Supporting Information

The molecular basis for inhibition of stem-like cancer cells by salinomycin

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Safety statement: No unexpected or unusually high safety hazards were encountered during this work. We recommend that conjugate **6** is handled following recommendations in the safety data sheet for salinomycin sodium salt.

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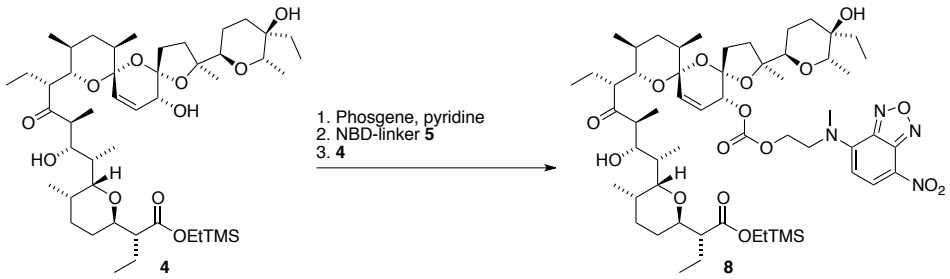
Safety:

I. General synthetic procedures.

Chemical synthesis. All reactions were carried out under an atmosphere of nitrogen in oven-dried glassware. Reactions were stirred using Teflon-coated magnetic stir bars. Reactions were monitored by thin layer chromatography (TLC) using aluminum-backed plates (Merck $60F_{254}$). TLC plates were visualized by UV-light (254 nm and 380 nm) and treatment with phosphomolybdic acid (PMA) (5% in EtOH) followed by gentle heating. Organic phases were dried using phase separators (International Sorbent Technology). Solvents were removed using Heidolph rotary evaporators. Products were purified by flash chromatography using silica gel 60 Å (40 – 60 μm), or by an automatic chromatography system (Biotage Isolera One) using the solvent systems indicated as mobile phase. Optical rotation data were obtained on a Perkin Elmer Model 341 polariometer and are reported as $[\alpha]_D^T$ (c = g/100 mL), where D indicates the sodium D line (589 nm) and T indicates the temperature. NMR spectra were recorded on a 400 MHz (^{1}H NMR

at 400 MHz and ^{13}C NMR at 101 MHz) or a 500 MHz (^{1}H NMR at 500 MHz and ^{13}C NMR at 126 MHz) magnetic resonance spectrometer. Spectra were processed using MestReNova v.8.1. ^{1}H NMR signals are reported in chemical shifts downfield from $SiMe_4$, using the respective residual solvent peak as internal standard (chloroform = 7.26 ppm) (1), and listed as follows: chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, q = quartet, dd = doublet of doublets, td = triplet of doublets, ddd = doublet of doublet of doublets, m = multiplet, app. = apparent), coupling constant(s) in Hz, and integration. Significant peaks are reported within the overlapping 2.5-0.5 ppm region of the ^{1}H NMR spectra. Acidic protons (O*H*) are assumed to reside in the 2.5-0.5 ppm region unless listed explicitly. ^{13}C NMR spectra are reported in chemical shifts using the respective residual solvent peak (chloroform = 77.16 ppm) as internal standard. Infrared spectra were recorded on a Fourier transform infrared spectrometer (FTIR) and are reported as follows: wave numbers (cm^{-1}), description (w = weak, m = medium, s = strong, br = broad). High-resolution mass spectra (HRMS) were obtained on a Waters Xevo Q-TOF mass spectrometer using electrospray ionization and lock mass correction. Anhydrous CH_2Cl_2 and *N*,*N*-dimethylformamide (DMF) were collected from a dry solvent system. Salinomycin (SA) was purified from tech. grade material, and salinomycin derivatives **2** and **3** were prepared as described previously by Strand and co-workers (2). The NBD-linker 2-(*N*-methyl-*N*-7-nitro-2,1,3-benzoxadiazol-4-ylamino) ethanol **5** was prepared according to the procedure of Kupryushkin et al. (3). All other reagents and solvents were purchased from commercial suppliers and used as received.

*II. Experimental procedures and characterization data of compounds **6**, 7, and **8**.*



Carbonate 8. To pyridine (3 mL) stirred at 0 °C was added phosgene (600 µL, ~1.21 mmol, ~20% in toluene) dropwise over 2 min resulting in a yellow suspension. A solution of NBD-linker **5** (276.1 mg, 1.175 mmol) in pyridine (2 mL) was then added dropwise over 3 min. The resulting

red suspension was stirred for 15 min, after which a solution of EtTMS ester **4** (206.1 mg, 0.235 mmol) in pyridine (1 mL) was added in one portion. The resulting mixture was stirred for 21 h while slowly warming to RT, then diluted with CH_2Cl_2 (20 mL) and washed with HCl (3 x 20 mL, 0.1 M aq.). The organic layer was separated, dried using a phase separator, and concentrated under reduced pressure. Purification by flash chromatography (25 – 50% EtOAc/*n*-heptane) gave yellow oil that was concentrated three times from *n*-pentane to give carbonate **8**.

Yield: 146 mg, 56%. Isolated as a red amorphous solid, >95% pure by NMR, and a single spot on TLC.

R*f*: 0.10 in 50% EtOAc/petroleum ether. Yellow spot, UV-active, fluorescent and stains brown with PMA.

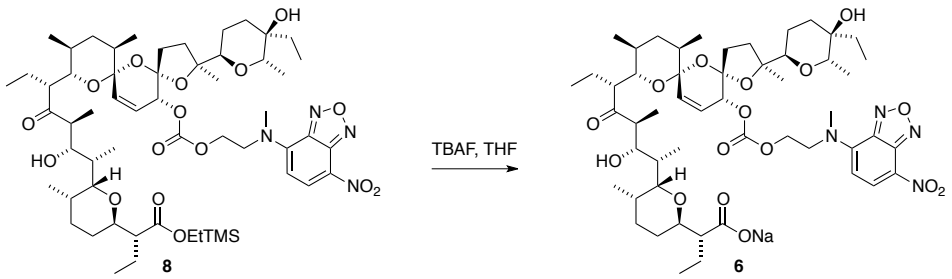
Optical rotation: $[\alpha]_D^{20}$ -73.0 (*c* = 1.0 CH_2Cl_2).

^{1}H–NMR ($CDCl_3$, 400 MHz) δ: 8.47 (d, *J* = 8.9 Hz, 1H), 6.22 (dd, *J* = 10.9, 2.6 Hz, 1H), 6.19 (d, *J* = 9.1 Hz, 1H), 5.80 (dd, *J* = 10.8, 1.5 Hz, 1H), 5.06 (dd, *J* = 2.5, 1.5 Hz, 1H), 4.63 – 4.30 (m, 6H), 4.11 – 3.98 (m, 2H), 3.74 – 3.62 (m, 2H), 3.53 – 3.42 (m, 4H), 3.38 (dd, *J* = 11.5, 1.7 Hz, 1H), 3.26 – 3.14 (m, 1H), 3.11 (d, *J* = 5.8 Hz, 1H), 2.99 (td, *J* = 10.9, 4.4 Hz, 1H), 2.80 – 2.64 (m, 2H), 1.45 (s, 3H), 2.10 – 0.64 (m, 50H), 1.17 (d, *J* = 6.8 Hz, 3H), 0.07 (s, 9H) ppm.

^{13}C–NMR ($CDCl_3$, 101 MHz) δ: 212.9, 175.9, 155.0, 144.9, 144.6, 135.1, 126.4, 124.4, 123.6, 104.2, 101.8, 99.2, 87.2, 81.1, 75.0, 72.6, 72.3, 71.7, 70.8, 69.1, 63.6, 57.3, 54.2, 48.8, 48.0, 42.2, 40.4, 39.1, 36.4, 35.3, 33.1, 32.6, 30.4, 29.7, 29.4, 28.1, 26.3, 23.7, 22.6, 22.4, 21.5, 20.9, 19.7, 17.5, 17.4, 15.6, 14.2, 14.1, 13.8, 12.9, 11.9, 11.0, 7.2, 6.5, -1.5 ppm.

