9H), 0.09 (s, 6H).

Compound S8.

$NaBH_4$ (54 mg, 1.4 mmol) was slowly added to a solution of compound **S5** (500 mg, 1.4 mmol) in THF/MeOH (16mL, 1:1). The reaction mixture was stirred at room temperature 0.5 h. At this point, TLC (silica, 1:2 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was evaporated, and then diluted with Et_2O (20 mL), washed with 1M HCl (10 mL), saturated $NaHCO_3$ (10 mL), and brine (10 mL). The aqueous layer was extracted with Et_2O (20 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography

(silica, EtOAc–hexanes, 1:4 → 1:1) gave compound **S6** (450 mg, 91%) as a yellow oil. A solution of compound **S6** (400 mg, 1.1 mmol), Et_3N (326 mg, 3.2 mmol) and DMAP (13 mg, 0.1 mmol) in THF (20 mL) was slowly added to Compound **S7** (325 mg, 1.6 mmol) in THF. The reaction mixture was stirred at 0 ^oC 3h. At this point, TLC (silica, 1:3 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was quenched with 5% HCl (20 mL), and the aqueous layer was extracted with Ethyl Acetate (20 mL), washed with saturated $NaHCO_3$ (10 mL), and brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–hexanes, 1: 4) gave desired compound **S8** (510 mg, 88%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, *J* = 9.2 Hz, 2H), 7.68 (s, 1H), 7.34 (d, *J* = 9.2 Hz, 2H), 6.75 (s, 1H), 6.53 (q, *J* = 6.4 Hz, 1H), 4.12 (t, *J* = 5.2 Hz, 2H), 4.03 (t, *J* = 5.2 Hz, 2H), 3.99 (s, 3H), 1.77 (d, *J* = 5.2 Hz, 3H), 0.89 (s, 9H), 0.09 (s, 6H).

Compound S10.

A solution of compound **S8** (200 mg, 0.37 mmol), Et_3N (101 mg, 1.0 mmol) in THF (6 mL) was slowly added to Compound **S9** (107 mg, 0.5 mmol) in THF (0.5 mL). The reaction mixture was stirred at room temperature 0.5 h. At this point, TLC (silica, 1:1 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was quenched with 5% HCl (5 mL), and the aqueous layer was extracted with Ethyl Acetate (20 mL), washed with saturated $NaHCO_3$ (10 mL), and brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–hexanes, 2: 1) gave desired compound **S10** (220 mg, 96%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H), 7.07 (s, 1H), 6.40 (q, *J* = 6.4 Hz, 1H), 4.30-4.24 (m, 2H), 4.22 (t, *J* = 5.2 Hz, 2H), 4.16 (t, *J* = 5.2 Hz, 2H), 4.02 (s, 3H). 3.72-3.65 (m, 12H), 3.40-3.36 (m, 2H). 1.66 (d, *J* = 5.2 Hz, 3H), 0.89 (s, 9H), 0.09 (s, 6H).

Compound S11.

2M HCl (3 mL) was slowly added to a solution of compound **S10** (200 mg, 0.33 mmol) in THF (6mL). The reaction mixture was stirred at room temperature 0.5 h. At this point, TLC (silica, 3:1 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was quenched with saturated $NaHCO_3$ (10 mL), and the aqueous layer was extracted with Ethyl Acetate (20 mL), washed with brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc) gave desired compound **S11** (152 mg, 92%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.63 (s, 1H), 7.09 (s, 1H), 6.38 (q, *J* = 6.4 Hz, 1H), 4.29-4.21 (m, 2H), 4.18 (t, *J* = 5.2 Hz, 2H), 4.01-3.99 (m, 2H), 3.97 (s, 3H). 3.71-3.64 (m, 12H), 3.39 (t, *J* = 5.2 Hz, 2H). 2.23 (t, *J* = 6.0 Hz,1H), 1.66 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.28, 154.04, 147.19, 139.67, 133.29, 109.72, 108.20, 72.09, 71.06, 70.65, 70.63(2), 70.51, 70.02, 68.85, 67.05, 60.96, 56,44, 50.66, 22.08

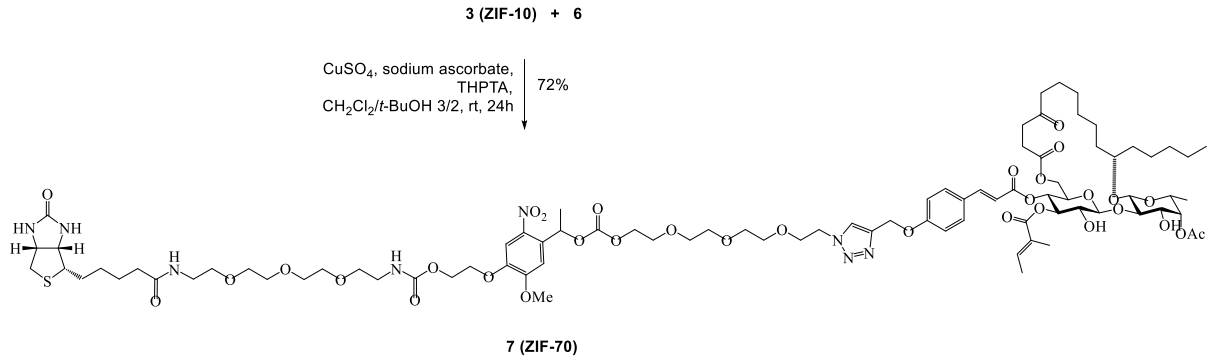
Compound S12.

A solution of compound **S11** (140 mg, 0.28 mmol), Et_3N (85 mg, 0.84 mmol) and DMAP (4 mg, 0.03 mmol) in THF (10 mL) was slowly added to Compound **S7** (85 mg, 0.42 mmol) in THF (5 mL). The reaction mixture was stirred at 0 ^oC 3 h. At this point, TLC (silica, 2:1 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was quenched with 5% HCl (10 mL), and the aqueous layer was extracted with Ethyl Acetate (20 mL), washed with saturated $NaHCO_3$ (10 mL), and brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–hexanes, 1: 1) gave desired compound **S12** (150 mg, 81%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, *J* = 9.2 Hz, 2H), 7.66 (s, 1H), 7.41 (d, *J* = 9.2 Hz, 2H), 7.12 (s, 1H), 6.40 (q, *J* = 6.4 Hz, 1H), 4.70 (t, *J* = 4.4 Hz, 2H), 4.41-4.4.39 (m, 2H), 4.33-4.21 (m, 2H), 3.99 (s, 3H). 3.71 (t, *J* = 4.8 Hz, 2H), 3.70-3.65 (m, 10H), 3.39 (t, *J* = 5.2 Hz, 2H). 1.67 (d, *J* = 6.4 Hz, 3H).

Compound 6.

A solution of compound **S12** (100 mg, 0.15 mmol), Et_3N (37 mg, 0.36 mmol) in THF (6 mL) was slowly added to Compound **S13** (75 mg, 0.18 mmol) in THF (0.5 mL). The reaction mixture was stirred at room temperature 0.5 h. At this point, TLC (silica, 5:1 EtOAc–MeOH) showed the reaction was complete. The reaction mixture was quenched with 5% HCl (5 mL), and the aqueous layer was extracted with Ethyl Acetate (30 mL), washed with saturated $NaHCO_3$ (10 mL), and brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–MeOH, 10: 1) gave desired compound **6** (58 mg, 41%) as a yellow oil. ^1H NMR (400 MHz, MeOD) δ 8.02 (brs, 1H, NH), 7.70 (s, 1H), 7.20 (s, 1H), 6.30 (q, *J* = 6.4 Hz, 1H), 4.53 (dd, J_1 = 4.4 Hz, J_2 = 7.6 Hz, 1H). 4.45 (brs, 2H), 4.36-4.33 (m, 3H), 4.30-4.26 (m, 2H), 4.00 (s, 3H). 3.74-3.65 (m, 22H), 3.59-3.56 (m, 4H), 3.26-3.22 (m, 1H), 2.97 (dd, J_1 = 5.2 Hz, J_2 = 12.8 Hz, 1H). 2.74 (d, *J* = 12.4 Hz, 1H), 2.26 (t, *J* = 6.4 Hz, 2H), 1.82-1.58 (m, 4H), 1.66 (d, *J* = 6.4 Hz, 3H), 1.52-1.43 (m, 2H). ^{13}C NMR (100 MHz, MeOD) δ 176.29, 166.16, 158.88, 155.92, 155.86, 148.88, 141.43, 134.18, 110.94, 109.87, 73. 32, 71.73, 71.67, 71.60, 71.59, 71.33, 71.30, 71.21, 70.87, 70.14, 69.44, 68.41, 64.37, 63.48, 61.75, 57.22, 57.12, 51.93,41.88, 41.02, 40.43, 36.88, 29.89, 29.65, 26.98, 22.25.

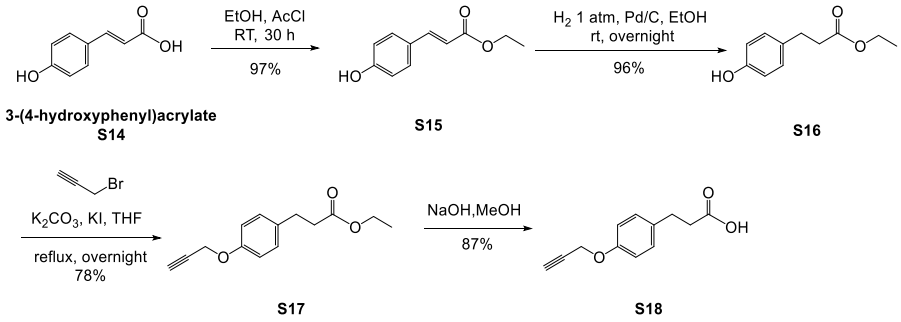
Photo Cleavable Biotin Probe 7



Compound **3** (28.0 mg, 0.0316 mmol) and the azide **6** (15.0 mg, 0.0158 mmol) were dissolved in a mixture of CH_2Cl_2/*t*-BuOH (3:2, w/w, 2 mL). To the mixture were added an aqueous solution of $CuSO_4$ (1 M, 1 eq, 32 μL) and an aqueous solution of sodium ascorbate (1 M, 2 eq, 63 μL) and the mixture was vigorously stirred without light for 24 h. TLC analysis (silica, 1:20 MeOH–CH_2Cl_2) showed the reaction was done. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (silica, MeOH–CH_2Cl_2, 1:30 → 1:20) gave compound **7** (20.9 mg, 72%) as a white foam. $[\alpha]_D^{25}$ –13.3° (*c* 1 $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (s, 1H, triazole-*H*), 7.63 (s, 1H, NO_2-Ar-*H*), 7.56 (d, *J* = 16.0 Hz, 1H, Ph-C*H*=C-), 7.44 (d, *J* = 8.8 Hz, 2H, Ar*H*), 7.06 (s, 1H, NO_2-Ar-*H*), 6.99 (d, *J* = 8.8 Hz, 2H, Ar*H*), 6.93 – 6.80 (m, 1H, Me-C*H*-C(Me)-C=O), 6.49 (br, 1H, N*H*), 6.36 (q, 1H, *J* = 6.4 Hz, NO_2-Ar-C*H*(CH_3)-O-), 6.20 (d, *J* = 16.0 Hz, 1H, Ph-CH=C*H*-), 5.94 (br, 1H, N*H*), 5.58 (br, 1H, N*H*), 5.28 (t, *J* = 9.6 Hz, 1H, H-4-Glu*p*), 5.23 (s, 3H, triazole-CH_2O-, N*H*), 5.18 – 5.10 (m, 2H, H-3-Glu*p*, H-4-Fuc*p*), 4.83 (br, 1H, O*H*), 4.62 (d, *J* = 8.0 Hz, 1H, H-1-Glu*p*), 4.56 (t, *J* = 5.2 Hz, 2H, PEG-CH_2), 4.53 – 4.42 (m, 5H, biotin-C*H*, PEG-CH_2, O*H*), 4.40 (d, *J* = 7.6 Hz, 1H, H-1-Fuc*p*), 4.35 – 4.10 (m, 6H, H-6-Glu*p*, PEG-CH_2), 3.94 (s, 3H, CH_3OAr), 3.92 – 3.84 (m, 3H, H-3-Fuc*p*, PEG-CH_2), 3.78 – 3.52 (m, 26H, H-2-Glu*p*, H-5-Glu*p*, H-2-Fuc*p*, H-5-Fuc*p*, -CH_2-C*H*-CH_2-, PEG-CH_2), 3.48 – 3.32 (m, 4H, PEG-CH_2), 3.18 – 3.10 (m, 1H), 2.95 – 2.86 (m, 1H), 2.83 – 2.39 (m, 6H), 2.26 – 2.15 (m, 5H), 1.88 (br, 1H), 1.78 – 1.59 (m, 16H), 1.58 – 1.20 (m, 18H), 1.17 (d, *J* = 6.4 Hz, 3H, H-6-Fuc*p*), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.0, 173.1, 171.7, 171.6, 168.6, 165.6, 163.5, 160.3, 156.2, 154.3, 154.1, 147.1, 145.5, 143.3, 139.7, 139.4, 133.1, 130.0(×2), 127.6, 127.2, 124.2, 115.1(×2), 114.5, 109.7, 108.4, 105.7, 100.4, 82.7, 79.8, 75.7, 74.0, 72.8, 72.6, 72.4, 72.1, 70.5, 70.4, 70.2, 70.1, 69.9, 69.8, 69.4, 68.8, 68.8, 68.1, 67.5, 67.0, 62.9, 62.0, 61.9, 61.8, 60.1, 56.5, 55.4, 50.3, 41.8, 40.5, 39.1, 37.6, 35.8, 34.4, 33.2, 31.9, 29.1, 29.0, 28.3, 28.1, 28.0, 25.4, 24.7, 24.5, 23.5, 22.6, 22.0, 20.9, 16.3, 14.5, 14.1, 12.0. HRMS (ESI) *m*/*z* calcd for $C_{86}H_{126}N_8NaO_{33}S$ $[M+Na]^+$ 1853.8046, found: 1853.8023.

Syntheses of Negative Controls 8 and 9

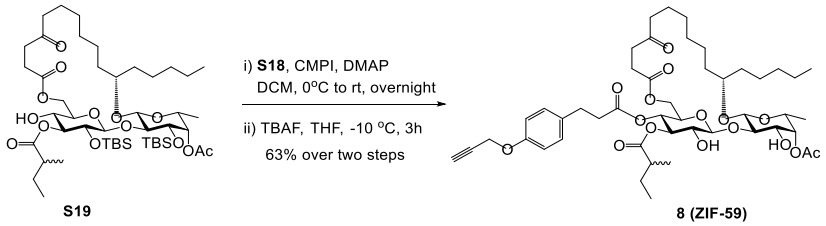
Compound S18.