FTIR (CH_2Cl_2 film): 3529 (s, br), 2969 (m), 2938 (m), 2876 (w), 2858 (w), 1749 (s), 1710 (s), 1614 (m), 1555 (s) cm^{-1}.

HRMS-ESI *(m/z)*: $[M+H]^+$ Calcd for $C_{57}H_{91}N_4O_{16}Si$ 1115.6194; Found 1115.6199.



8 6

Conjugate 6. To a stirred solution of ester **8** (73.6 mg, 0.066 mmol) in THF (10 mL) at RT was added TBAF (66 µL, 0.340 mmol, 1.0 M in THF) dropwise over 60 s. The resulting yellow solution was stirred for 8 h in the dark during which time it turned dark yellow. The reaction mixture was then diluted with EtOAc (100 mL) and washed with Na_2CO_3 (3 x 20 mL, 0.1 M aq.). The organic layer was separated, dried using a phase separator, and concentrated under reduced pressure. Purification by flash chromatography (75 – 100% EtOAc/*n*-heptane) gave the product as a mixture of the acid and salt form. The product mixture was re-dissolved in EtOAc (10 mL) and washed with Na_2CO_3 (3 x 10 mL, 0.1 M aq.). The organic layer was separated, dried using a phase separator, and lyophilized from 20% acetonitrile/water to give conjugate **6**.

Yield: 12.8 mg, 19% (based on recovered starting material 61%). Isolated as a yellow amorphous solid, >95% pure by NMR, and a single spot on TLC.

R_f: 0.22 in 100% EtOAc. Yellow spot, UV-active, fluorescent and stains brown with PMA.

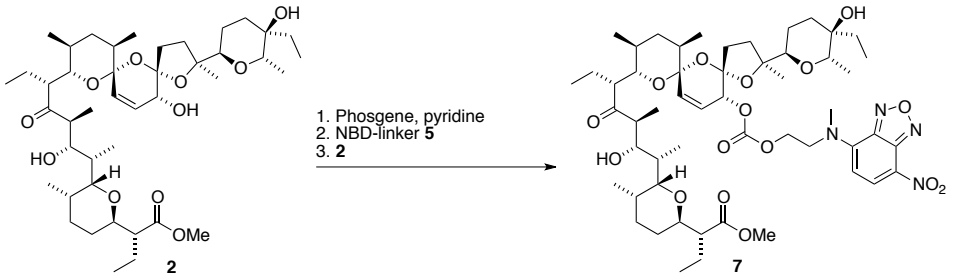
Optical rotation: $[\alpha]_D^{20}$ - 3.0 (*c* = 1.05 CH_2Cl_2).

^{1}H–NMR ($CDCl_3$, 400 MHz) δ: 8.46 (dd, *J* = 9.0, 1.1 Hz, 1H), 6.21 (dd, *J* = 11.1, 2.7 Hz, 1H), 6.18 (d, *J* = 9.0 Hz, 1H), 5.96 (dd, *J* = 10.8, 1.5 Hz, 1H), 5.07 (s, 1H), 4.56 – 4.40 (m, 3H), 4.37 (q, *J* = 6.6 Hz, 1H), 4.33 – 4.21 (m, 2H), 3.91 (dd, *J* = 10.8, 4.6 Hz, 1H), 3.70 (d, *J* = 10.2 Hz, 1H), 3.57 – 3.47 (m, 4H), 3.37 (dd, *J* = 12.1, 2.1 Hz, 1H), 2.81 (td, *J* = 11.0, 3.2 Hz, 1H), 2.68 (dd, *J* = 11.5, 2.9 Hz, 1H), 2.65 – 2.52 (m, 1H), 2.24 (ddd, *J* = 25.0, 12.6, 5.3 Hz, 1H), 1.73 (s, 3H), 2.18 – 0.55 (m, 49H), 1.21 (d, *J* = 6.8 Hz, 3H) ppm.

^{13}C–NMR ($CDCl_3$, 101 MHz) δ: 216.4, 183.6, 154.5, 145.3, 144.9, 144.7, 135.5, 126.1, 124.6, 123.0, 104.4, 101.9, 100.0, 89.0, 76.3, 75.9, 75.8, 74.6, 71.8, 71.7, 69.8, 67.3, 55.1, 54.3, 51.4, 50.3, 43.2, 40.7, 38.9, 36.1, 35.8, 32.7, 32.5, 32.4, 30.0, 29.8, 28.1, 28.0, 27.0, 23.8, 20.2, 19.6, 17.5, 16.1, 15.9, 14.8, 13.3, 12.8, 12.0, 10.8, 6.9, 6.8 ppm.

FTIR (CH_2Cl_2 film): 3336 (s, br), 2970 (s), 2935 (s), 2873 (w), 2856 (w), 1750 (s), 1713 (s), 1613 (m), 1557 (s) cm^{-1}.

HRMS-ESI *(m/z)*: $[M+H]^+$ Calcd for $C_{52}H_{78}N_4NaO_{16}$ 1037.5311; Found 1037.5330.



Conjugate 7. To pyridine (1 mL) stirred at 0 °C was added phosgene (335 µL, ~0.673 mmol, ~20% in toluene) dropwise over 1 min resulting in a yellow suspension. A solution of NBD-linker **5** (156 mg, 0.654 mmol) in pyridine (5 mL) was then added dropwise over 5 min. The resulting red suspension was stirred for 15 min after which methyl ester **2** (100 mg, 0.131 mmol) was added in one portion. The resulting mixture was stirred for 22 h while slowly warming to RT, then diluted with EtOAc (100 mL) and washed with Na_2CO_3 (3 x 20 mL, 0.1 M aq.). The organic layer was separated, dried using a phase separator, and concentrated under reduced pressure. Purification by flash chromatography (25 – 50% EtOAc/*n*-heptane, and 1 – 5% MeOH/CH_2Cl_2) gave conjugate **7** that was lyophilized from 20% acetonitrile/water.

Yield: 40.0 mg, 30%. Isolated as a yellow amorphous solid, >95% pure by NMR, and a single spot on TLC.

R_f: 0.10 in 50% EtOAc. Yellow spot, UV-active, fluorescent and stains brown with PMA.

Optical rotation: $[\alpha]_D^{25}$ - 61.6 (*c* = 0.97 CH_2Cl_2).

^{1}H–NMR ($CDCl_3$, 400 MHz) δ: 8.47 (d, *J* = 8.9 Hz, 1H), 6.21 (app. m, 2H), 5.80 (dd, *J* = 10.8, 1.5 Hz, 1H), 5.06 (s, 1H), 4.63 – 4.32 (m, 4H), 4.11 – 3.97 (m, 2H), 3.87 (s, 3H), 3.70 (q, *J* = 6.9 Hz, 1H), 3.64 (dd, *J* = 9.9, 2.1 Hz, 1H), 3.54 (dd, *J* = 10.6, 2.5 Hz, 1H), 3.47 (s, 3H), 3.37 (dd, *J* = 11.5, 2.2 Hz, 1H), 3.21 – 3.07 (m, 1H), 3.07 – 2.93 (m, 2H), 2.84 – 2.67 (m, 2H), 1.44 (s, 3H), 1.17 (d, *J* = 6.9 Hz, 3H), 2.08 – 0.63 (m, 42H), 0.94 (d, *J* = 6.6 Hz, 3H), 0.70 (d, *J* = 6.8 Hz, 3H) ppm.

^{13}C–NMR ($CDCl_3$, 101 MHz) δ: 213.9, 176.4, 155.1, 145.1, 144.8, 144.7, 135.3, 126.6, 124.4, 123.7, 104.3, 102.0, 99.3, 87.4, 80.2, 77.1, 75.2, 72.8, 72.5, 71.9, 70.9, 69.2, 65.8, 57.4, 54.3, 52.8, 48.9, 48.4, 42.3, 40.6, 39.1, 36.6, 35.5, 33.4, 32.7, 30.6, 29.6, 28.2, 26.4, 23.9, 22.8, 21.0, 20.7, 19.9, 17.6, 15.7, 14.3, 13.9, 13.0, 12.0, 11.1, 7.3, 6.6 ppm.

FTIR (CH_2Cl_2 film): 3527 (s, br), 2947 (s), 2876 (m), 1747 (s), 1718 (s), 1614 (m), 1555 (s) cm^{-1}.

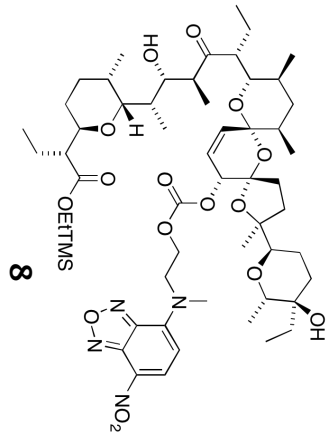
HRMS-ESI *(m/z)*: $[M+Na]^+$ Calcd for $C_{53}H_{80}N_4NaO_{16}$ 1051.5467; Found 1051.5469.

*III. Structural elucidation of conjugates **6** and **7**.*

Structural assignment of conjugate 6. The NBD carbonate linker of conjugate **6** was assigned to the C20 position of salinomycin using the diagnostic downfield shift of the C20 proton in the ^{1}H NMR spectrum relative to that of the C20 proton of salinomycin sodium salt (**1**). In $CDCl_3$: **6** C20*H* δ: 5.07 ppm, Δδ **1**: 1.00 ppm (4). The ^{1}H NMR signals of the allylic C20 proton of **6** were in turn assigned by a $^3J_{HH}$ coupling to the characteristic olefinic C18 and C19 protons in the COSY spectrum. For ^{1}H NMR, ^{13}C NMR, and the partly assigned COSY spectrum of **6**, see pages S11–S13.

Structural assignment of conjugate 7. The NBD carbonate linker of conjugate **7** was assigned to the C20 position of salinomycin using a diagnostic downfield shift of the of C20 proton in the ^{1}H NMR spectrum relative to that of the C20 proton of salinomycin methyl ester (**2**). In $CDCl_3$: **7** C20*H* δ: 5.06 ppm, Δδ **2**: 1.05 ppm (4). The ^{1}H NMR signals of the allylic C20 proton was assigned by a $^3J_{HH}$ coupling to the characteristic olefinic C18 and C19 protons in the COSY spectrum. For ^{1}H NMR, ^{13}C NMR, and the partly assigned COSY spectrum of **7**, see pages S14–S16.

*IV. NMR spectra of compounds **8**, **6**, and **7**.*



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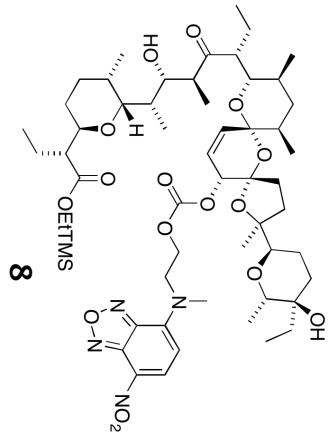

8

212.9
175.9
155.0
144.9
144.6
135.1
126.4
124.4
123.6
104.2
101.8
99.2
87.2
81.1
75.0
72.6
72.3
71.7
70.8
69.1
63.6
57.3
54.2
48.8
48.0
40.4
36.4
33.1
32.6
30.4
29.4
28.1
26.3
23.7
22.6
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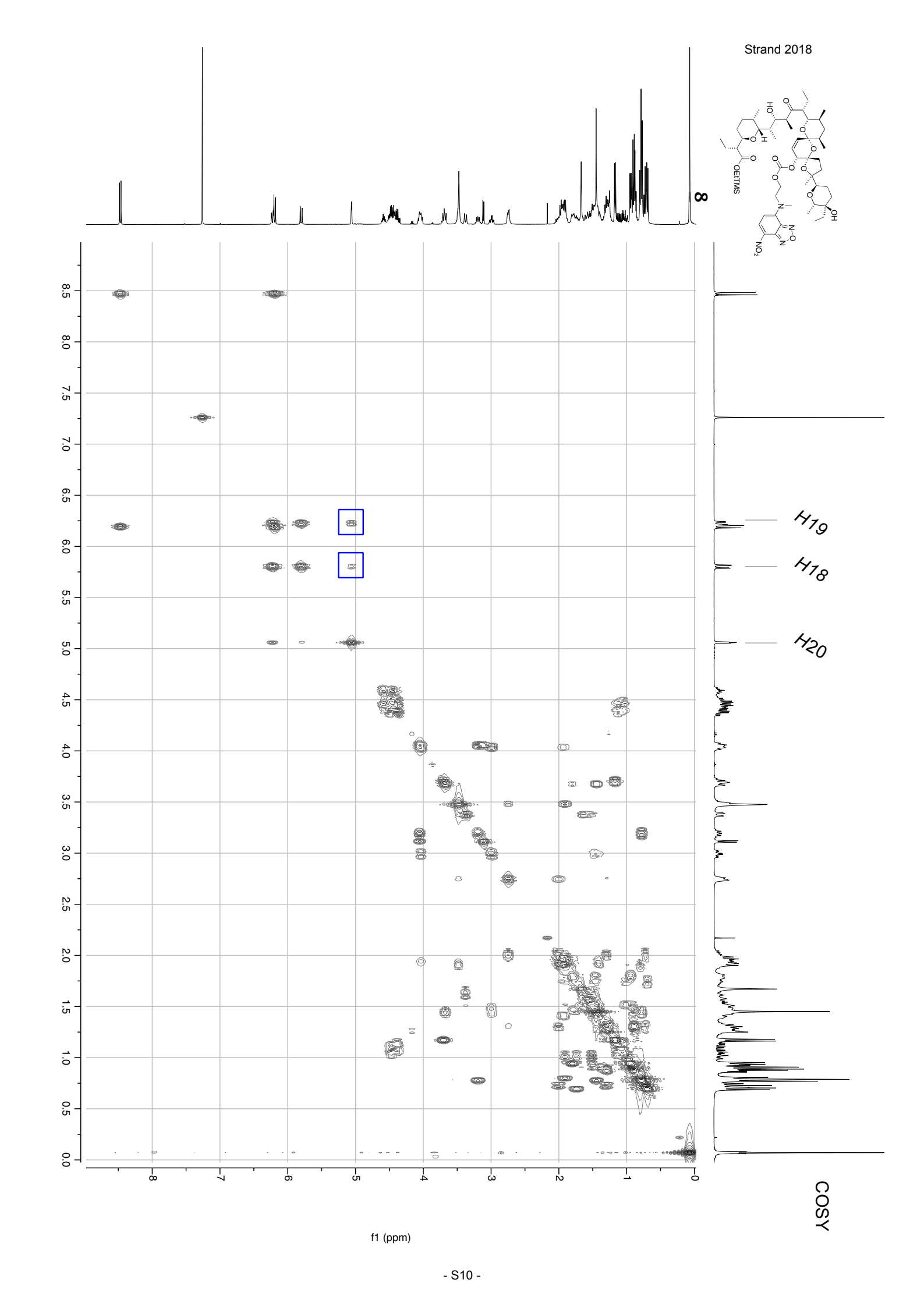
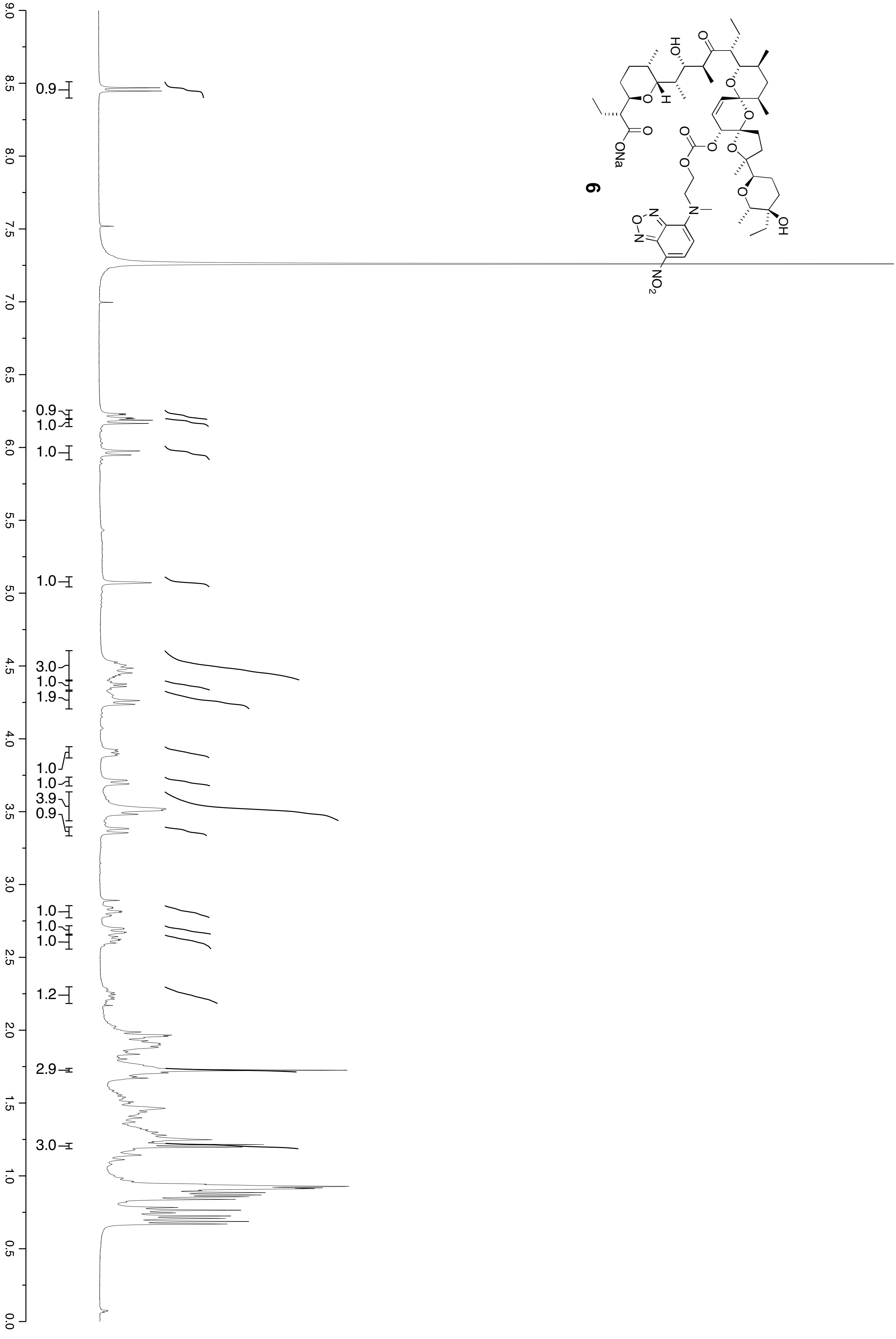
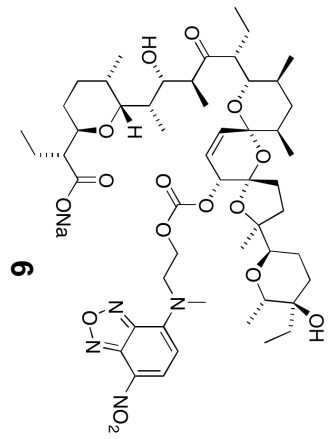
11.9
11.0
7.2
6.5
1.5