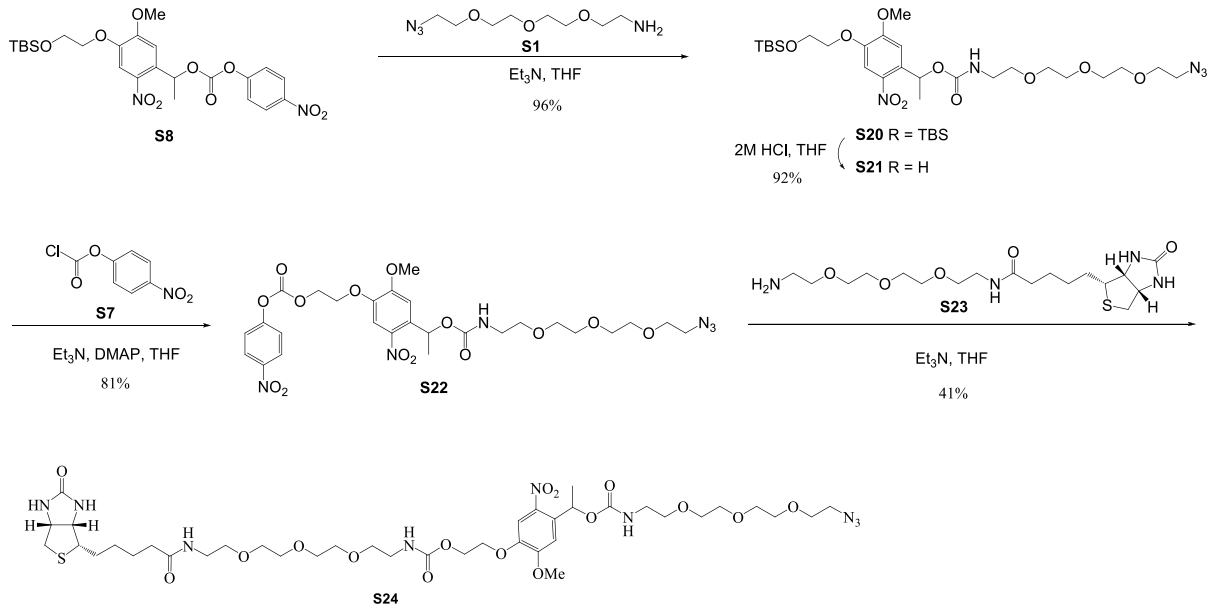
Compound **S16** was prepared from 3-(4-hydroxyphenyl)acrylate following the literature reported procedure[2] with a yield of 93% over two steps. Propargyl bromide (0.28 mL, 2.55 mmol, 80wt.% solution in toluene) was added to a mixture of **S16** (247.7 g, 1.275 mmol), K_2CO_3 (352.5 mg, 2.55 mmol) and KI (21.2 mg, 0.128 mmol) in THF (10 mL). The reaction mixture was stirred at reflux overnight. TLC (silica, 1:4 EtOAc–hexanes) showed it was complete. The reaction mixture was concentrated in vacuo and the residue was diluted with CH_2Cl_2 (20 mL), washed with water (30 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–hexanes, 1:5 →1:4) gave compound **S17** (231.4 mg, 78%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.14 (d, *J* = 8.4 Hz, 2H, Ar*H*), 6.91 (d, *J* = 8.4 Hz, 2H, Ar*H*), 4.67 (d, *J* = 2.4 Hz, 2H, alkyne CH_2), 4.13 (q, *J* = 7.2 Hz, 2H, CH_2CH$_3$), 2.91, 2.59 (2t, *J* = 7.6 Hz, 4H, Ar-CH_2CH_2-), 2.52 (t, *J* = 2.4 Hz, 1H, alkyne C*H*), 1.24 (t, *J* = 7.2 Hz, 3H, CH$_2$CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 156.0, 133.6, 129.2(×2), 114.9(×2), 78.7, 75.4, 60.3, 55.8, 36.1, 30.0, 14.2.

To a solution of ethyl ester **S17** (204.3 mg, 0.880 mmol) in MeOH (5 mL) was added NaOH (2 M solution in H_2O, 2.2 mL, 4.40 mmol) at 0 °C. The mixture was slowly warmed to RT and stirred overnight. TLC analysis (silica, 1:2 EtOAc–hexanes) showed it was complete. MeOH was evaporated and the resulting mixture was acidified with 10% w/v aq. HCl until pH = 4. White precipitate was formed. Filtration following washing with water (5 mL) and then dry in high vacuum gave **S18** (155.4 mg, 87%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.45 (br, 1H, COO*H*), 7.16 (d, *J* = 8.4 Hz, 2H, Ar*H*), 6.92 (d, *J* = 8.4 Hz, 2H, Ar*H*), 4.68 (d, *J* = 2.4 Hz, 2H, alkyne CH_2), 2.92, 2.67 (2t, *J* = 7.6 Hz, 4H, Ar-CH_2CH_2-), 2.53 (t, *J* = 2.4 Hz, 1H, alkyne C*H*). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.0, 156.1, 133.2, 129.2(×2), 115.0(×2), 78.7, 75.4, 55.8, 35.7, 29.7. The ^{1}H NMR data was in accordance with the literature.[3]

Compound 8.



2-Chloro-1-methylpyridinium iodide (CMPI, 64.1 mg, 0.251 mmol), N,N-dimethylaminopyridine (DMAP, 10.2 mg, 0.0837 mmol) and Et_3N (117 μL, 0.837 mmol) were added to a solution of **S19**[4] (155.9 mg, 0.167 mmol) and acid **S18** (51.3 mg, 0.251 mmol) in dry CH_2Cl_2 (4 mL) at 0 °C. The reaction was warmed to ambient temperature and stirred for 12 h. At this point, TLC (silica, 1:2 EtOAc–hexanes) showed the reaction was complete. Evaporation of the solvent followed by purification of the residue by flash column chromatography (silica, EtOAc−hexanes, 1:5 → 1:3) to give protected compound (161.7 mg, 89%) as a colorless syrup. To a solution of the protected compound (135.5 mg, 0.148 mmol) in THF (5 mL) was added TBAF (1M solution in THF, 740 μL, 740 μmol, 5 equiv) at –10 °C. The reaction mixture was stirred at the same temperature for 3 h at which point TLC (silica, 1:1 EtOAc–hexanes) showed it was complete. The reaction mixture was diluted with Et_2O (40 mL), washed with 1M HCl (20 mL), saturated $NaHCO_3$ (20 mL), brine (20 mL). The aqueous layer was extracted with Et_2O (40 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–hexanes, 1:2 → 1:1) gave compound **8** (79.6 mg, 71%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15 – 7.07 (m, 2H, Ar*H*), 6.91 – 6.84 (m, 2H, Ar*H*), 5.14 – 5.05 (m, 3H, H-3-Glu*p*, H-4-Glu*p*, H-4-Fuc*p*), 4.66 (d, *J* = 2.4 Hz, 2H, alkyne CH_2), 4.52 (d, *J* = 8.0 Hz, 1H, H-1-Glu*p*), 4.48 – 4.35 (m, 2H, H-6-Glu*p*, H-1-Fuc*p*), 4.03 – 3.95 (m, 1H, H-6-Glu*p*), 3.90 (dd, *J* = 9.6, 3.6 Hz, 1H, H-3-Fuc*p*), 3.73 – 3.54 (m, 5H, H-2-Glu*p*, H-5-Glu*p*, H-2-Fuc*p*, H-5-Fuc*p*, -CH_2-C*H*-CH_2-), 2.90 – 2.31 (m, 12H), 2.17 (s, 3H, CH_3-C=O), 1.80 – 1.13 (m, 23H), 1.09, 1.06 (2d, *J* = 7.2, 6.8 Hz, 3H), 0.96 – 0.82 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.0, 177.0, 177.0, 172.0, 172.0, 171.7, 171.7, 171.5, 171.4, 156.1, 133.2, 133.2, 129.2, 129.2, 114.9, 114.9, 105.7, 100.0, 82.6, 79.5, 78.7, 75.4, 74.7, 73.8, 73.7, 72.9, 72.7, 72.7, 72.5, 68.8, 67.2, 67.1, 61.3, 55.8, 41.8, 41.2, 41.0, 37.6, 35.6, 35.6, 34.4, 33.1, 31.9, 29.6, 29.6, 29.1, 29.0, 28.3, 26.5, 26.4, 24.7, 24.5, 23.5, 22.6, 20.9, 16.5, 16.4, 16.3, 14.1, 11.5, 11.5. HRMS (ESI) *m*/*z* calcd for $C_{47}H_{68}NaO_{16}$ $[M+Na]^+$ 911.4405, found: 911.4409.



Compound S21.

A solution of compound **S8** (200 mg, 0.37 mmol), Et_3N (101 mg, 1.0 mmol) in THF (6 mL) was slowly added to Compound **S1** (107 mg, 0.5 mmol) in THF (0.5 mL). The reaction mixture was stirred at room temperature 0.5 h. At this point, TLC (silica, 1:1 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was quenched with 5% HCl (5 mL), and the aqueous layer was extracted with Ethyl Acetate (20 mL), washed with saturated $NaHCO_3$ (10 mL), and brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–hexanes, 2:1) gave desired compound **S20** (220 mg, 96%) as a yellow oil. 2M HCl (3 mL) was slowly added to a solution of compound **S20** (200 mg, 0.33 mmol) in THF (6mL). The reaction mixture was stirred at room temperature 0.5 h. At this point, TLC (silica, 3:1 EtOAc–hexanes) showed the reaction was complete. The reaction mixture was quenched with saturated $NaHCO_3$ (10 mL), and the aqueous layer was extracted with Ethyl Acetate (20 mL), washed with brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc) gave desired compound **S21** (152 mg, 92%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (s, 1H), 7.04 (s, 1H), 6.38 (q, J = 6.4 Hz, 1H), 5.34 (brs, 1H, NH), 4.18 (t, J = 5.2 Hz, 2H), 4.00 (t, J = 5.2 Hz, 2H), 3.97 (s, 3H). 3.70-3.62 (m, 10H), 3.54-3.53 (m, 2H), 3.41-3.35 (m, 4H), 1.61 (d, J = 6.4 Hz, 3H).

Compound S22.

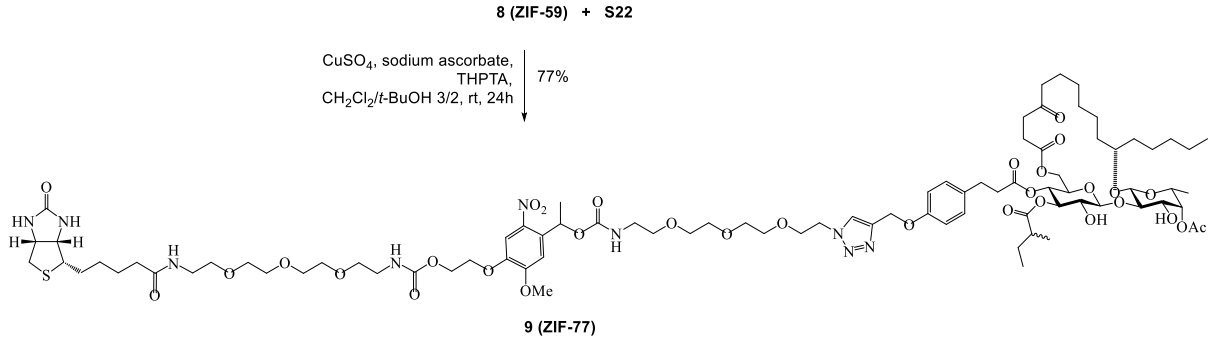
A solution of compound **S21** (140 mg, 0.28 mmol), Et_3N (85 mg, 0.84 mmol) and DMAP (4 mg, 0.03 mmol) in THF (10 mL) was slowly added to Compound **S7** (85 mg, 0.42 mmol) in THF (5 mL). The reaction mixture was stirred at 0 ^{o}C 3 h. At this point, TLC (silica, 2:1 EtOAc–hexanes) showed the

reaction was complete. The reaction mixture was quenched with 5% HCl (10 mL), and the aqueous layer was extracted with Ethyl Acetate (20 mL), washed with saturated $NaHCO_3$ (10 mL), and brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–hexanes, 1: 1) gave desired compound **S22** (150 mg, 81%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, *J* = 9.2 Hz, 2H), 7.63 (s, 1H), 7.39 (d, *J* = 9.2 Hz, 2H), 7.05 (s, 1H), 6.35 (q, *J* = 6.4 Hz, 1H), 5.37 (t, *J* = 4.8 Hz 1H, NH), 4.67 (t, *J* = 4.4 Hz, 2H), 4.36 (t, *J* = 4.4 Hz, 2H), 3.96 (s, 3H). 3.69-3.61 (m, 10H), 3.52 (brs, 2H), 3.39-3.29 (m, 4H), 1.59 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.33, 154.18, 152.28, 146.33, 145.37, 139.40, 135.26, 125.94, 125.23, 121.68, 115.47, 109.95, 108.44, 70.58, 70.52, 70.48, 70.15, 69.96, 69.86, 68.74, 66.95, 66.86, 56.34, 50.56, 40.72, 22.10.

Compound S24.

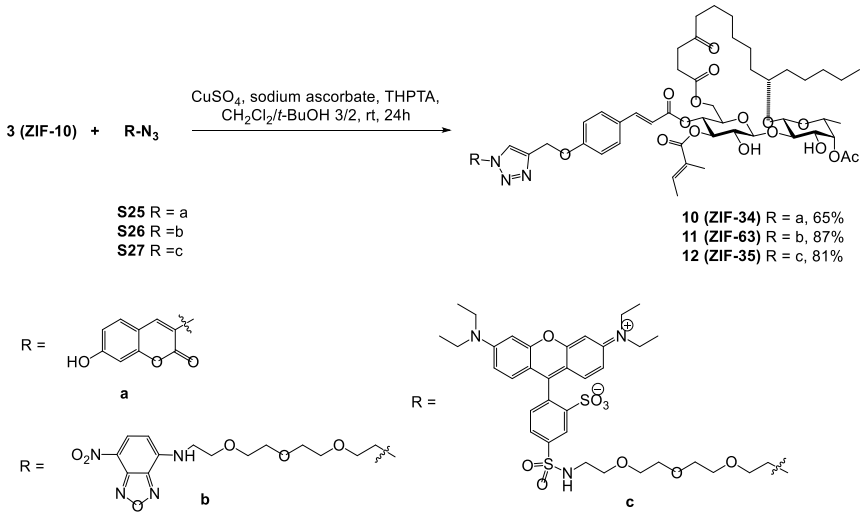
A solution of compound **S22** (100 mg, 0.15 mmol), Et_3N (37 mg, 36 mmol) in THF (6 mL) was slowly added to Compound **S23** (75 mg, 0.18 mmol) in THF (0.5 mL). The reaction mixture was stirred at room temperature 0.5 h. At this point, TLC (silica, 5:1 EtOAc–MeOH) showed the reaction was complete. The reaction mixture was quenched with 5% HCl (5 mL), and the aqueous layer was extracted with Ethyl Acetate (30 mL), washed with saturated $NaHCO_3$ (10 mL), and brine (10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, EtOAc–MeOH, 10: 1) gave desired compound **S24** (58 mg, 41%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H), 7.20 (s, 1H), 6.28 (q, *J* = 6.4 Hz, 1H), 4.48 (dd, J_1 = 4.4 Hz, J_2 = 7.6 Hz, 1H). 4.41-4.39 (m, 2H), 4.31-4.28 (m, 3H), 3.96 (s, 3H). 3.66-3.51 (m, 22H), 3.49-3.25 (m, 4H), 3.26-3.22 (m, 1H), 2.93 (dd, J_1 = 5.2 Hz, J_2 = 12.8 Hz, 1H). 2.70 (d, *J* = 12.8 Hz, 1H), 2.20 (t, *J* = 7.2 Hz, 2H), 1.74-1.60 (m, 4H), 1.60 (d, *J* = 6.4 Hz, 3H), 1.482-1.41 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.10, 166.05, 158.67, 157.87, 155.79, 148.41, 140.95, 134.89, 110.91, 109.81, 71.60, 71.58, 71.54, 71.52, 71.46, 71.21, 71.18, 71.08, 70.96, 70.61, 69.75, 69.29, 64.25, 63.34, 61.61, 57.02, 56.98, 51.77 ,41.75, 41.64, 41.04, 40.32, 36.73, 29.75, 29.49, 26.83, 22.46.

Compound 9.