Strand 2018
H19
H18
H20
COSY
f1 (ppm)


6



6

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145.3
144.9
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135.5
126.1
124.6
123.0
104.4
101.9
100.0
89.0
76.3
75.9
75.8
74.6
71.8
71.7
69.8
67.3
55.1
54.3
51.4
50.3
43.2
40.7
38.9
36.1
35.8
32.7
32.5
32.4
30.0
29.8
28.1
28.0
27.0
23.8
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16.1
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14.8
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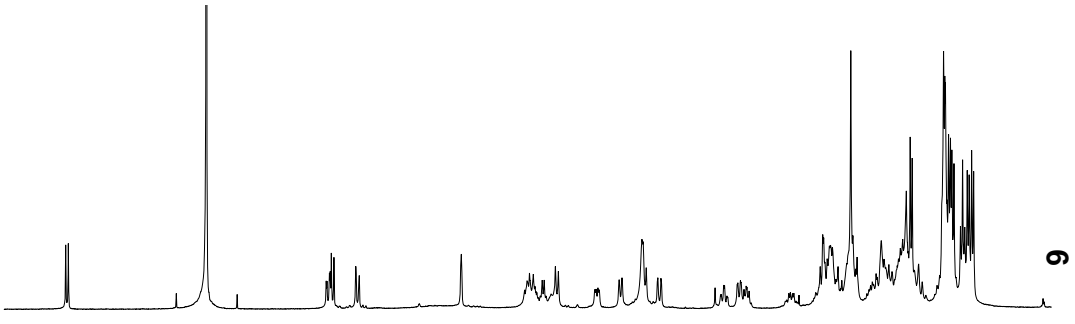
12.8
12.0
10.8
6.9
6.8



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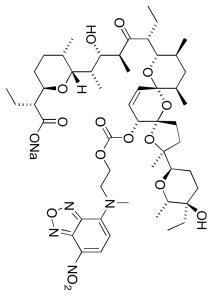

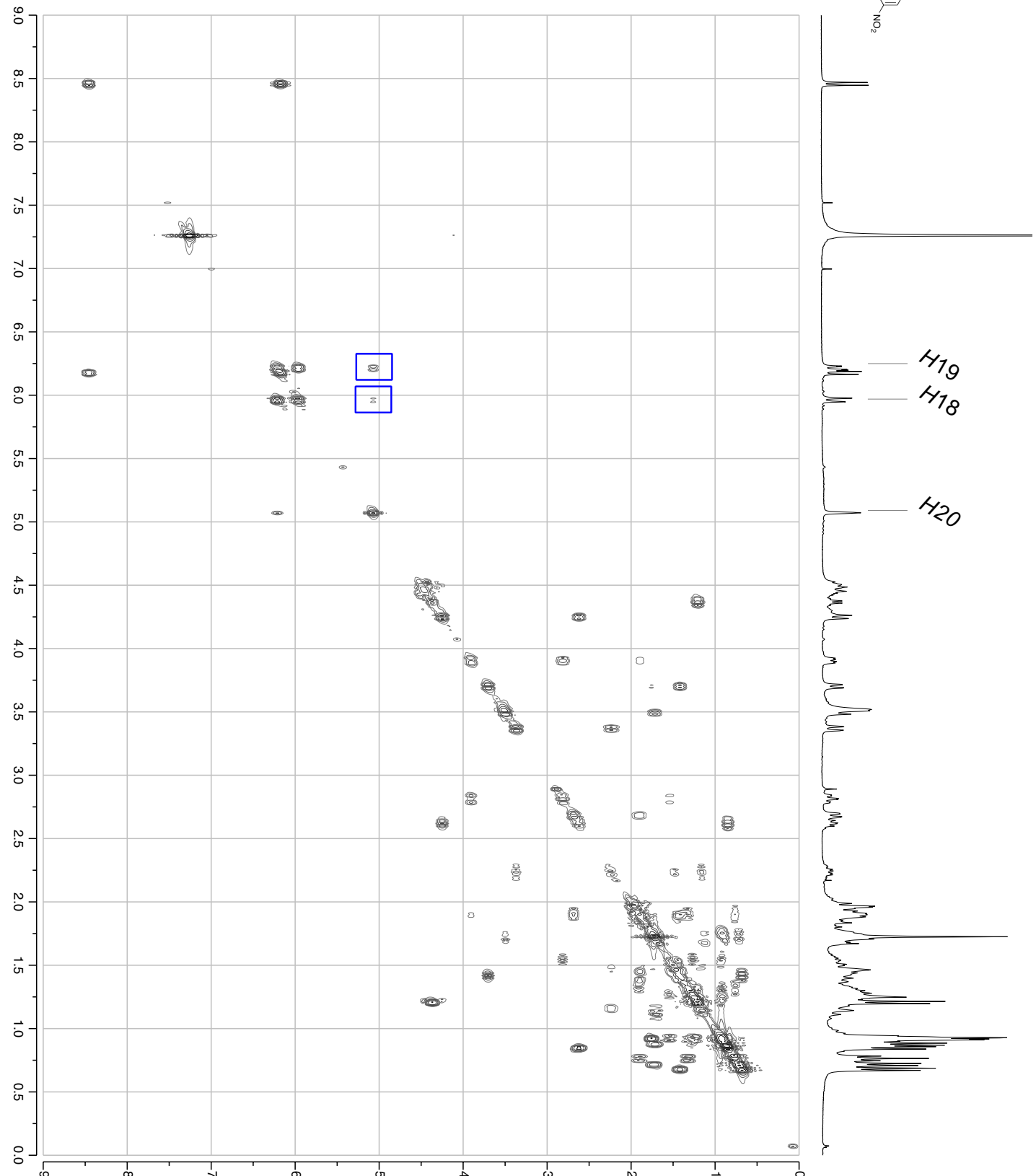

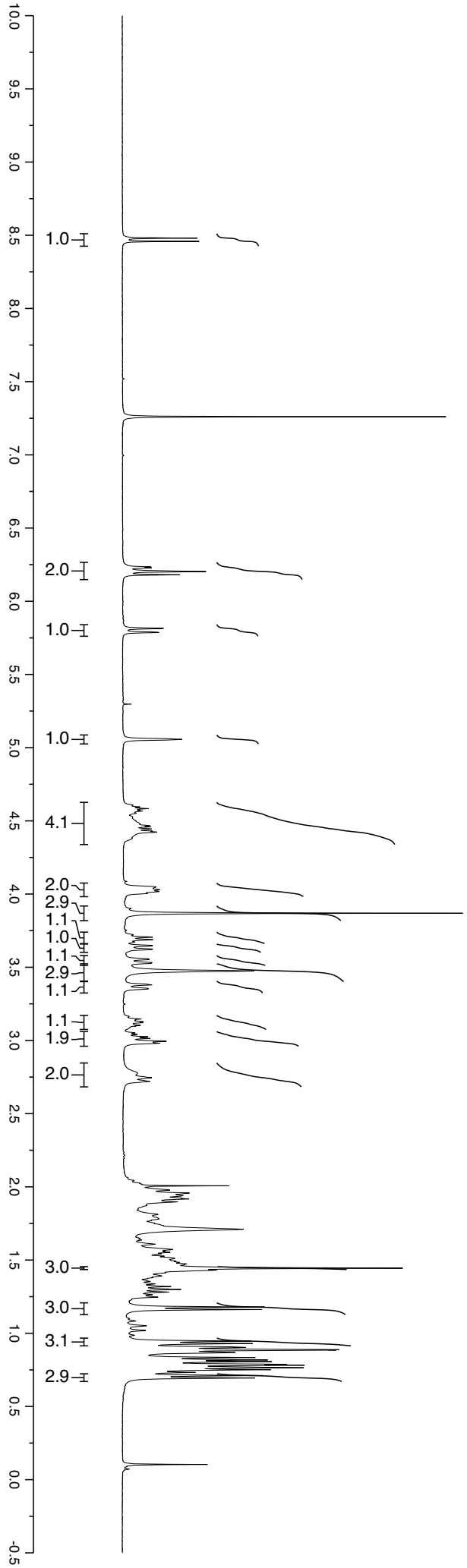
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
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4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
1.0
2.0
1.0
1.0
4.1
2.0
2.9
1.1
1.0
1.1
2.9
1.1
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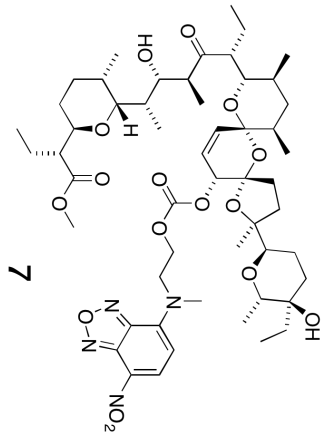
3.0
3.0
3.1
2.9


HO
O
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O
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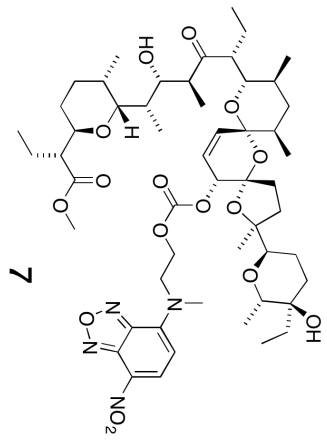
O
H
N
N
O
N
NO2
OH

7



7

213.9
176.4
155.1
145.1
144.8
144.7
135.3
126.6
124.4
123.7
104.3
102.0
99.3
87.4
80.2
77.1
75.2
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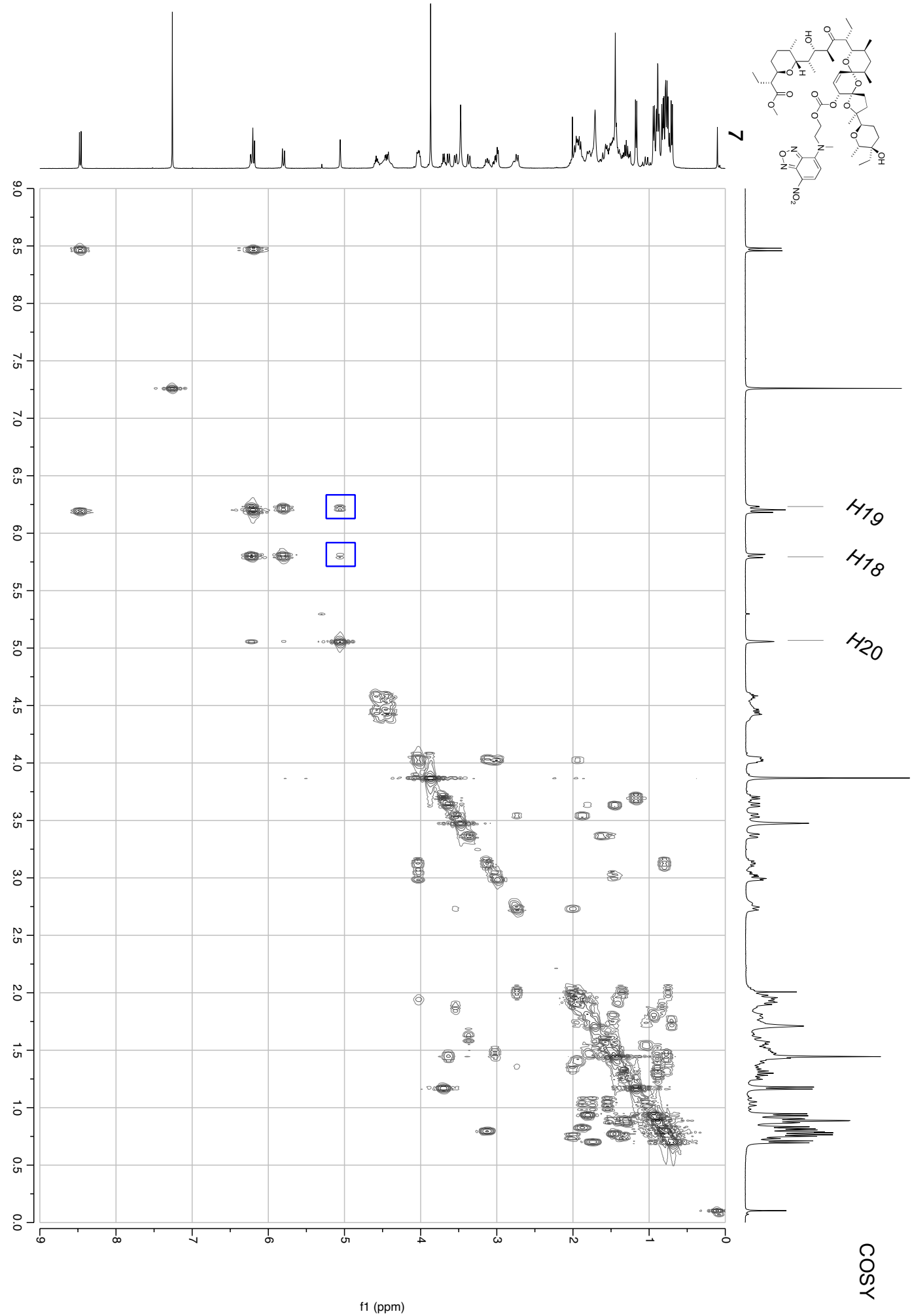
7.3
6.6