Compound **8** (21.5 mg, 0.0242 mmol) and azide **S22** (13.7 mg, 0.0145 mmol) were dissolved in a mixture of CH_2Cl_2/*t*-BuOH (3:2, w/w, 2 mL). To the mixture were added an aqueous solution of $CuSO_4$ (1 M, 1 eq, 24 μL), an aqueous solution of sodium ascorbate (1 M, 2 eq, 48 μL) and ligand THPTA (2.1 mg, 0.2 eq) and the mixture was vigorously stirred in dark for 24 h. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (silica, MeOH–CH_2Cl_2, 1:20 → 1:10) gave compound **9** (20.5 mg, 77%) as a white film. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H, triazole-*H*), 7.63 (s, 1H, NO_2-Ar-*H*), 7.08 (d, *J* = 8.4 Hz, 2H, Ar*H*), 7.03 (s, 1H, NO_2-Ar-*H*), 6.89 (d, *J* = 8.4 Hz, 2H, Ar*H*), 6.45 (br, 1H, N*H*), 6.35 (q, 1H, *J* = 6.0 Hz, NO_2-Ar-C*H*(CH_3)-O-), 5.79 (br, 1H, N*H*), 5.59 (br, 1H, N*H*), 5.45 (br, 1H, N*H*), 5.22 – 5.14 (m, 3H, triazole-CH_2O-, N*H*), 5.13 – 5.06 (m, 3H, H-3-Glu*p*, H-4-Glu*p*, H-4-Fuc*p*), 4.63 – 4.42 (m, 8H, H-1-Glu*p*, H-6-Glu*p*, biotin-C*H*, PEG-CH_2, O*H*), 4.38 (d, *J* = 7.6 Hz, 1H, H-1-Fuc*p*), 4.35 – 4.20 (m, 4H), 4.01 – 3.85 (m, 7H, H-6-Glu*p*, H-3-Fuc*p*, PEG-CH_2, CH_3OAr), 3.70 – 3.22 (m, 32H, H-2-Glu*p*, H-5-Glu*p*, H-2-Fuc*p*, H-5-Fuc*p*, -CH_2-C*H*-CH_2-, PEG-CH_2), 3.18 – 3.11 (m, 1H), 2.95 – 2.64 (m, 6H), 2.62 – 2.30 (m, 7H), 2.24 – 2.13 (m, 5H), 1.82 –1.21 (m, 30H), 1.17 (d, *J* = 6.4 Hz, 3H, H-6-Fuc*p*), 1.09, 1.05 (2d, *J* = 6.8, 7.2 Hz, 3H), 0.90 – 0.82 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.0, 176.8, 176.7, 173.1, 171.9, 171.7, 171.6, 171.5, 171.4, 156.8, 156.2, 155.4, 154.1, 146.8, 139.5, 134.7, 132.9, 129.3, 114.8, 109.9, 108.5, 105.7, 100.3, 82.5, 79.6, 74.6, 73.7, 73.6, 73.0, 72.7, 72.7, 72.4, 70.5, 70.5, 70.4, 70.4, 70.2, 70.0, 70.0, 69.8, 69.4, 68.7, 68.1, 67.4, 67.3, 63.0, 62.1, 61.8, 61.4, 60.2, 56.4, 55.4, 50.4, 41.8, 41.2, 41.0, 40.8, 40.7, 40.5, 39.1, 37.6, 35.8, 35.7, 34.4, 33.2, 31.9, 29.6, 29.6, 29.2, 29.0, 28.3, 28.1, 26.5, 26.4, 25.4, 24.7, 24.5, 23.6, 22.6, 22.2, 21.0, 16.5, 16.4, 16.3, 14.1, 11.5, 11.5. HRMS (ESI) *m*/*z* calcd for $C_{86}H_{132}N_9O_{32}S$ $[M+H]^+$ 1834.8699, found: 1834.8657.

Syntheses of Fluorescent Probes 10, 11 and 12.



Compound 10.

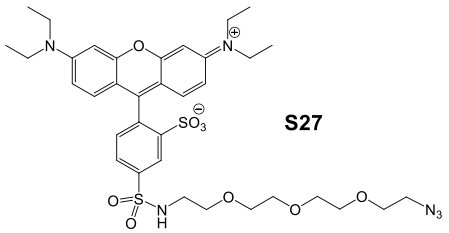
Compound **3** (23.2 mg, 0.0262 mmol) and the azide **S25**[5] (5.3 mg, 0.0262 mmol) were dissolved in a mixture of CH_2Cl_2/*t*-BuOH (3:2, w/w, 2 mL). To the mixture were added an aqueous solution of $CuSO_4$ (1 M, 1 eq, 26 μL) and an aqueous solution of sodium ascorbate (1 M, 2 eq, 52 μL) and the mixture was vigorously stirred without light for 24 h. TLC analysis (silica, 1:20 MeOH–CH_2Cl_2) showed the reaction was done. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (silica, MeOH–CH_2Cl_2, 1:30 → 1:20) gave compound **10** (18.5 mg, 65%) as a colorless film. $[\alpha]_D^{25}$ –15.1° (*c* 0.3 $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (s, 1H, Ar*H*), 8.43 (s, 1H, Ar*H*), 7.63 – 7.55 (m, 1H, Ar*H*), 7.52 (d, *J* = 15.6 Hz, 1H, Ph-C*H*=C-), 7.43 (d, *J* = 8.8 Hz, 1H, Ar*H*), 7.37 – 7.31 (m, 2H, Ar*H*), 7.01 – 6.85 (m, 3H, Ar*H*, Me-C*H*-C(Me)-C=O), 6.82 (d, *J* = 2.0 Hz, 1H, Ar*H*), 6.15 (d, *J* = 15.6 Hz, 1H, Ph-CH=C*H*), 5.28 (t, *J* = 10.0 Hz, 1H, H-4-Glu*p*), 5.24 (s, 2H, triazole-CH_2O-), 5.17 – 5.10 (m, 2H, H-3-Glu*p*, H-4-Fuc*p*), 4.68 – 4.62 (m, 3H, H-1-Glu*p*, H-6-Glu*p*, O*H*), 4.41 (d, *J* = 7.6 Hz, 1H, H-1-Fuc*p*), 4.17 – 4.05 (m, 2H, H-6-Glu*p*, O*H*), 3.98 – 3.90 (m, 1H, H-3-Fuc*p*), 3.84 – 3.75 (m, 1H, H-5-Glu*p*), 3.74 – 3.59 (m, 4H, H-2-Glu*p*, H-2-Fuc*p*, H-5-Fuc*p*, -CH_2-C*H*-CH_2-), 2.93 – 2.38 (m, 6H), 2.19 (s, 3H, CH_3-C=O), 1.79 – 1.71 (m, 6H, CH_3-CH-C(CH_3)-C=O), 1.71 – 1.60 (m, 2H), 1.55 – 1.22 (m, 16H), 1.20 (d, *J* = 6.4 Hz, 3H, H-6-Fuc*p*), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.2, 172.7, 171.9, 169.1, 165.7, 161.3, 160.2, 156.2, 154.6, 145.7, 140.2, 134.2, 130.2, 129.9(×2), 127.5, 127.1, 119.7, 114.9(×2), 114.6, 114.2, 111.0, 105.9, 103.2, 100.3, 83.2, 79.9, 77.7, 76.2, 74.0, 72.7, 72.6, 68.8, 66.9, 61.8, 61.7, 41.8, 37.7, 34.4, 33.3, 31.9, 29.7, 29.2, 29.1, 28.4, 24.8, 24.6, 23.6, 22.6, 20.9, 16.3, 14.6, 14.1, 12.0. HRMS (ESI) *m*/*z* calcd for $C_{56}H_{69}N_3NaO_{19}$ $[M+Na]^+$ 1110.4423, found: 1110.4420.

Compound 11.

Compound **3** (40.0 mg, 0.0452 mmol) and the azide **S26**[6] (15.5 mg, 0.0407 mmol) were dissolved in a mixture of CH_2Cl_2/*t*-BuOH (3:2, w/w, 2 mL). To the mixture were added an aqueous solution of $CuSO_4$ (1 M, 1 eq, 45 μL) and an aqueous solution of sodium ascorbate (1 M, 2 eq, 90 μL) and the mixture was vigorously stirred without light for 24 h. TLC analysis (silica, 1:10 MeOH–EtOAc) showed the reaction was done. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (Ialtra beads, MeOH–EtOAc, 1:20 → 1:10) gave compound **11** (44.8 mg, 87%) as colorless syrup. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, *J* = 8.8 Hz, 1H, NBD-*H*), 7.82 (s, 1H, Triazole-*H*), 7.52 (d, *J* = 16.0 Hz, 1H, Ph-C*H*=C-), 7.39 (d, *J* = 8.8 Hz, 2H, Ar*H*), 7.21 (br, 1H, N*H*), 6.94 (d, *J* = 8.8 Hz, 1H, Ar*H*), 6.92 – 6.80 (m, 1H, Me-C*H*-C(Me)-C=O), 6.18 (d, *J* = 16.0 Hz, 1H, Ph-CH=C*H*-), 6.18 (d, *J* = 8.8 Hz, 1H, NBD-*H*), 5.26 (t, *J* = 9.6 Hz, 1H, H-4-Glu*p*), 5.24 (s, 2H, triazole-CH_2O-), 5.18 – 5.11 (m, 2H, H-3-Glu*p*, H-4-Fuc*p*), 4.71 (br, 1H, O*H*), 4.60 (d, *J* = 7.6 Hz, 1H, H-1-Glu*p*), 4.56 (t, *J* = 4.8 Hz, 2H, PEG-CH_2), 4.47 (dd, *J* = 12.8, 3.2 Hz, 1H, H-6-Glu*p*), 4.38 (d, *J* = 7.6 Hz, 1H, H-1-Fuc*p*),

4.20 (br, 1H, O*H*), 4.15 – 4.04 (m, 2H, H-6-Glu*p*, PEG-C*H*), 3.90 (t, *J* = 4.8 Hz, 2H, PEG-CH_2), 3.85 – 3.70 (m, 3H, H-5-Glu*p*, H-3-Fuc*p*, PEG-C*H*), 3.69 – 3.55 (m, 14H, H-2-Glu*p*, H-2-Fuc*p*, H-5-Fuc*p*, -CH_2-C*H*-CH_2-, PEG-CH_2), 2.82 – 2.35 (m, 6H), 2.15 (s, 3H, CH_3-C=O), 1.79 – 1.70 (m, 6H, CH_3-CH-C(CH_3)-C=O), 1.69 – 1.41 (m, 6H), 1.40 – 1.20 (m, 12H), 1.16 (d, *J* = 6.4 Hz, 3H, H-6-Fuc*p*), 0.87 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.1, 171.7, 171.6, 171.09, 168.6, 165.5, 160.1, 145.4, 144.2, 144.1, 143.9, 139.5, 136.5, 129.9(×2), 127.6, 127.2, 124.0, 123.6, 115.0(×2), 114.5, 105.7, 100.2, 98.8, 82.7, 79.7, 75.7, 73.9, 72.7, 72.6, 72.3, 70.5, 70.4, 70.4, 70.4, 69.3, 68.7, 68.1, 67.3, 61.9, 61.7, 60.3, 50.3, 43.6, 41.8, 37.6, 34.3, 33.1, 31.8, 29.1, 29.0, 28.2, 24.6, 24.4, 23.5, 22.6, 21.0, 20.9, 16.3, 14.5, 14.1, 14.0, 11.9. HRMS (ESI) *m*/*z* calcd for $C_{61}H_{84}N_7O_{22}$ $[M+H]^+$ 1266.5669, found: 1266.5660.

Compound 12.



Compound **3** (50.1 mg, 0.0566 mmol) and azide **S27**[7] (30.0 mg, 0.0396 mmol) were dissolved in a mixture of CH_2Cl_2/*t*-BuOH (3:2, w/w, 3 mL). To the mixture were added an aqueous solution of $CuSO_4$ (1 M, 1 eq, 57 μL), sodium ascorbate (1 M, 2 eq, 113 μL) and ligand THPTA (5 mg). The mixture was vigorously stirred without light for 24 h. TLC analysis (silica, 1:20 MeOH–CH_2Cl_2) showed the reaction was done. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (silica, MeOH–CH_2Cl_2, 1:30 → 1:20) gave compound **12** (52.7 mg, 81%) as a purple solid and starting material **3** (10.8 mg) was recovered. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (d, *J* = 2.0 Hz, 1H, Ar*H*), 8.03 (s, 1H, Ar*H*), 7.98 (dd, *J* = 7.6, 1.6 Hz, 1H, Ar*H*), 7.57 (d, *J* = 16.0 Hz, 1H, Ph-C*H*=C-), 7.43 (d, *J* = 8.8 Hz, 2H, Ar*H*), 7.27 – 7.23 (m, 2H, Ar*H*), 7.20 (d, *J* = 8.0 Hz, 1H, Ar*H*), 6.99 (d, *J* = 8.4 Hz, 2H, Ar*H*), 6.93 – 6.83 (m, 1H, Me-C*H*-C(Me)-C=O), 6.82 – 6.75 (m, 2H, Ar*H*), 6.66 (d, *J* = 2.4 Hz, 2H, Ar*H*), 6.25 – 6.15 (m, 2H, Ph-CH=C*H*-, SO_2N*H*), 5.29 (t, *J* = 9.6 Hz, 1H, H-4-Glu*p*), 5.21 (s, 2H, triazole-CH_2O-), 5.18 – 5.10 (m, 2H, H-3-Glu*p*, H-4-Fuc*p*), 4.69 (br, 1H, O*H*), 4.65 (d, *J* = 7.6 Hz, 1H, H-1-Glu*p*), 4.60 (t, *J* = 5.2 Hz, 2H, PEG-CH_2), 4.45 (dd, *J* = 12.4, 3.6 Hz, 1H, H-6-Glu*p*), 4.39 (d, *J* = 7.6 Hz, 1H, H-1-Fuc*p*), 4.23 – 4.07 (m, 2H, H-6-Glu*p*, O*H*), 3.95 (t, *J* = 4.8 Hz, 2H, CH_2), 3.90 (dd, *J* = 9.6, 3.6 Hz, 1H, H-3-Fuc*p*), 3.78 – 3.72 (m, 1H, H-5-Glu*p*), 3.72 – 3.45 (m, 22H, H-2-Glu*p*, H-2-Fuc*p*, H-5-Fuc*p*, -CH_2-C*H*-CH_2-, PEG-CH_2, N(C$H_2$$CH_3$)$_2$), 3.28 – 3.21 (m, 2H, SO_2NHC$H_2$$CH_2$O), 2.86 – 2.36 (m, 6H), 2.15 (s, 3H, CH_3-C=O), 1.93 – 1.20 (m, 36H), 1.17 (d, *J* = 6.4 Hz, 3H, H-6-Fuc*p*), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.1, 171.7, 171.6, 168.9, 165.7, 160.5, 159.0, 157.9(×2), 155.5(×2), 148.4, 145.8, 143.0, 141.9, 139.8, 133.6, 133.4(×2), 129.9(×2), 129.6, 127.6, 127.2, 126.9,

124.8, 115.3, 114.3, 114.2, 113.5, 105.6, 100.4, 95.6, 82.6, 79.9, 76.0, 73.9, 72.8, 72.5, 72.4, 70.4, 70.2, 70.2, 69.4, 69.3, 68.8, 67.4, 61.9, 50.3, 45.8, 43.2, 41.8, 37.6, 34.4, 33.2, 31.9, 29.1, 29.0, 28.3, 24.7, 24.5, 23.5, 22.6, 20.9, 16.4, 14.6, 14.1, 12.5, 11.9. HRMS (ESI) *m/z* calcd for $C_{82}H_{111}N_6O_{25}S_2$ $[M+H]^+$ 1643.7040, found: 1643.7048.

BIOLOGY.

General Methods

All reagents were purchased from commercial sources listed below and were used without further purification unless otherwise noted. DMEM high glucose culture medium was purchased from GE Healthcare Life Sciences (SH30243.02). Protease inhibitor (0993C286), Resazurin sodium (0695), Deoxycholic acid sodium salt (0613) and Glycerol(M152) were from Amresco. Tris(2-carboxyethyl) phosphine (TCEP) was from Combi-blocks (OR-5119). High capacity streptavidin agarose beads and Alamar Blue cell viability reagent (DAL1025) were from Thermo scientific (20361). Thiazoyl blue tetrazolium bromide (00697), N-dodecyl-ß-D-maltoside (21950) and Sodium Dodecyl Sulfate (00270) were from Chem Implex Intl Inc. Hepes (HB0264), Sodium orthovanadate (SB0869) and Micro BCA protein assay kit (SK3061) were from Bio Basic. Tergitol NP40 was from Spectrum (T1279). Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (TBTA) was from TCI Chemicals (T2993). Copper (II) Sulfate Pentahydrate was from Alpha Aesar (14178). Sodium fluoride was from Avantor (3688-01). β-Glycerophosphoric acid was from Acros (410991000). Fibronectin (Bovine) was from Akron Biotech (AK8350-0001). Mountain medium was from Biotium (23001). Coverslip was from Electron Microscopy Sciences (72296-08). ER-Tracker™ Blue-White DPX was from Invitrogen (E12353). 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) was from MP Biomedicals (157574). Expre35s35s protein labelling mix (NEG072002MC) was from Perkin Elmer. Concanavalin A aragrose beads were from Sigma-Aldrich (C7555). Antibody against Sec61α was from Abcam (ab15575). Anti-rabbit IgG-HRP (C2818), anti-goat IgG-HRP (sc-2020) and antibody against actin (I-19) (sc-1616) were from Santa Cruz. IRDye® 800CW StreptavidinSynergy™ (92632230) and Odyssey® Blocking Buffer (92640000) was from Licor. Synergy™ H1 plate reader was from BioTek Instruments. Typhoon™ FLA 9500 was from GE Healthcare Life Sciences. Leica TCS SP5 was from Leica Microsystems. Odyssey infrared imager was from Licor. Synthetic mycolactone used in the pulse-chase analysis of protein secretion in HepG2 cells was a gift from Yoshito Kishi, Harvard University, Cambridge, MA.