7
H19
H18
H20
COSY
f1 (ppm)

*V. Fluorescence properties of NBD conjugates **6** and **7**.*

General procedures. UV-VIS spectra in DMSO were recorded using a Probe Drum spectrometer at 25 °C averaging 16 spectral recordings to obtain the spectra. Fluorescence spectra in DMSO were recorded using a Varian Cary Eclipse fluorescence spectrophotometer at room temperature with the excitation wavelength set to 348 nm.

Fluorescence lifetime, accumulation in cells, and fluorescence spectra in cells and buffer were recorded using a custom-built wide-field fluorescence microscope based on Olympus X71 that is explained in detail elsewhere (5, 6). The samples were excited by an Ar-ion laser at 458 nm for video-rate fluorescence imaging, or by a diode laser (PicoQuant) at 485 nm for fluorescence lifetime measurements. The fluorescence kinetics were measured by time-correlated single photon counting (PicoHarp) with an avalanche photodiode (Micro Photonic devices) as a detector. The system instrumental response function width was about 100 ps. The excitation spot had a diameter of approximately 30 μm at the sample plane. Fluorescence intensity was collected by an objective lens (Olympus LUCPLanFL, 40X/0.6 or UPLanFLN 10X/0.3) and imaged by an EMG CCD Camera (ProEM-512, Princeton Instrument). The exposure time of the camera was around 0.1 s per image and was varied depending on the excitation power density. The excitation power density was varied from 10^{-2} to 100 W cm^{-2} (for labeled and unlabeled cells respectively) using a set of neutral density filters placed in the excitation beam. The experiments were carried out at room temperature. Fluorescence images and videos were processed using the ImageJ (1.49v) software.

For imaging experiments, samples of JIMT-1 cells, cells were seeded with a density of 20000 cells/cm^2 on quartz covers slips (14 x 14 x 0.25 mm). One to two days after seeding when the cells were attached, the cells were washed once with warm HBSS containing 10% fetal bovine serum (FBS), sodium pyruvate (1 mM), nonessential amino acids (1 mM), and L-glutamine (2 mM). The cells were then maintained in the same HBSS until placed under the microscope.

Spectral measurements. The UV-VIS absorbance spectra of both conjugate **6** and **7** were recorded in DMSO (1 mg/mL in a quartz cuvette). Both conjugates display similar spectra with two absorbance maxima (Figure S1). The fluorescence spectra of the conjugates in DMSO (1 mg/mL in a glass cuvette) were recorded using a fluorescence spectrophotometer with irradiation of the lower maxima. Fluorescence spectra in JIMT-1 cells were recoded using a custom-built wide-field fluorescence microscope with irradiation at 458 nm. A quartz cover slip on which cells

had been grown was placed in the microscope beam path and the cells were kept covered by a thin layer of HBSS. Cells were localized and the focus adjusted based on cellular auto-fluorescence. A single drop of conjugate (10 µL, 200 µM in HBSS with 2% DMSO) was added to the buffer layer covering the cells and spectra recorded after the fluorescence in the cells had reached a stable intensity. Fluorescence spectra in HBSS were recorded in the same experiment by measuring the fluorescence spectrum in cell-free areas of the slide. The emission spectra in cells, HBSS, and DMSO showed small differences (Figure S1).

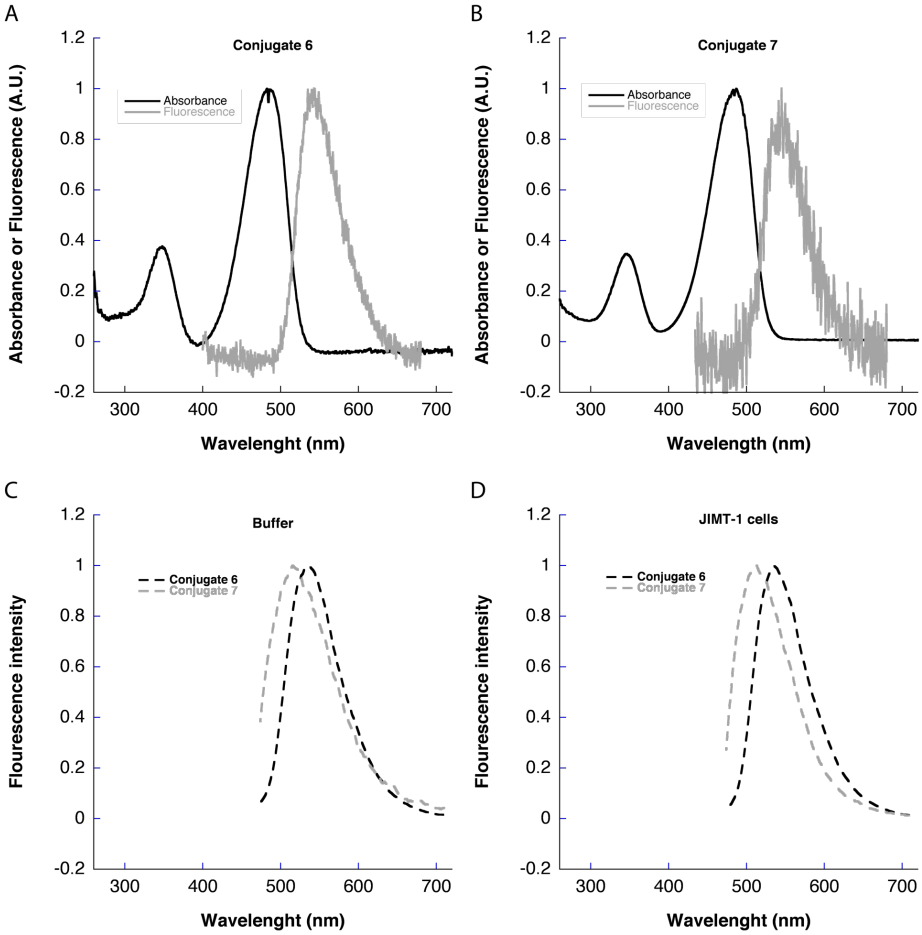
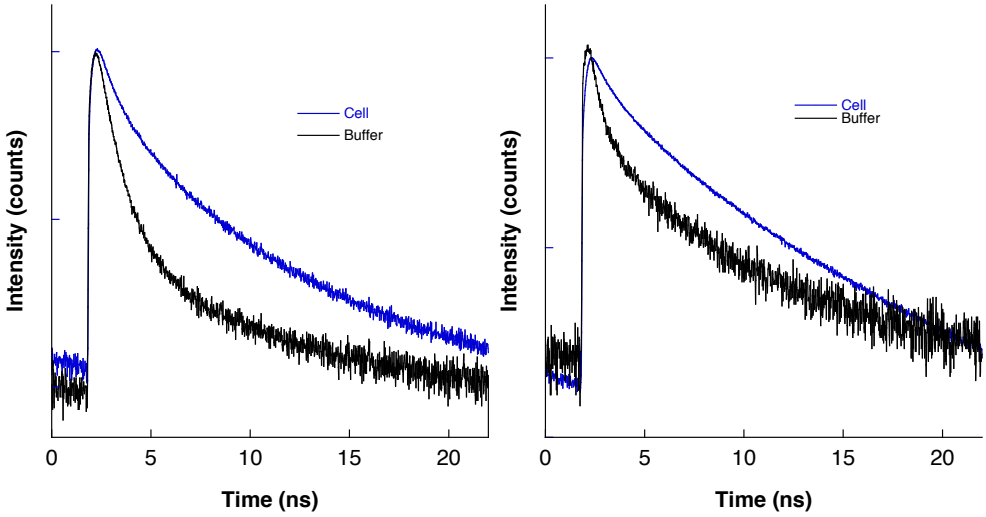