Experimental Procedures

Cytotoxicity Assay

Cell culture

Two human breast cancer cell lines (MDA-MB-231 and MCF7) and one human breast non-tumorigenic epithelial cell line (MCF-10A) were maintained in a DMEM high glucose culture medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. The cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 ºC. The culture medium was changed every 2–4 days depending on cell density. Cell cultures were passaged once a week using 0.125% trypsin-EDTA to detach the cells from culture flasks/dishes.

The human colorectal cancer cell line HCT116 was maintained in McCoy's 5A (modified) medium supplemented with 10% fetal bovine serum (FBS). The human embryonic kidney (HEK293T and HEK293 FRT TRex) cell line was maintained in DMEM high glucose culture medium supplemented with 10% fetal bovine serum (FBS).

HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37°C under 5% CO_2.

MTT Cytotoxicity Assay

Viable cells were counted with a hemocytometer immediately before each experiment. Experiments were done in triplicate. First, 100 µL of MCF7 cells at the density of 50,000 cells/mL were seeded in a 96-well plate (5,000 cells/well), which was incubated at 37 ºC and 5% CO_2 atmosphere for 24 h. The compounds were dissolved in DMSO (dimethyl sulfoxide) to make drug stocks (10 mM). The stock solutions were diluted with the complete DMEM high glucose culture medium to make a series of gradient fresh working solutions right before each test. Subsequently, the cells were treated with 100 µL of the freshly made gradient working solution in a total volume of 200 µL/well for 72 h. After that, the media were discarded and 200 µL of the fresh complete medium containing 10% of MTT stock solution (5 mg/mL) was added to each well. The plate was then incubated at 37 ºC and 5% CO_2 for 3 h. Next, 180 µL of the medium was discarded from each well, and 180 µL of DMSO was added to each well to dissolve formazan crystals. Absorbance of formazan was detected by a microplate reader (BioTek Synergy H1) at 570 nm with 650 nm as the reference wavelength. The percentage of viability compared to the negative control (DMSO-treated cells) was determined. GraphPad Prism 6 software was used to make a plot of % viability versus sample concentration and to calculate the concentration at which a compound exhibited 50% cytotoxicity (IC_{50}).

AlamarBlue Cytotoxicity Assay

MDA-MB-231, MCF-10A and HCT116. Viable cells were counted with a hemocytometer immediately before each experiment. Experiments were done in triplicate. First, 100 μL of MDA-MB-231 cells or MCF-10A cells at the density of 50,000 cells/mL was seeded in a 96-well plate (5,000 cells/well) and then incubated at 37 ºC and 5% CO_2 for 24 h. The compounds were dissolved in DMSO (dimethyl sulfoxide) to make drug stocks (10 mM). The stock solutions were diluted with the complete DMEM high glucose culture medium to make a series of gradient fresh working solutions right before each test. Subsequently, the cells were treated with 100 μL of the freshly made gradient working solution in a total volume of 200 μL/well for 72 h. After that, the media were discarded and 200 μL of the fresh complete medium containing 10% of AlamarBlue (resazurin) stock solution was added to each well. The plate was then incubated at 37 ºC and 5% CO_2 for another 3 h. Emission of each well at 620 nm was detected by a microplate reader (BioTek Synergy H1) at excitation 580 nm. For HCT116 WT and mutant lines, cells were seeded at 1000 cells/ well in complete McCoys medium. Compounds were diluted from a DMSO stock into culture medium as described above. Cells were treated for 96 h, then viability measured with AlamarBlue, detected using the conditions described but with a BMG Fluostar Optima Microplate Reader. The percentage of viability compared to the negative control (DMSO-treated cells) was determined. GraphPad Prism 6 software was used to make a plot of % viability versus sample concentration and to calculate the concentration at which a compound exhibited 50% cytotoxicity (IC_{50}).

HCT116 and HEK293T. Viable cells were counted using an automated cell counter (BioRad TC20) before each experiment. Experiments were performed either in quadruplicate or duplicate sets. First 100μl of HCT116 or HEK293T cells at a density of 25,000 cells/ml was seeded in black wall, clear bottom, 96-well plates (2500 cells/well) (Viewplate-96 Perkin Elmer). HEK293 FRT Trex cells were seeded in complete medium supplemented to 1 μg/ml Doxycycline to induce expression of Sec61a constructs. Cells were then incubated at 37 ºC and 5% CO_2 for 24 h. The compounds were dissolved in DMSO to make drug stocks (10 mM). The stock solutions were diluted with the complete DMEM or McCoy's 5A culture media supplemented with DMSO to make a series of gradient fresh working solutions at equal DMSO percentage immediately prior to each test. Subsequently, the cells were treated with 25 μl of the freshly made gradient in a total volume of 125 μl/well for 72 h. After that, the media were supplemented with 10% of AlamarBlue (resazurin) stock solution. The plate was then incubated at 37 ºC and 5% CO_2 for another 4 h. Emission of each well at 620 nm was detected by a microplate reader (Perkin Elmer EnSpire) at excitation 580 nm. The percentage of viability compared to the negative control (DMSO-treated cells) was determined. GraphPad Prism 7 software was used to make a plot of % viability versus sample concentration and to calculate the concentration at which a compound exhibited 50% cytotoxicity (IC_{50}).

Activity Based Protein Profiling

MDA-MB-231 cells were seeded in five groups of 6 cm petri dishes with 3 ml DMEM high glucose culture and incubated at 37 ºC and 5% CO_2 until 90% confluency. Each group was treated with fresh medium containing 20 µM competitor or an equal volume of DMSO vehicle for 30 min at 37 ºC and 5% CO_2, followed by addition of 0.2 µM probe or an equal volume of DMSO. After 1 h incubation at 37 ºC and 5% CO_2, the cells were harvested by scraping and washed with cold PBS for 3 times. The cells were then lysed in 100 µl of lysis buffer 1 (50 mM Hepes, pH 9.0, 100 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% NP40, 1x protease inhibitor) using ultrasound sonication. The supernatant of each group was collected after centrifugation at 21,100 g and 4 ºC for 10 min, and the protein concentration was quantified with a BCA kit. 10% SDS was then added into protein solution to make a final SDS concentration of 0.4%, and the solution was incubated at 95 ºC for 5 min. 1 µl TCEP (100 mM stock solution in H_2O) was mixed with 1 µl TBTA (10 mM stock solution in a 4:1 ratio of *t*-butanol and DMSO), 1 µl $CuSO_4$ (100 mM stock solution in H_2O), and 1 µl rhodamine-azide (1 mM stock solution in DMSO). The mixed solution was added into the previously prepared protein solution containing 0.25 mg protein. The total volume was adjusted to 100 µl with the lysis buffer 1 to make a final protein concentration of 2.5 mg/ml, and the click reaction was performed at room temperature for 1.5 h. Proteins in 15 µl of the reactant were fractionated by an SDS-PAGE gel. The protein gel was scanned with a typhoon scanner (Typhoon™ FLA 9500) at 532 nm excitation with 700 mV gain setting and then stained with Coomassie blue.

Biotin Affinity Pulldown

Live Cell-based Pulldown

According to the experimental needs, MDA-MB-231 cells were seeded into 3-7 groups of 15-cm tissue-culture dishes and incubated at 37 ºC and 5% CO_2 until 90% confluency. Each group was treated with fresh medium containing a competitor or an equal volume of DMSO vehicle for 30 min at 37 ºC and 5% CO_2, followed by addition of a probe or an equal volume of DMSO. After 1 h incubation at 37 ºC and 5% CO_2, the cells were harvested by scraping and washed with cold PBS for 3 times and lysed with the lysis buffer 2 (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM Na_3VO_4). The supernatant of each group was collected after centrifugation at 4 ºC and 21,100 g for 10 min, and the protein concentration was quantified with the BCA kit. After adjusting protein concentration with lysis buffer 2, each protein solution with equal amount of total protein in the same volume was incubated with 20 µl of washed streptavidin bead at 4 ºC for 2 h with

rotation. After incubation, the beads were washed with washing buffer (50 mM Tris, pH 7.4, 150mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM Na_3VO_4) for 3 times and boiled at 95ºC for 5 min in 2x sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 2% β-mercaptoethanol and 0.02% bromophenol blue). After centrifugation at 21,100 g and 4 ºC for 1 min, the cleared eluted proteins were divided into two parts. One part was fractionated by a 12% SDS-PAGE and visualized by silver staining. The other part was analyzed by Western blotting, in which proteins on the nitrocellulose membrane were visualized by either fluorescently labeled streptavidin or an anti-Sec61α antibody.

Cell Lysate-based Pulldown

MDA-MB-231 cells from seven 15-cm tissue culture dishes were harvested and washed with PBS for 3 times and lysed in buffer 2 using ultrasound sonication. The supernatant of each group was collected after centrifugation at 21,100 g and 4 ºC for 10 min, and the protein concentration was quantified with the BCA kit and adjusted to be between 3.5–4 mg/mL with lysis buffer 2. Next, the cell lysate was equally divided into 7 fractions, 1 ml each. Each fraction was treated with either a competitor or an equal volume of DMSO vehicle for 30 min at 4 ºC, followed by the addition of a probe or an equal volume of DMSO. All the samples were then incubated at 4 ºC for 1 h. After incubation, 20 µl of streptavidin washed bead was added to each sample followed by 2 h incubation at 4 ºC with rotation. The remaining procedures were the same as the live cell-based pulldown (see above).

ER Microsome-based Pulldown

1 ul of Ipomoeassin F (2 mM stock concentration) or DMSO vehicle was added to 198.6 ul ER microsome suspension in one of the three Eppendorf tubes to produce a final ipomoeassin F concentration of 10 µM and two blank tubes. The resulting mixtures were incubated at 4 ºC for 30 min, followed by addition of 0.2 µM probe (final concentration) to two tubes or an equal volume of DMSO to a blank tube. After 1 h incubation at 4 ºC, 10% DDM (*n*-dodecyl-ß-D-maltoside) was added to each tube to give the final concentration of 1%, and the mixtures were further incubated at 4 ºC for another 1 h. The supernatant of each group was collected after centrifugation at 4 ºC and 21,100 g for 10 min. 20 µl of washed streptavidin beads was added to each sample followed by 2 h incubation at 4 ºC with rotation. After washing with washing buffer (50 mM Tris, pH 7.4, and 150 mM NaCl) for 3 times, the bound proteins were eluted by boiling the beads in 20 µl of 2x sample buffer at 95 ºC for 5 min. The eluted proteins were analyzed by SDS-PAGE or Western blotting as described above.

Delayed/Reverse Competition in Live Cell-based Pulldown

The procedure is almost identical to that of the regular pulldown except for the switched order of addition for the biotin probe and ipomoeassin F.

Mass Spectrometry (MS) Analysis and MS Data Processing

In the pulldown, the bound proteins were eluted via boiling the high capacity streptavidin agarose beads in 1x sample buffer. The eluted proteins were fractionated with a 12% SDS-PAGE and the fractionated proteins were visualized by blue silver staining.[8] The ~40 kDa protein band from the probe **7**-enriched sample and the corresponding regions of the gel for the negative control and the ipomoeassin F competition sample were cut, in-gel digested, the resulting peptides were analyzed by an Orbitrap Fusion mass spectrometer (*Thermo Fisher Scientific*), and the MS/MS spectra were searched against a composite target-decoy UniProtkB human protein database (2018_02, 20317 entries) using Mascot (Matrix Science, London, UK; version 2.5.1) as described previously.[9-10] The parameters for database searching were as follows: MS tolerance of 3.0 ppm and MS/MS tolerance of 0.5 Da; tryptic enzyme specificity with a maximum of 2 missed cleavages; fixed modification, carbamidomethyl of cysteine; and variable modifications, oxidation of methionine and acetylation of the N-terminus. Search results were further processed by Scaffold software (version 4.8.6; Proteome Software, Portland, OR) for viewing protein and peptide identification information. In the Scaffold analysis, peptide identifications were accepted if they could achieve a false discovery rate (FDR) of <1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could achieve an FDR of <1.0% and contained at least 2 identified peptides. Total spectral counts were exported from Scaffold and used to represent relative protein abundance from different samples based on the positive, linear correlation between spectral count and the abundance of a protein in complex samples.[11-13] Contaminant proteins such as keratins were discarded. For reliable representation of protein abundance, proteins identified with <4 spectra in the probe **7**-enriched sample were discarded.[12]

Cell Imaging

MDA-MB-231 cells were seeded on fibronectin-coated coverslips at 70% confluency in a 48-well plate and cultured at 37 ºC and 5% CO_2. After 24 h, culture medium was replaced with fresh medium containing a 20 µM competitor or an equal volume of DMSO vehicle for 30 min at 37 ºC and 5% CO_2, followed by addition of 0.2 µM (final concentration) fluorescent probe **12**. After 1 h incubation at 37 ºC and 5% CO_2, the coverslips were mounted onto a glass slide with a mounting medium with or without 0.5 µg/ml DAPI. The fluorescent images were acquired with a confocal microscope (Leica TCS SP5) using 405 nm excitation and 562 nm excitation. For the ER co-localization studies, 1 uM ER-Tracker™ Blue-White DPX was added to the medium after 1 h incubation with probe **12**. After 5 min incubation, the coverslips were mounted onto a glass slide and the images were acquired as described above.

Photo-Affinity Labeling.

CT7 photo-affinity labeling and click chemistry were done as previously described.[14] Briefly, SRMs (sheep rough microsomes, isolated as described in reference[15]) containing 100 nM Sec61 were treated with 10 µM ipomoeassin F/mycolactone or 1% DMSO for 30 min at 0°C, followed by incubation with 100 nM CT7 for 10 min RT. Samples were then photolyzed for 10 min and crosslinked proteins were detected by click chemistry, SDS-PAGE, and in-gel fluorescent scanning (Typhoon Trio, GE Healthcare).

***In Vitro* Membrane Translocation Assays.**

DNA constructs

Unless otherwise stated, all cDNAs are of human origin and as previously described: CytB5OPG2,[16] GypC,[17] ppαF (*Saccharomyces cerevisiae*),[16] ppcecAOPG2 (*Hyalophora cecropia*),[16] PPL (*Bos Taurus*),[16] Sec61βOPG2[16] and VCAM1.[17] Ii encodes residues 17 to 232 of the short form of human HLA class II histocompatibility antigen gamma chain (Ii; P04232, isoform 2). Linear DNA templates were generated by PCR and transcribed into RNA using T7 or SP6 polymerase (Promega).