Figure S1. Spectroscopic properties of conjugates **6** and **7** in various environments. (*A*) Normalized absorbance and fluorescence spectra of **6** in DMSO. Maxima for **6**: λ(Abs$_1$) = 348 nm, λ(Abs$_2$) = 483 nm, λ(Em) = 543 nm. (*B*) Normalized absorbance and fluorescence spectra of **7** in DMSO. Maxima for **7**: λ(Abs$_1$) = 345 nm, λ(Abs$_2$) = 487 nm, λ(Em) = 545 nm. (*C*) Normalized fluorescence spectra of **6** and **7** in HBSS. Maximum for **6**: λ(Em) = 535 nm; **7**: λ(Em) = 515 nm. (*D*) Normalized fluorescence spectra of **6** and **7** in JIMT-1 cells. Maximum for **6**: λ(Em) = 535 nm; **7**: λ(Em) = 513 nm.

Lifetime measurements. The excited state fluorescence lifetimes of **6** and **7** were measured in cells and in HBSS (Figure S2). The cells and the conjugates were handled in the same way as for recording of fluorescence spectra. The fluorescence decay was fitted using a double exponential model. The amplitude averaged lifetime in the cells was about 2-3 times longer than in the surrounding buffer. In pure DMSO, the lifetimes were in between that found in cells and buffer.



Compound	Location	$\langle\tau\rangle_{amp}$ (ns)
Conjugate **6**	DMSO	1.5710
	Cell	2.6815
	Buffer	1.2490
Conjugate **7**	Cell	1.5043
	Buffer	0.5674

Figure S2. Fluorescence lifetimes ($\langle\tau\rangle_{amp}$) of conjugate **6** (left) and **7** (right) in JIMT-1 cells and buffer.

Accumulation rate of conjugates 6 and 7 in JIMT-1 cells. A solution of either conjugate **6** or **7** (10 μL of a 2, 20, or 200 μM solution in HBSS with 2% DMSO) was added into a buffer layer covering JIMT-1 cells immobilized on a quartz cover slip placed on the fluorescence microscope (Figure 3*A*). The addition was done at a position outside of the irradiated area. The dye spread rapidly throughout the buffer solution (<1 s).

Images of cells irradiated by the Ar laser were continuously recorded as a video with 0.1 s exposure time per image. The recording was started right before addition and the increase in fluorescence was monitored for 100 s using either a 40x or a 10x objective. The intensity of the recorded

fluorescence was adjusted by setting the optical density (OD) to 2 or 3 and the multiplication gain to 5 or 100. Generally, setting the OD to 2 and the gain to 100 gave good results across all concentrations. The background intensity recorded outside the illuminated area was subtracted from the fluorescence intensities in both the cell and buffer. The accumulation of fluorescence was characterized by the difference of the intensities between equally sized areas in the cell and the surrounding buffer. Aggregates were observed for conjugate **7** across all concentrations, while aggregates of conjugate **6** were only observed at a 200 µM concentration. Sonication did not fully dissolve the visible aggregates but reduced their number and size.

Exposing JIMT-1 cells to each conjugate resulted in an accumulation in a distinct pattern (Figure S3).

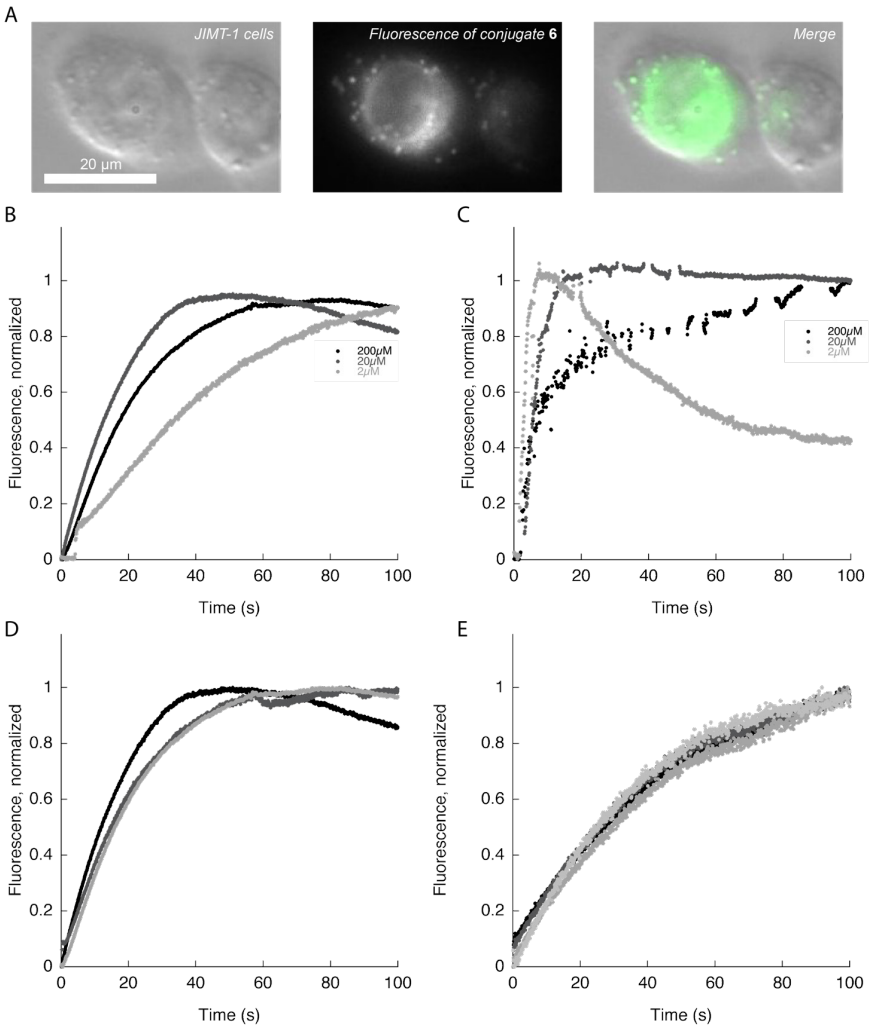


Figure S3. Uptake of conjugate **6** and **7** in JIMT-1 cells. (*A*) (left) Morphology of JIMT-1 cells when viewed in bright field. (center) The fluorescence of conjugate **6** when excited by a 458 nm Ar laser line. (right) Merged bright field and fluorescence image (green). Bar = 20 μm. (*B*) Accumulation of conjugate **6** in JIMT-1 cells. Representative data from experiments using three different concentrations of **6** (2, 20, and 200 μM). (*C*) Accumulation of conjugate **7** in JIMT-1 cells. Representative data from experiments using three different concentrations of **7** (2, 20, and 200 μM). Fluorescence intensity flashes due to aggregates diffusing through the excited sample (seen as gaps in the curve) were removed for clarity. (*D*) Data from uptake experiments of **6** (20 μM) using cells grown on three different quartz cover slips (variability between experiments). (*E*) Data from uptake experiments of **6** (20 μM) from three cells grown on the same quartz cover slip (variability between cells within a single experiment).