In vitro translation and translocation into ER microsomes

Translation reactions (20 µL) were performed in nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of EasyTag EXPRESS ^{35}S Protein Labelling Mix containing [^{35}S] methionine (Perkin Elmer) (0.533 MBq; 30.15 TBq/mmol). Amino acids minus methionine (Promega) were added to 25 µM each, 5% (v/v) compound (concentration as indicated in figures, from stock solutions in DMSO), or an

equivalent volume of DMSO, and nuclease-treated ER microsomes (from stock with OD_{280} = 44/mL) were added to 6.5% (v/v). Lastly, 10% volume of *in vitro* transcribed mRNA (from 200-1000 ng/μL stocks) encoding the relevant precursor protein was added and translation reactions performed for 20 min at 30°C. Following incubation with 0.1 mM puromycin for 5 min at 30°C to ensure translational termination and ribosome release, membrane-associated fractions were isolated by centrifugation through an 80 μL high-salt cushion (0.75 M sucrose, 0.5 M KOAc, 5 mM Mg(OAc)2, 50 mM Hepes-KOH, pH 7.9) at 100,000 x g for 10 min at 4°C in a TLA100 rotor (Beckmann). Membrane pellets were suspended directly in SDS (sodium dodecyl sulphate) sample buffer (0.02% bromophenol blue, 62.5 mM, 4% (w/v) SDS, 10% (v/v) glycerol, pH 7.6, 1 M dithiothreitol) and, where indicated, treated with 1,000 U of Endoglycosidase H (New England Biolabs) for 30 min at 37°C prior to analysis. All samples were denatured for 10 min at 70°C prior to resolution by SDS-PAGE (12% or 16% polyacrylamide gels, 120 V, 120 min), gels were fixed (20% MeOH, 10% AcOH) for 5 min, dried (BioRad gel dryer) for 2 h at 65°C and, following exposure to a phosphorimaging plate for 24-72 h, radiolabelled products were visualised using a Typhoon FLA-7000 (GE Healthcare).

Statistical Analysis

Quantitative analysis of Ii membrane insertion was performed using AIDA v.5.0 (Raytest Isotopenmeβgeräte) whereby the ratio of the signal intensity for the 2Gly/0Gly forms was used as a read-out to estimate efficiency of integration into the ER membrane. This value was then expressed relative to the matched DMSO control in order to derive the mean relative ER insertion from three independent experiments (n=3). Statistical significance of the effectiveness of Ipom-F analogues (RM one-way ANOVA) was determined using Tukey's multiple comparisons test. IC_{50} value estimates were determined using non-linear regression to fit data to a curve of variable slope (four parameters) using the least squares fitting method in which the bottom and top plateaus of the curve were defined as 0% and 100% respectively. The differences between the estimated IC_{50} values for the three compounds analysed were statistically significant (****) according to the extra-sum-of-squares F test. Statistical analyses and IC_{50} estimates were performed using Prism 7.0d (GraphPad) with statistical significance given as n.s., non-significant and ****, $P < 0.0001$.

U2-OS secNLuc-ATZ Secretion and Cell Viability Assays

The secNLuc-ATZ assay has been described elsewhere.[18] Briefly, U2-OS cells stably expressing secNLuc-ATZ were cultured in McCoy's 5A supplemented with 10% bovine growth serum (Hyclone SH30540.03), 50 units/mL penicillin, and 50 µg/mL streptomycin and plated into 1536-well black, clear-bottom treatment plates (Aurora Microplates #ABI121000A) at 1200 cells/well in 5 µL volume using a Multidrop Combi dispenser (ThermoFisher). Cells were returned to 37 ˚C for 1 h to adhere and compound or vehicle control was added to wells using a 1536-well pin-tool transfer device (Wako Automation, San Diego). Cells were incubated for 24 h and secNLuc-ATZ-containing media was transferred using an ATS-100 acoustic dispenser (EDC Biosystems, Fremont, CA) from the treatment plate to a white, solid-bottom 1536-well recipient plate (Greiner Bio One #789175-F) containing 3 µL/well of 1X PBS. Next, recipient plates received 2 µL/well Nano-Glo luciferase assay reagent by BioRAPTR FRD (Beckman Coulter), incubated for 10 min then imaged on a ViewLux uHTS Microplate Imager (PerkinElmer).

For the multiplexed cytotoxicity assay (24 h), after acoustic media transfer 3 µL/well CellTiter-Glo reagent (Promega) was added by BioRAPTR FRD to the black, clear-bottom treatment plate containing treated cells, incubated for 10 min, and then imaged with a ViewLux. Imaging conditions for the multiplexed cytotoxicity assay were: clear filter with 10 sec integration, slow speed, high sensitivity, and 2X binning.

SH-SY5Y GLuc Secretion and Cell Viability Assays.

SH-SY5Y human neuroblastoma cells stably expressing a constitutively secreted variant of Gaussia luciferase (GLuc-'No Tag') were previously described.[19] Cells were cultured in DMEM (4.5 g/L D-glucose, 1 mM sodium pyruvate) supplemented with 10% bovine growth serum (Hyclone SH30540.03) 20 mM Hepes, 50 units/mL penicillin, and 50 µg/mL streptomycin. Cells were plated into white 1536-well tissue culture treated plates (Corning cat. #7464) at 1,000 cells per well in a 5 µL volume using a Multidrop Combi dispenser. Cells were returned to 37 ˚C for 4 h to adhere and compound or vehicle control was added to wells using a 1536-well pin-tool transfer device (Wako Automation, San Diego). Cells were incubated at 37 ˚C for 24 h and secreted luciferase was detected by adding 1 µL of coelenterazine substrate to each well using a BioRAPTR FRD workstation. The substrate was prepared from a Pierce Gaussia Luciferase Glow Assay Kit (ThermoFisher) by adding 1 volume of coelenterazine to 99 volumes of Gaussia Glow Assay Buffer (final concentration of coelenterazine = 0.17X). Luminescence was measured using a ViewLux microplate imager equipped with clear filters.

For cell viability assays, cells were cultured, plated, and treated with test compounds as outlined for the secretion assays. 48 h after treatment, viability was assessed using a CellTiter-Glo assay by adding 3

µL of reagent to each well, incubating 15 min at room temperature, and reading luminescence using a ViewLux microplate imager.

Metabolic Labelling Assays in HCT116 Cells

HCT116 cells were counted using an automated cell counter (BioRad TC20) immediately prior to each experiment. 3 ml of HCT116 cells at a density of $0.166x10^6$ cells/mL were seeded to 6-well plates ($0.5x10^6$ cells/well) and then incubated at 37 ºC and 5% CO_2 for 24 h. The compounds were dissolved in DMSO to make drug stocks (10 mM). The stock solutions were diluted with DMEM high glucose culture medium lacking methionine and cysteine to make a fresh working solution immediately prior to each test. Cells were washed twice and 1ml of fresh DMEM lacking methionine and cysteine was added to each well. Subsequently, the cells were treated with 250 µl of compound solutions in a total volume of 1,250 µl/well. The plate was then incubated at 37 ºC and 5% CO_2 for 30 min before 100 µCi ^{35}S-labelled methionine/cysteine was added (Expre^{35}s^{35}s protein labelling mix). Plates were incubated for a further 30 min, then media collected and cells harvested by scraping after four washes in ice-cold PBS. Total protein was acquired by RIPA extraction from the cell pellet, cytoplasmic fraction was acquired by partial permeabilization of the outer cell membranes with 0.15% Digitonin followed by centrifugation, secreted proteins were acquired by TCA precipitation of the media.

Pulse-Chase Analysis of Protein Secretion in HepG2 Cells

The secretion assay was performed using a pulse–chase approach as described previously.[20] HepG2 cells were treated with DMSO, 100 nM compound **8**, 100 nM Ipom-F or 31.3 ng/µl mycolactone for 13.5 h before initiating the experiment, whereas pre-treatment with bortezomib 100 nM (Stratech) or 2.5 µg/ml brefeldin A were performed for 2 h and 1 h, respectively. Cells were starved in Met- and Cys-free DMEM (Invitrogen) for 20 min at 37°C. Labeling was initiated by addition of fresh starvation medium containing 22 mCi/ml [^{35}S]Met and [^{35}S]Cys protein labeling mix (PerkinElmer; specific activity >1,000 Ci/mmol) for 15 min. After washing with PBS, cells were incubated in serum-free DMEM supplemented with excess unlabeled L-Met and L-Cys (2 mM final concentration) for 2 h. The compounds were included throughout the starvation, pulse and chase. Secreted proteins in the media were recovered by precipitation with 13% trichloroacetic acid, washed in acetone and dissolved in 2x sample buffer. Cells were solubilized with Triton X-100 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% TX-100, 1 mM EDTA, 1 mM EGTA) supplemented with protease inhibitor cocktail and phenylmethylsulfonyl fluoride. Equal amounts

of precipitated proteins in the media and clarified lysates were analyzed by SDS-PAGE and phosphorimaging (FLA-3000; Fuji).

T Cell Assays

Jurkat E6.1 (ATCC TIB-152TM) T cells were cultured in RPMI GlutamaxTM (Life Technologies), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen) and penicillin/streptomycin (100 U/ml, 100 µg/ml). Cells were treated with Ipomoeassin F or mycolactone for 24 h, then pelleted and stained with FITC-conjugated anti-human CD62L (clone DREG-56 (BioLegend #304804) at 500 ng/ml) during 40 min at 4°C. Fluorescence signals were acquired on an Accuri CD (BD) and analyzed with the FlowJo X software. Immunosuppressive activity on T cells was measured through the inhibition of IL-2 production by Jurkat T cells incubated with mycolactone for 1 h prior to 24 h of activation with phytohemagglutinin-ionomycin, as described.[21] In both assays, blanks corresponded to cells treated with the vehicle volume of the highest concentration of sample tested (DMSO for IpoF; EtOH for mycolactone).

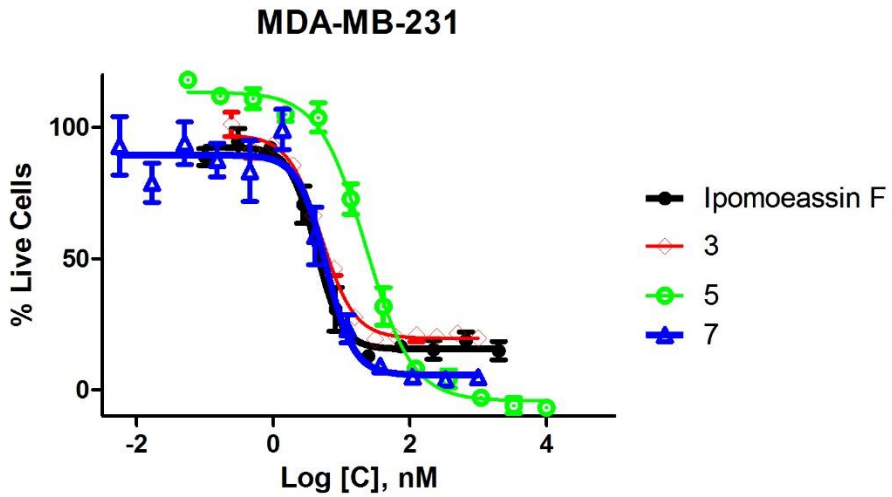
Washout assay

The reversibility of the inhibition of secreted protein production by ipomoeassin F was determined by following the production of TNF in RAW264.7 cells as described before.[22] Briefly, RAW264.7 cells were maintained at 37 °C and 5% CO_2 in high glucose DMEM (Merck), supplemented with 10% heat-inactivated FBS (GIBCO). Cells were plated into 24 well plates at a concentration of 1 x 10^5/ml 24 hour prior to assay, then incubated for 1 hour in the presence or absence of 125 ng/ml mycolactone, 250 nM Ipomoeassin F or DMSO carrier. Cells were either stimulated directly with 100 ng/ml TLR-grade LPS (Enzo) or washed 3 times with medium and incubated 24 hour, then stimulated with LPS. In each case, cells were incubated with LPS for 4 hour then the supernatants were removed. The concentration of TNF in supernatants was quantified by ELISA (eBioscience).

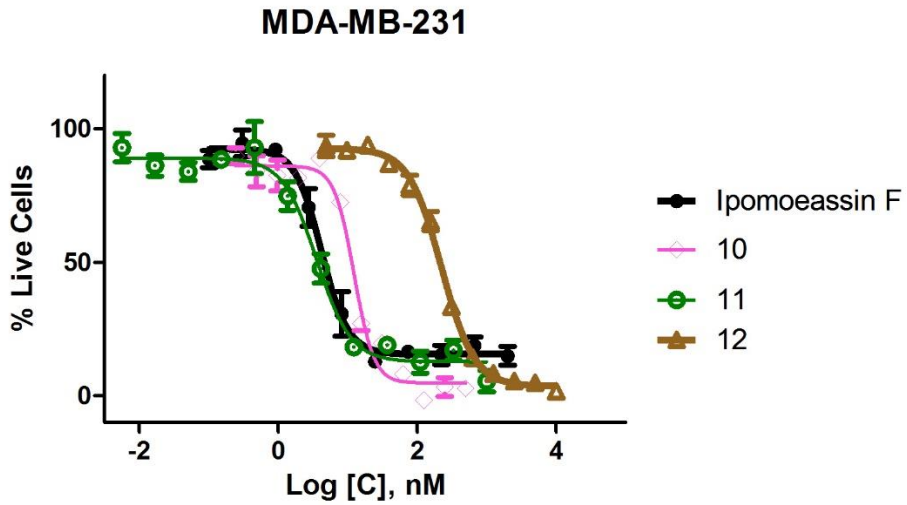
Figures:

Cell Cytotoxicity Assay

A.



B.



C.

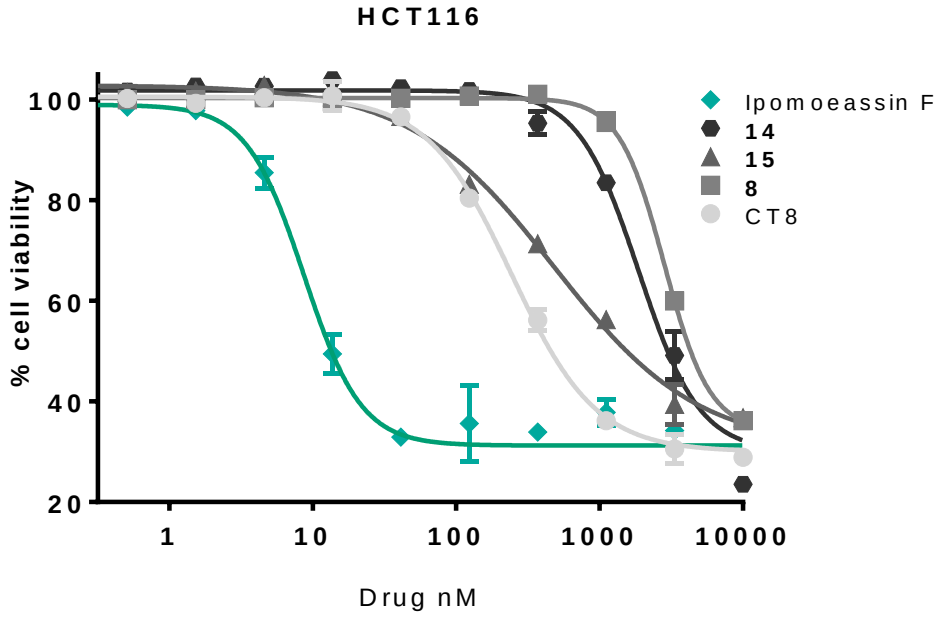
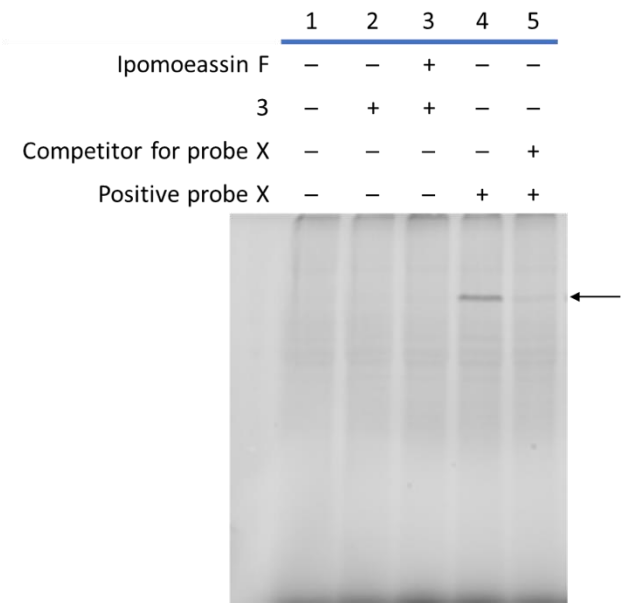


Figure S1. Cytotoxicity in MDA-MB-231 and HCT116 cells. (A) Cell viability curves of analogues **3**, **5**, **7** and ipomoeassin F (72 h). (B) Cell viability curves of analogues **10**, **11**, **12** and ipomoeassin F (72 h). (C) HCT116 colon carcinoma cells were treated with increasing concentrations of analogues **8**, **14**, **15**, CT8, ipomoeassin F or carrier (0.1% DMSO) and cell viability was assessed at 72 h by the Alamar Blue assay (mean ± S.D., n = 4).

A.



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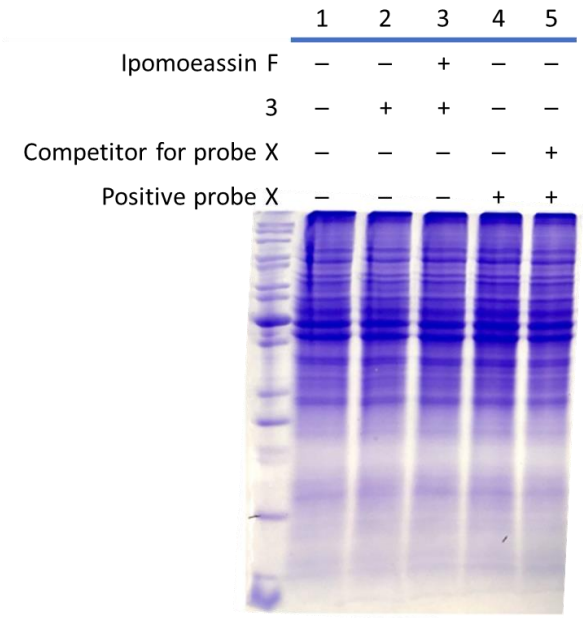
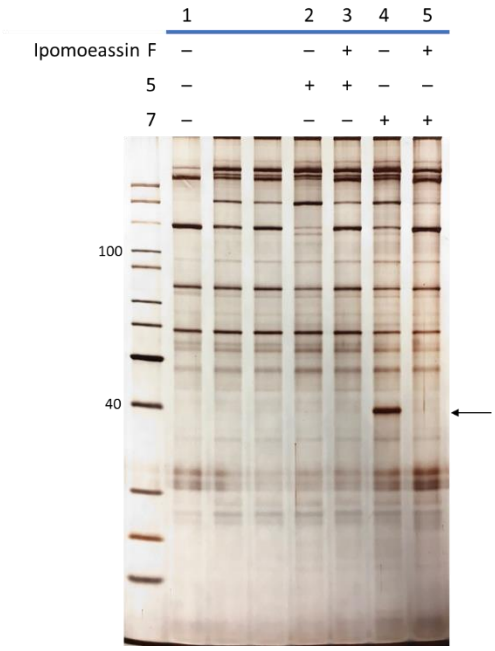


Figure S2. Click reaction performed after incubating cells with competitor and its corresponding alkyne probe. (A) Fluorescent image by a Typhoon scanner. (B) Coomassie Blue stain of the gel. (The arrow indicates a positive result for probe X, where a protein band appeared in lane 4 but was absent in lanes 1 and 5.)

Biotin Affinity Pulldown

A.



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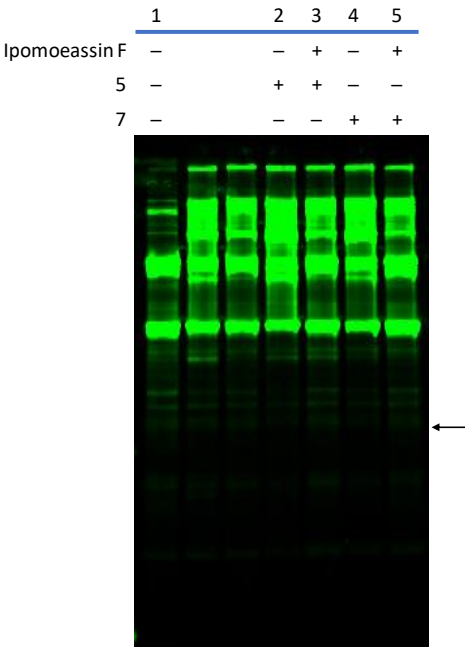

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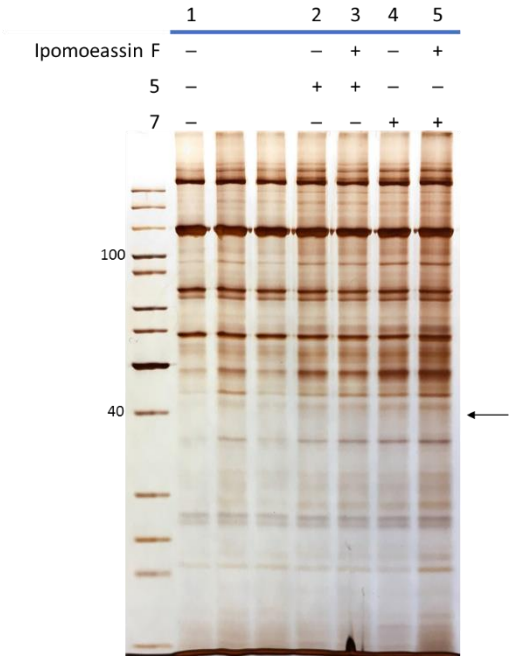


Figure S3. Biotin affinity pulldown with probes 5 and 7. Cells were incubated with ipomoeassin F and probes, then lysed and subjected to biotin affinity pulldown with streptavidin beads. (A) SDS-PAGE and silver staining analysis of pulldown samples from live cells. (B) SDS-PAGE analysis of pulldown samples

from live cells using fluorescently labeled streptavidin. (C) SDS-PAGE and silver staining analysis of pulldown samples from cell lysates. Arrows indicate the ~40 kDa protein position.

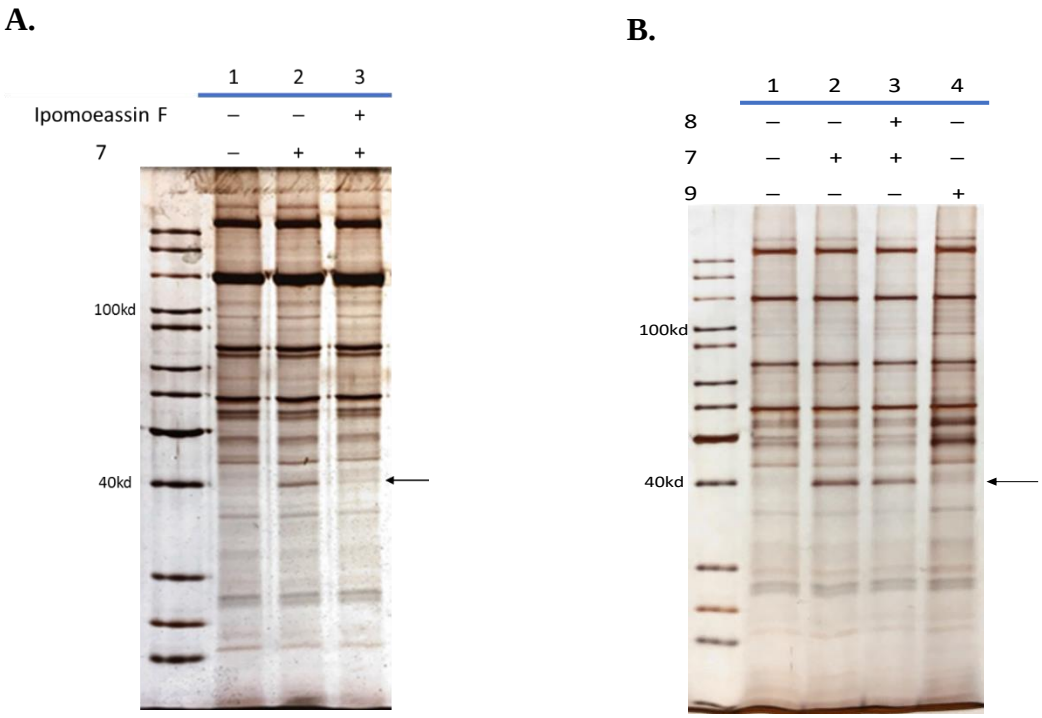
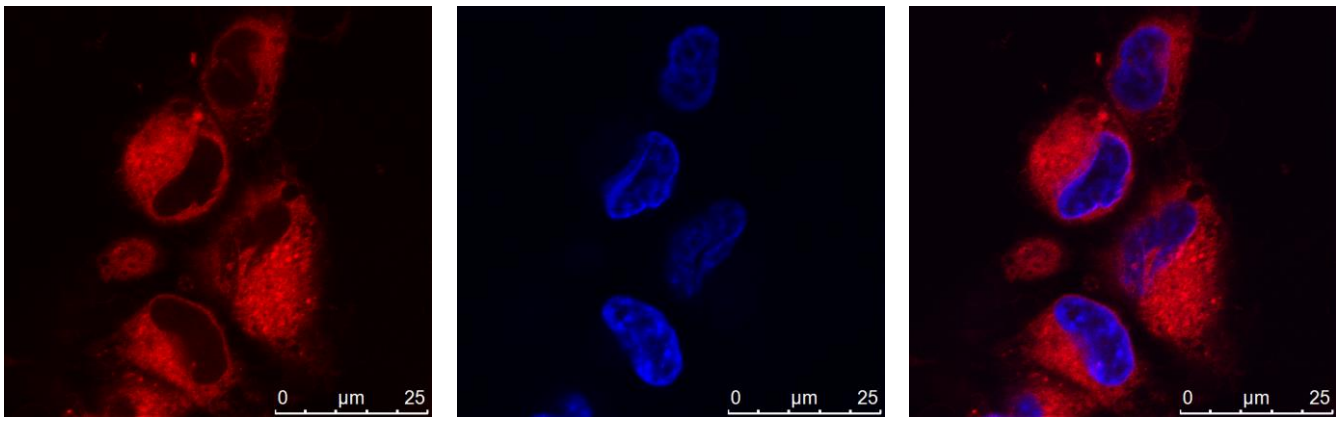


Figure S4. Biotin affinity pulldown with probe 7 or 9 in the presence of the competitor ipomoeassin F or 8. (A) Pulldown experiment with 10 nM **7** and 50 nM ipomoeassin F. (B) Pulldown experiments with either 20 nM **7** and 1 μM **8** or 500 nM **9** without competition. Arrows indicate the ~40 kDa protein position.

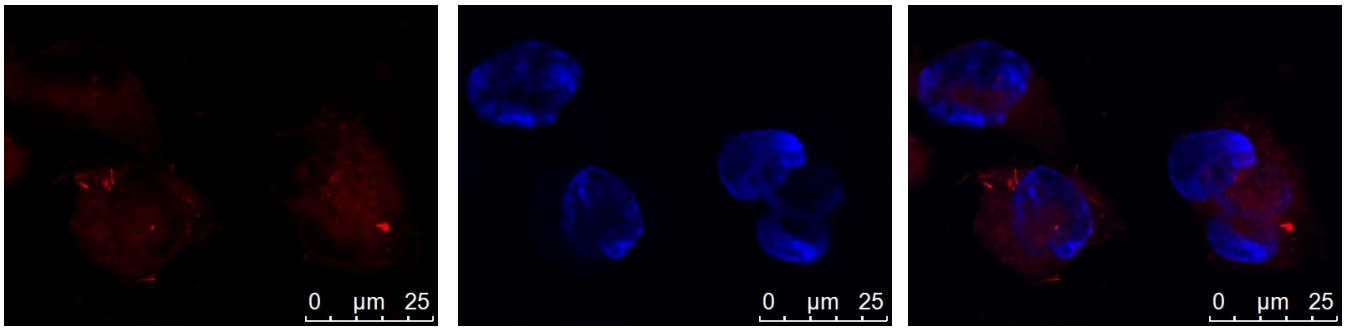
Cell Imaging

A.



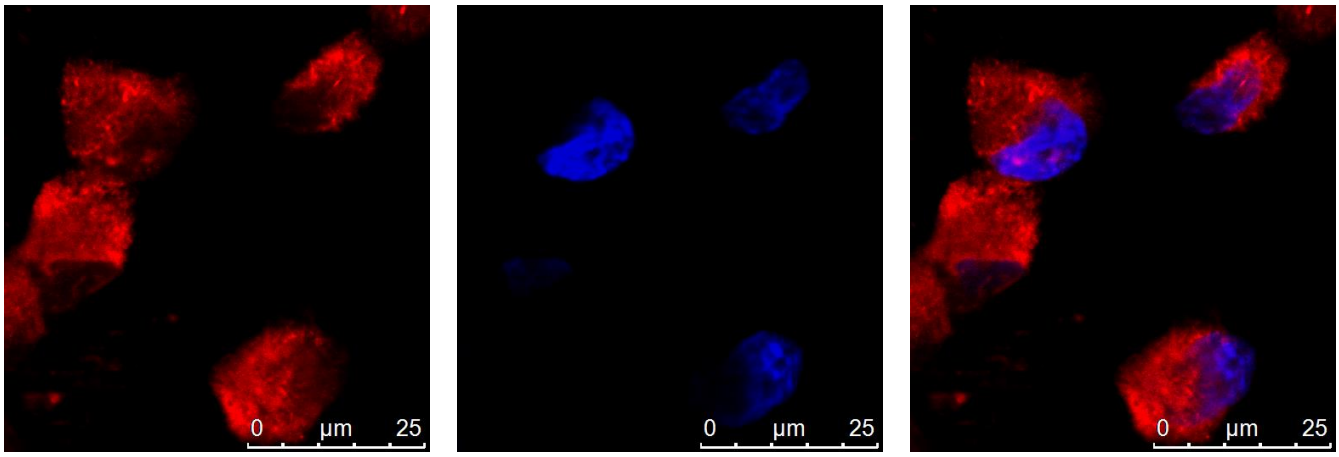
12, 0.2 µM DAPI Merged

B.



12 (0.2 µM) + Ipom-F (20 µM) DAPI Merged

C.

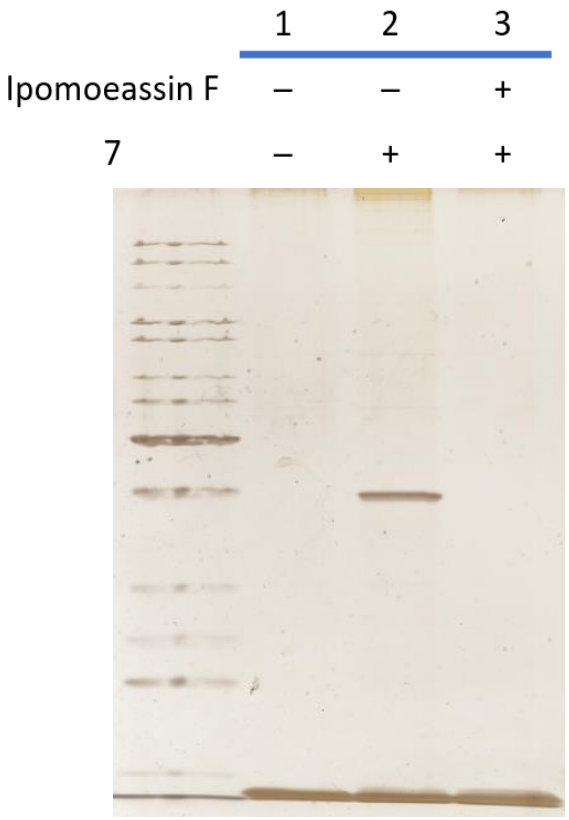


12 (0.2 µM) + **8** (20 µM) DAPI Merged

Figure S5. Live cell imaging-based competition experiments. (A) Cells were treated with 1 µM **12**. (B) Cells were treated with ipomoeassin F (100-fold) and **12**; (C) Cells were treated with **8** (100-fold) and **12**.

Pulldown in ER Microsomes.

A.



B.

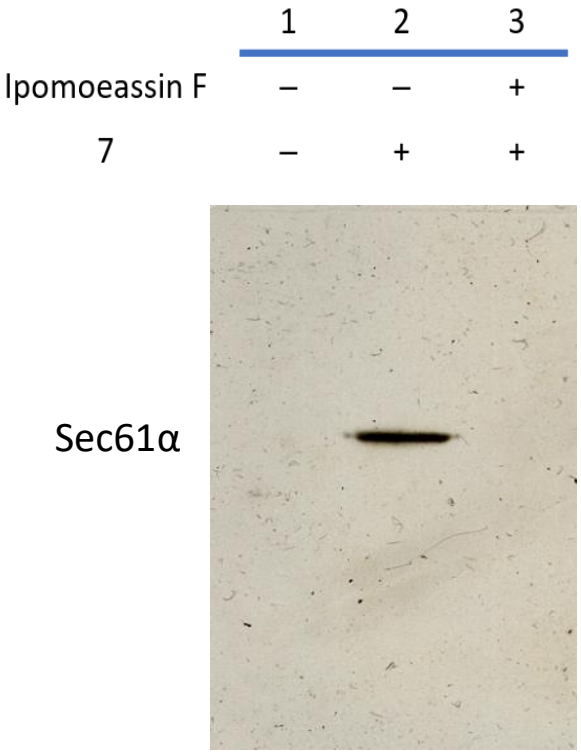


Figure S6. Biotin affinity pulldown with probe 7 in ER microsomes. (A) The proteins were visualized by silver staining. The arrow indicates the positive result. (B) The proteins were transferred onto a nitrocellulose membrane and visualized by western blot using an anti-Sec61α antibody.

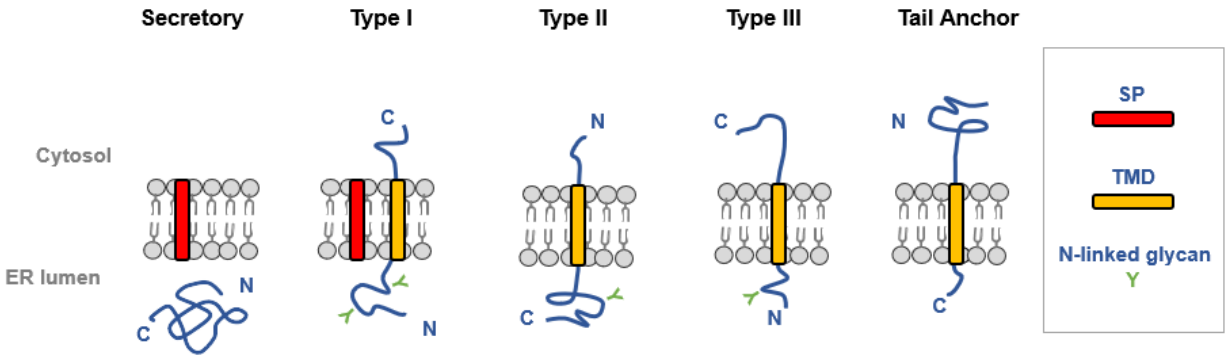


Figure S7. Membrane topology schematic.

***In Vitro* Membrane Insertion Assays of a Model Type II Protein.**

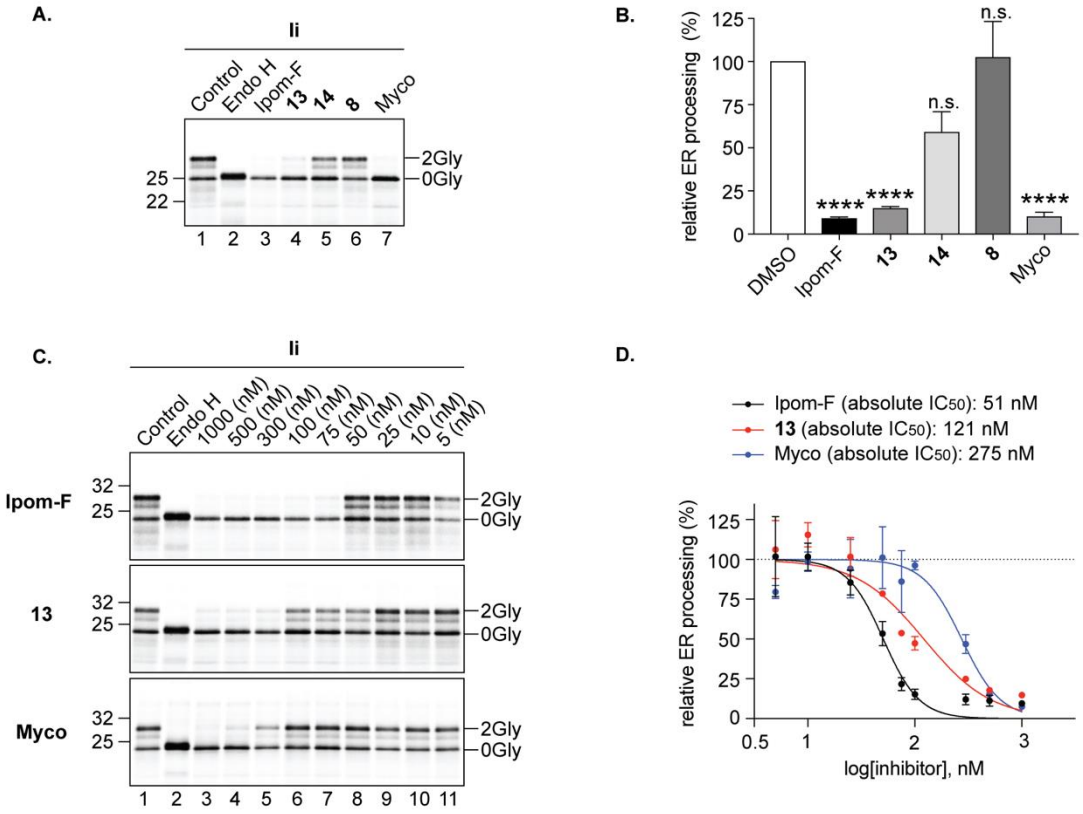


Figure S8. Comparison of the effectiveness of ipomoeassin F and related structural variants on the membrane insertion of Ii. (A) Phosphorimage of the type II integral membrane protein Ii synthesized in the presence of ipomoeassin F (Ipom-F), the open-chain analogue **13**, inactive analogues **14** and **3** and mycolactone (Myco). (B) Quantitative analysis of A whereby the efficiency of Ii membrane integration was estimated using the ratio of signal intensity for the 2Gly/0Gly forms and expressed relative to the control. (C) Ipom-F, **13** and Myco were included at 1000-5 nM concentrations during the translation of Ii and (D) quantitative analysis of the efficiency of Ii membrane integration was performed as described in B. IC_{50} values were estimated using non-linear regression as described in materials and methods. Each phosphorimage depicts experiments performed using a translation master mix, to which different compounds and Ii mRNA were then added prior to translation and analysis of the membrane-associated fraction from three independent experiments (*n*=3).

Pulse-Chase Analysis of Protein Secretion in HepG2 Cells

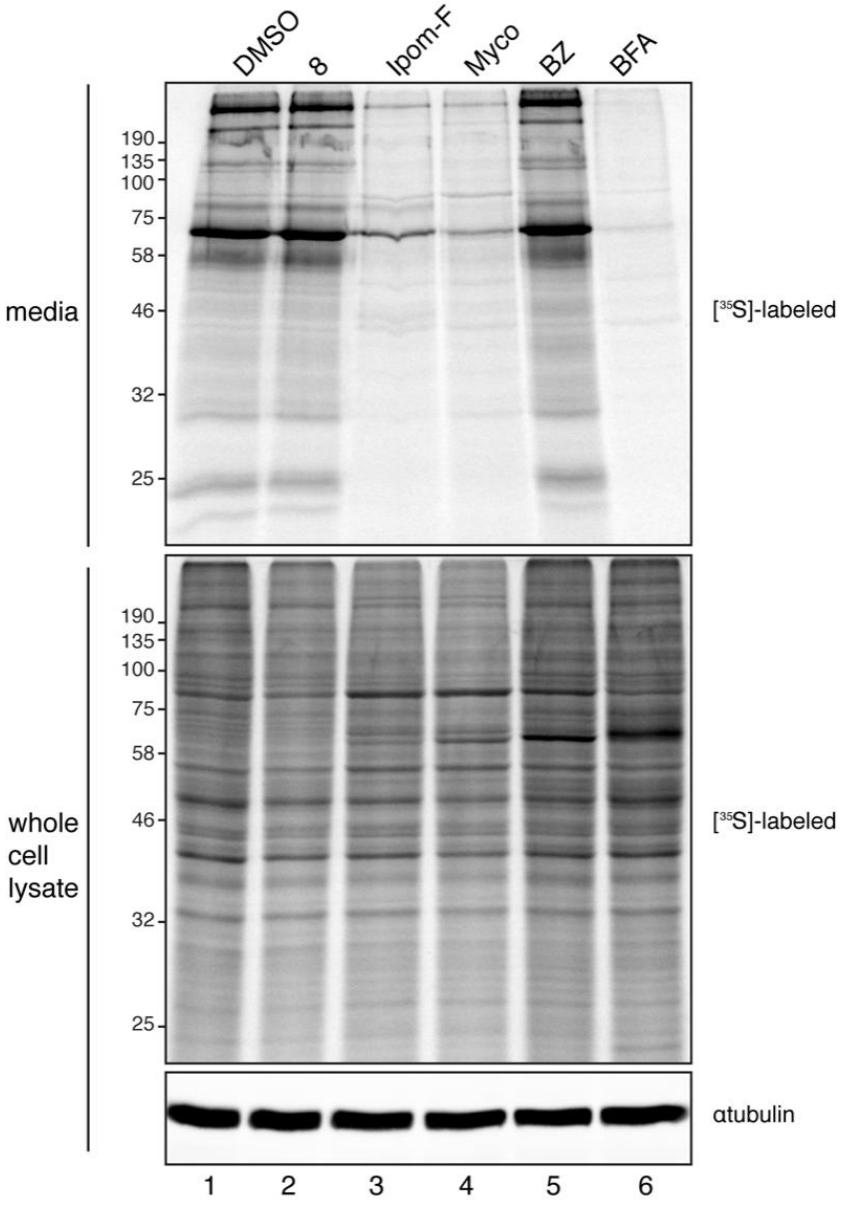


Figure S9. Ipomoeassin-F inhibits protein secretion. HepG2 cells were treated with DMSO, 100 nM compound **8**, 100 nM Ipom-F, or 42 nM mycolactone (Myco) for 13.5 h. HepG2 cells were treated with 100 nM bortezomib (BZ) for 2 h or 2.5 μg/ml BFA for 1 h. Newly synthesized proteins were pulse-labeled for 15 min and then chased in medium containing unlabeled Met and Cys for 2 h. The compounds were included throughout the pulse and chase. At the end of chase, radiolabeled proteins in media (upper panel) and whole cell lysates (middle panel) were resolved by SDS-PAGE and visualized by phosphorimaging. Whole cell lysates of treated cells were also analyzed by immunoblotting with anti-α-tubulin antibodies (lower panel). Molecular masses are in kDa.

Reversed Competition in Live Cell-based Pulldown

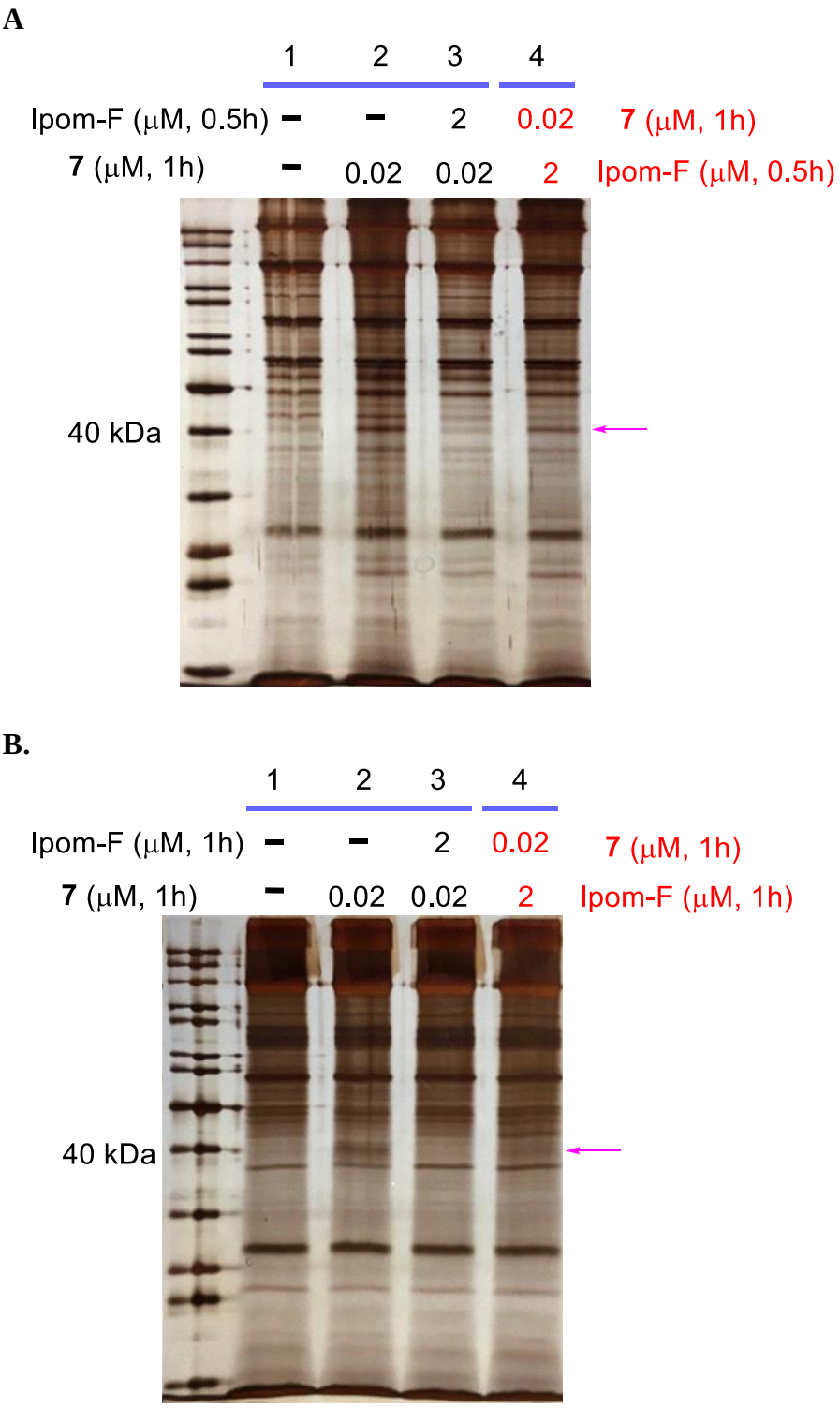


Figure S10. Ipomoeassin-F binds Sec61α strongly yet reversibly. SDS-PAGE images (silver staining) for affinity pulldown using probe **7** with 0.5 h (A) or 1 h (B) competition in both regular (lane 3) and reversed (lane 4) order. Pink arrows indicate the protein band for Sec61α.

Table S1. Spectral Counts for Proteins Identified in ~40 kDa Bands

Identified Proteins	Accession Number	Molecular Weight	Spectral count			
			DMSO	Probe 7	7 + Ipom-F	delta (Probe/probe + Ipom-F)
Vimentin	VIME_HUMAN	54 kDa	108	88	126	**0.70**
Protein transport protein Sec61 subunit alpha isoform 1	S61A1_HUMAN	52 kDa	11	80	16	**5.00**
Pyruvate carboxylase, mitochondrial	PYC_HUMAN	130 kDa	97	68	109	**0.62**
Poly(rC)-binding protein 1	PCBP1_HUMAN	37 kDa	45	45	82	**0.55**
mRNA export factor	RAE1L_HUMAN	41 kDa	50	41	53	**0.77**
Actin, cytoplasmic 1	ACTB_HUMAN	42 kDa	45	40	54	**0.74**
Stomatin-like protein 2, mitochondrial	STML2_HUMAN	39 kDa	42	37	68	**0.54**
Enoyl-CoA delta isomerase 2, mitochondrial	ECI2_HUMAN	44 kDa	34	36	51	**0.71**
Plectin	PLEC_HUMAN	532 kDa	30	29	65	**0.45**
Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial	MCCA_HUMAN	80 kDa	42	28	44	**0.64**
26S proteasome regulatory subunit 10B	PRS10_HUMAN	44 kDa	27	25	45	**0.56**
Acetyl-CoA acetyltransferase, mitochondrial	THIL_HUMAN	45 kDa	24	25	37	**0.68**
Core histone macro-H2A.1	H2AY_HUMAN	40 kDa	30	24	50	**0.48**
Heterogeneous nuclear ribonucleoprotein A3	ROA3_HUMAN	40 kDa	35	24	47	**0.51**
Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	MCCB_HUMAN	61 kDa	36	23	37	**0.62**
DnaJ homolog subfamily B member 11	DJB11_HUMAN	41 kDa	25	21	28	**0.75**
Fructose-bisphosphate aldolase A	ALDOA_HUMAN	39 kDa	55	21	37	**0.57**
Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial	SUCB2_HUMAN	47 kDa	25	21	28	**0.75**
Poly(rC)-binding protein 2	PCBP2_HUMAN	39 kDa	23	20	34	**0.59**
Cytosolic acyl coenzyme A thioester hydrolase	BACH_HUMAN	42 kDa	23	19	23	**0.83**
Transmembrane protein 43	TMM43_HUMAN	45 kDa	19	18	21	**0.86**

Galactokinase	GALK1_HUMAN	42 kDa	15	17	29	**0.59**
Leukocyte elastase inhibitor	ILEU_HUMAN	43 kDa	35	17	34	**0.50**
Acetyl-CoA carboxylase 1	ACACA_HUMAN	266 kDa	32	16	34	**0.47**
Caveolae-associated protein 1	CAVN1_HUMAN	43 kDa	17	16	23	**0.70**
Perilipin-3	PLIN3_HUMAN	47 kDa	16	16	23	**0.70**
RNA-binding protein 4	RBM4_HUMAN	40 kDa	17	16	27	**0.59**
28S ribosomal protein S31, mitochondrial	RT31_HUMAN	45 kDa	14	15	21	**0.71**
Heterogeneous nuclear ribonucleoprotein D0	HNRPD_HUMAN	38 kDa	23	15	36	**0.42**
Inhibitor of nuclear factor kappa-B kinase-interacting protein	IKIP_HUMAN	39 kDa	14	15	19	**0.79**
Protein CYR61	CYR61_HUMAN	42 kDa	17	15	10	**1.50**
Tubulin beta chain	TBB5_HUMAN	50 kDa	16	15	25	**0.60**
Tubulin beta-4B chain	TBB4B_HUMAN	50 kDa	16	15	25	**0.60**
Erlin-2	ERLN2_HUMAN	38 kDa	16	14	20	**0.70**
Guanine nucleotide-binding protein subunit alpha-13	GNA13_HUMAN	44 kDa	15	14	18	**0.78**
Macrophage-capping protein	CAPG_HUMAN	38 kDa	22	14	24	**0.58**
Serpin B6	SPB6_HUMAN	43 kDa	20	14	28	**0.50**
Endophilin-B1	SHLB1_HUMAN	41 kDa	12	13	17	**0.76**
Mitogen-activated protein kinase 1	MK01_HUMAN	41 kDa	16	13	20	**0.65**
Prelamin-A/C	LMNA_HUMAN	74 kDa	19	13	25	**0.52**
Eukaryotic translation initiation factor 3 subunit M	EIF3M_HUMAN	43 kDa	16	12	21	**0.57**
Mitochondrial import receptor subunit TOM40 homolog	TOM40_HUMAN	38 kDa	14	12	19	**0.63**
Protein FAM50A	FA50A_HUMAN	40 kDa	12	12	22	**0.55**
Reticulocalbin-1	RCN1_HUMAN	39 kDa	6	12	16	**0.75**
RNA-binding protein 4B	RBM4B_HUMAN	40 kDa	0	12	23	**0.52**
Septin-2	SEPT2_HUMAN	41 kDa	20	12	21	**0.57**
26S proteasome non-ATPase regulatory subunit 13	PSD13_HUMAN	43 kDa	12	11	28	**0.39**
28S ribosomal protein S29, mitochondrial	RT29_HUMAN	46 kDa	11	11	19	**0.58**
Elongation factor 1-alpha 1	EF1A1_HUMAN (+1)	50 kDa	11	11	21	**0.52**
Heterogeneous nuclear ribonucleoproteins A2/B1	ROA2_HUMAN	37 kDa	12	11	22	**0.50**

Heterogeneous nuclear ribonucleoproteins C1/C2	HNRPC_HUMAN	34 kDa	13	11	17	**0.65**
Immunoglobulin-binding protein 1	IGBP1_HUMAN	39 kDa	8	11	15	**0.73**
Nuclear pore complex protein Nup153	NU153_HUMAN	154 kDa	11	11	21	**0.52**
Protein quaking	QKI_HUMAN	38 kDa	10	11	17	**0.65**
28S ribosomal protein S35, mitochondrial	RT35_HUMAN	37 kDa	10	10	15	**0.67**
Activator of 90 kDa heat shock protein ATPase homolog 1	AHSA1_HUMAN	38 kDa	7	10	14	**0.71**
ATP synthase subunit alpha, mitochondrial	ATPA_HUMAN	60 kDa	20	10	28	**0.36**
Centrosomal protein of 170 kDa	CE170_HUMAN	175 kDa	11	10	8	**1.25**
DNA-directed RNA polymerases I and III subunit RPAC1	RPAC1_HUMAN	39 kDa	14	10	18	**0.56**
Lamina-associated polypeptide 2, isoform alpha	LAP2A_HUMAN	75 kDa	8	10	12	**0.83**
60 kDa heat shock protein, mitochondrial	CH60_HUMAN	61 kDa	6	9	16	**0.56**
Actin-related protein 2	ARP2_HUMAN	45 kDa	22	9	18	**0.50**
Aspartate aminotransferase, mitochondrial	AATM_HUMAN	48 kDa	23	9	18	**0.50**
Developmentally-regulated GTP-binding protein 1	DRG1_HUMAN	41 kDa	13	9	15	**0.60**
DnaJ homolog subfamily B member 1	DNJB1_HUMAN	38 kDa	5	9	16	**0.56**
DnaJ homolog subfamily B member 6	DNJB6_HUMAN	36 kDa	10	9	10	**0.90**
Hemoglobin subunit gamma-1	HBG1_HUMAN (+1)	16 kDa	13	9	3	**3.00**
Lamina-associated polypeptide 2, isoforms beta/gamma	LAP2B_HUMAN	51 kDa	7	9	12	**0.75**
Lysophospholipid acyltransferase 7	MBOA7_HUMAN	53 kDa	7	9	10	**0.90**
Nuclear distribution protein nudE homolog 1	NDE1_HUMAN	39 kDa	8	9	16	**0.56**
Hemoglobin subunit beta	HBB_HUMAN	16 kDa	12	8	6	**1.33**
Heterogeneous nuclear ribonucleoprotein M	HNRPM_HUMAN	78 kDa	7	8	19	**0.42**

HLA class I histocompatibility antigen, A-2 alpha chain	1A02_HUMAN	41 kDa	8	8	16	**0.50**
Mevalonate kinase	KIME_HUMAN	42 kDa	10	8	15	**0.53**
Mitotic checkpoint protein BUB3	BUB3_HUMAN	37 kDa	8	8	15	**0.53**
Nucleolar protein 7	NOL7_HUMAN	29 kDa	9	8	10	**0.80**
Nucleolysin TIAR	TIAR_HUMAN	42 kDa	9	8	13	**0.62**
Protein KTI12 homolog	KTI12_HUMAN	39 kDa	4	8	12	**0.67**
Protein SGT1 homolog	SGT1_HUMAN	41 kDa	8	8	22	**0.36**
Transcriptional activator protein Pur-alpha	PURA_HUMAN	35 kDa	15	8	19	**0.42**
Tropomodulin-3	TMOD3_HUMAN	40 kDa	15	8	19	**0.42**
40S ribosomal protein SA	RSSA_HUMAN	33 kDa	7	7	13	**0.54**
Cytoskeleton-associated protein 4	CKAP4_HUMAN	66 kDa	5	7	12	**0.58**
Erlin-1	ERLN1_HUMAN	39 kDa	8	7	9	**0.78**
Mitochondrial inner membrane protein OXA1L	OXA1L_HUMAN	49 kDa	8	7	9	**0.78**
Polymerase delta-interacting protein 3	PDIP3_HUMAN	46 kDa	8	7	11	**0.64**
Rab11 family-interacting protein 5	RFIP5_HUMAN	70 kDa	7	7	10	**0.70**
T-complex protein 1 subunit delta	TCPD_HUMAN	58 kDa	14	7	13	**0.54**
tRNA methyltransferase 10 homolog C	TM10C_HUMAN	47 kDa	13	7	14	**0.50**
Ubiquitin-60S ribosomal protein L40	RL40_HUMAN	15 kDa	7	7	12	**0.58**
V-type proton ATPase subunit C 1	VATC1_HUMAN	44 kDa	10	7	15	**0.47**
28S ribosomal protein S5, mitochondrial	RT05_HUMAN	48 kDa	7	6	15	**0.40**
Activity-dependent neuroprotector homeobox protein	ADNP_HUMAN	124 kDa	6	6	9	**0.67**
Aspartate aminotransferase, cytoplasmic	AATC_HUMAN	46 kDa	21	6	16	**0.38**
COP9 signalosome complex subunit 4	CSN4_HUMAN	46 kDa	7	6	9	**0.67**
Eukaryotic translation initiation factor 3 subunit H	EIF3H_HUMAN	40 kDa	6	6	17	**0.35**
Ferrochelatase, mitochondrial	HEMH_HUMAN	48 kDa	9	6	13	**0.46**
Filamin-B	FLNB_HUMAN	278 kDa	8	6	10	**0.60**

Glutaminyl-peptide cyclotransferase-like protein	QPCTL_HUMAN	43 kDa	7	6	6	**1.00**
Heat shock protein HSP 90-beta	HS90B_HUMAN	83 kDa	14	6	15	**0.40**
Hemoglobin subunit alpha	HBA_HUMAN	15 kDa	10	6	2	**3.00**
Mannose-1-phosphate guanyltransferase beta	GMPPB_HUMAN	40 kDa	6	6	13	**0.46**
Nucleolin	NUCL_HUMAN	77 kDa	5	6	6	**1.00**
Proline-rich protein 11	PRR11_HUMAN	40 kDa	11	6	14	**0.43**
Protein arginine N-methyltransferase 1	ANM1_HUMAN	42 kDa	11	6	14	**0.43**
Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	ACDSB_HUMAN	47 kDa	4	6	11	**0.55**
SUMO-activating enzyme subunit 1	SAE1_HUMAN	38 kDa	11	6	12	**0.50**
Ubiquitin-conjugating enzyme E2 Z	UBE2Z_HUMAN	38 kDa	8	6	10	**0.60**
Alpha-actinin-1	ACTN1_HUMAN	103 kDa	3	5	8	**0.63**
Alpha-actinin-4	ACTN4_HUMAN	105 kDa	11	5	11	**0.45**
Choline-phosphate cytidylyltransferase A	PCY1A_HUMAN	42 kDa	2	5	5	**1.00**
DnaJ homolog subfamily B member 2	DNJB2_HUMAN	36 kDa	5	5	3	**1.67**
Fructose-bisphosphate aldolase C	ALDOC_HUMAN	39 kDa	21	5	13	**0.38**
GDP-mannose 4,6 dehydratase	GMDS_HUMAN	42 kDa	9	5	11	**0.45**
Heat shock cognate 71 kDa protein	HSP7C_HUMAN	71 kDa	14	5	19	**0.26**
Hsp70-binding protein 1	HPBP1_HUMAN	39 kDa	6	5	8	**0.63**
Matrin-3	MATR3_HUMAN	95 kDa	2	5	7	**0.71**
Minor histocompatibility antigen H13	HM13_HUMAN	41 kDa	6	5	7	**0.71**
Nucleoside diphosphate kinase 7	NDK7_HUMAN	42 kDa	9	5	10	**0.50**
Probable ATP-dependent RNA helicase DDX5	DDX5_HUMAN	69 kDa	6	5	11	**0.45**
Probable methyltransferase-like protein 15	MET15_HUMAN	46 kDa	5	5	10	**0.50**
Propionyl-CoA carboxylase alpha chain, mitochondrial	PCCA_HUMAN	80 kDa	11	5	13	**0.38**
Propionyl-CoA carboxylase beta chain, mitochondrial	PCCB_HUMAN	58 kDa	7	5	6	**0.83**

Replication factor C subunit 2	RFC2_HUMAN	39 kDa	4	5	25	**0.20**
RNA 3'-terminal phosphate cyclase	RTCA_HUMAN	39 kDa	6	5	9	**0.56**
Serine/threonine-protein kinase MARK2	MARK2_HUMAN	88 kDa	3	5	4	**1.25**
Serpin B9	SPB9_HUMAN	42 kDa	11	5	9	**0.56**
Twinfilin-2	TWF2_HUMAN	40 kDa	10	5	10	**0.50**
7-dehydrocholesterol reductase	DHCR7_HUMAN	54 kDa	3	4	2	**2.00**
Alpha-2-macroglobulin receptor-associated protein	AMRP_HUMAN	41 kDa	2	4	4	**1.00**
Apoptosis-inducing factor 2	AIFM2_HUMAN	41 kDa	5	4	13	**0.31**
ATPase ASNA1	ASNA_HUMAN	39 kDa	3	4	5	**0.80**
BRISC and BRCA1-A complex member 1	BABA1_HUMAN	37 kDa	6	4	11	**0.36**
cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_HUMAN	41 kDa	16	4	13	**0.31**
Cdc42 effector protein 1	BORG5_HUMAN	40 kDa	6	4	9	**0.44**
Desmocollin-1	DSC1_HUMAN	100 kDa	0	4	0	**#DIV/0!**
Filamin-A	FLNA_HUMAN	281 kDa	7	4	8	**0.50**
Flap endonuclease 1	FEN1_HUMAN	43 kDa	3	4	7	**0.57**
Glucose-fructose oxidoreductase domain-containing protein 1	GFOD1_HUMAN	43 kDa	4	4	5	**0.80**
HAUS augmin-like complex subunit 4	HAUS4_HUMAN	42 kDa	1	4	11	**0.36**
Heat shock 70 kDa protein 1A	HS71A_HUMAN (+1)	70 kDa	6	4	7	**0.57**
HLA class I histocompatibility antigen, alpha chain E	HLAE_HUMAN	40 kDa	5	4	4	**1.00**
HLA class I histocompatibility antigen, B-41 alpha chain	1B41_HUMAN	41 kDa	2	4	4	**1.00**
Isovaleryl-CoA dehydrogenase, mitochondrial	IVD_HUMAN	46 kDa	8	4	8	**0.50**
MAP7 domain-containing protein 3	MA7D3_HUMAN	98 kDa	4	4	8	**0.50**
Monocarboxylate transporter 4	MOT4_HUMAN	49 kDa	4	4	7	**0.57**
Muscleblind-like protein 1	MBNL1_HUMAN	42 kDa	3	4	5	**0.80**
Pinin	PININ_HUMAN	82 kDa	2	4	5	**0.80**

Polyhomeotic-like protein 2	PHC2_HUMAN	91 kDa	4	4	4	**1.00**
RelA-associated inhibitor	IASPP_HUMAN	89 kDa	1	4	7	**0.57**
Serum albumin	ALBU_HUMAN	69 kDa	4	4	4	**1.00**
Stress-70 protein, mitochondrial	GRP75_HUMAN	74 kDa	5	4	6	**0.67**
Synaptic vesicle membrane protein VAT-1 homolog	VAT1_HUMAN	42 kDa	3	4	10	**0.40**
Testis-expressed protein 264	TX264_HUMAN	34 kDa	7	4	10	**0.40**
Transcription factor jun-B	JUNB_HUMAN	36 kDa	4	4	9	**0.44**
Transformer-2 protein homolog beta	TRA2B_HUMAN	34 kDa	5	4	6	**0.67**
UBX domain-containing protein 1	UBXN1_HUMAN	33 kDa	4	4	5	**0.80**

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