Accumulation of **6** into the cells was rapid and the fluorescence intensity stabilized within a time range of 40-80 s, while the dissolved part of conjugate **7** was taken up within 10-20 s.

A more accurate determination of the accumulation time was prohibited by the experimental conditions. The main contributors to the experimental uncertainty were likely the distance between the observation point and the position where the conjugate was added to the sample, the droplet size and the initial thickness of the buffer layer – all these factors influenced spreading and mixing of the solution of the conjugates in the sample. There was only minor variation of the time until a steady fluorescence intensity was reached between different cells of the same sample. At OD 2 (excitation power <1 W/cm^2) photo-bleaching was not pronounced.

*VI. Antiproliferative and selective phenotype activity of conjugates **6** and **7** in JIMT-1 and MCF-7 cell lines.*

Cell line and culturing conditions. The human breast carcinoma cell lines JIMT-1, MCF-7, and HCC1937 were cultured at 37 °C in a humidified incubator with 5% CO_2 in air. The JIMT-1 cell line was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). MCF-7 and HCC1937 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). JIMT-1 cells were routinely cultured in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 medium (1:1) while MCF-7 and HCC1937 cells were maintained in RPMI 1640 medium. All cell lines were cultured with the addition of 10% FBS, nonessential amino acids (1 mM), insulin (10 μg/mL), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cell culture medium of HCC1937 was also supplemented with

epidermal growth factor (20 ng/mL). The JIMT-1, MCF-7, and HCC1937 cells were seeded at a density of 20000, 25000, and 20000 cells/cm^2 in 0.2 mL medium/cm^2 surface area, respectively.

Compounds and treatment. Stock solutions of the compounds (10 mM in DMSO) were kept at 4 °C until use. The fluorescent conjugates were kept protected from light. At 24 h after seeding, the compounds were added to the final concentrations shown in figures and described in results. Control cells received the same final DMSO concentration as cells treated with compound dissolved in DMSO. The final DMSO concentration was never above 0.2%.

MTT assay and colony formation assay in soft agar. The MTT assay and the colony formation assay were performed as described previously (2, 7).

Luciferase reporter assay in Leading light® Wnt reporter cells. Leading light® Wnt reporter cells (Enzolifesciences, ENZ-61002-0001) (2 x 10^4 cells) were plated in 100 µL of DMEM medium containing 10% FBS and 1% PEST per well of a 96-well solid bottom white assay plate. Cells were then treated for 24 h with vehicle control (0.02% DMSO), salinomycin, conjugate **6**, or conjugate **7** (1 µM).[1] Firefly and Renilla signals were detected using Dual-Glo luciferase detection reagents (Promega) according to the manufacturer's instructions. Firefly luciferase levels were normalized to Renilla luciferase levels to generate a measurement of relative luciferase units. All experiments were performed eight times with three replications in each experiment.

Luciferase assay in JIMT-1 cells transfected with TOP/FOPFlash and Renilla plasmids. JIMT-1 cells (2 x 10^4 cells) were plated in 100 µL of medium containing 10% FBS per well of a 96 well solid bottom white assay plate. After 24 h, the cells were transfected overnight using Lipofectamine 2000 transfection reagent (Thermo scientific) according to the manufacturer's instructions with 150 ng TOP/FOPFlash reporter plasmid and 20 ng of Renilla plasmid. Cells were then treated for 24 h with DMSO, salinomycin, conjugate **6**, or conjugate **7** (1 µM).[1] Firefly and Renilla signals were detected using Dual-Glo luciferase detection reagents (Promega) according to the manufacturer's instructions. Renilla luciferase was used as the internal transfection control. Firefly luciferase levels were normalized to Renilla luciferase levels to generate a measurement of relative luciferase units. The results are presented as percentage luciferase activities normalized to

[1] To control for excitation by energy transfer from bioluminescence, a combination of salinomycin (1 µM) and the inactive conjugate **7** (1 µM) was also evaluated. The result was similar within experimental error to that of salinomycin alone leading us to conclude that at the concentration used, interference from the conjugate is limited.

transfected JIMT-1 cells (DMSO control). All experiments were performed three times with three replications in each experiment.

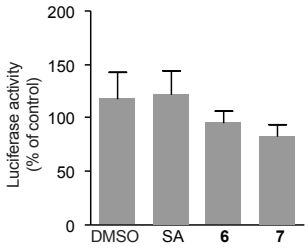


Figure S4. Luciferase reporter assay in JIMT-1 cells transfected with FOPFlash and Renilla plasmids. Cells were treated for 24 h. Columns show mean ± SE (n = 3). DMSO control = 100%. Firefly luciferase levels were normalized to Renilla luciferase levels.

VII. Cellular imaging

Confocal microscopy. Cells were seeded in glass bottom Petri dishes (MatTek Corporation, Ashland, MA, USA) and allowed to attach for at least 24 h before the experiments were performed. To visualize the ER together with conjugate **6** or **7**, cells were first washed with and then kept in HBSS, whereupon ER-Tracker™ Red was added to a final concentration of 1 μM and the cells were incubated for 30 min at 37 °C. Conjugate **6** and **7** were added to the cell culture dishes for 5 and 30 min, respectively before imaging in the confocal microscope. As Nile Red and Rhodamine 123 had excitation and emission wavelengths in the same ranges as the conjugates, they could not be imaged simultaneously. Instead the cells were stained with 0.5 μg/mL Nile Red for 15 min or 10 μg/mL Rhodamine 123 for 10 min at 37 °C following imaging in the confocal microscope. The Nile Red or Rhodoamine 123 staining, in the imaging area, were then bleached completely (confirmed by sub-sequent imaging). Conjugate **6** was then added, at a concentration of 2 μM, to the cells that were then imaged again (around 5 and 10 min after the conjugate was added to the culture dishes).

The cells seen in Figure S6 were imaged using a LSM-510 confocal laser-scanning microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) equipped with a Hamamatsu R6357 photomultiplier (Hamamatsu Photonics K.K., Hamamatsu, Japan). Images of the cells were obtained by capturing high-resolution images of individual optical planes at high magnification. Conjugate **6**, Nile Red (AAT Bioquest®, Inc., Sunnyvale, CA, USA) and Rhodamine 123 (Sigma-Aldrich, Stockholm, Sweden) were excited using an argon ion laser, at a wavelength of 488 nm.

A band-pass filter of 515-530 nm was used for detection of emission from conjugate **6** and Rhodamine 123, whereas a band pass filter of 530-600 nm was used for the Nile Red emission. ER-Tracker™ Red (Life Technologies, Carlsbad, CA, USA) was excited using a diode-pumped solid-state laser, at a wavelength of 561 nm, and a long-pass filter with a cut-off of 575 nm was used for the emission. An X-Cite® 120 PC fluorescence illumination system was used for bleaching the Rhodamine 123 and Nile Red dyes. Confocal microscopy images and co–localization experiments with ER Tracker, Nile Red, and Mito Tracker are shown in Figures S5, S6, and S7.

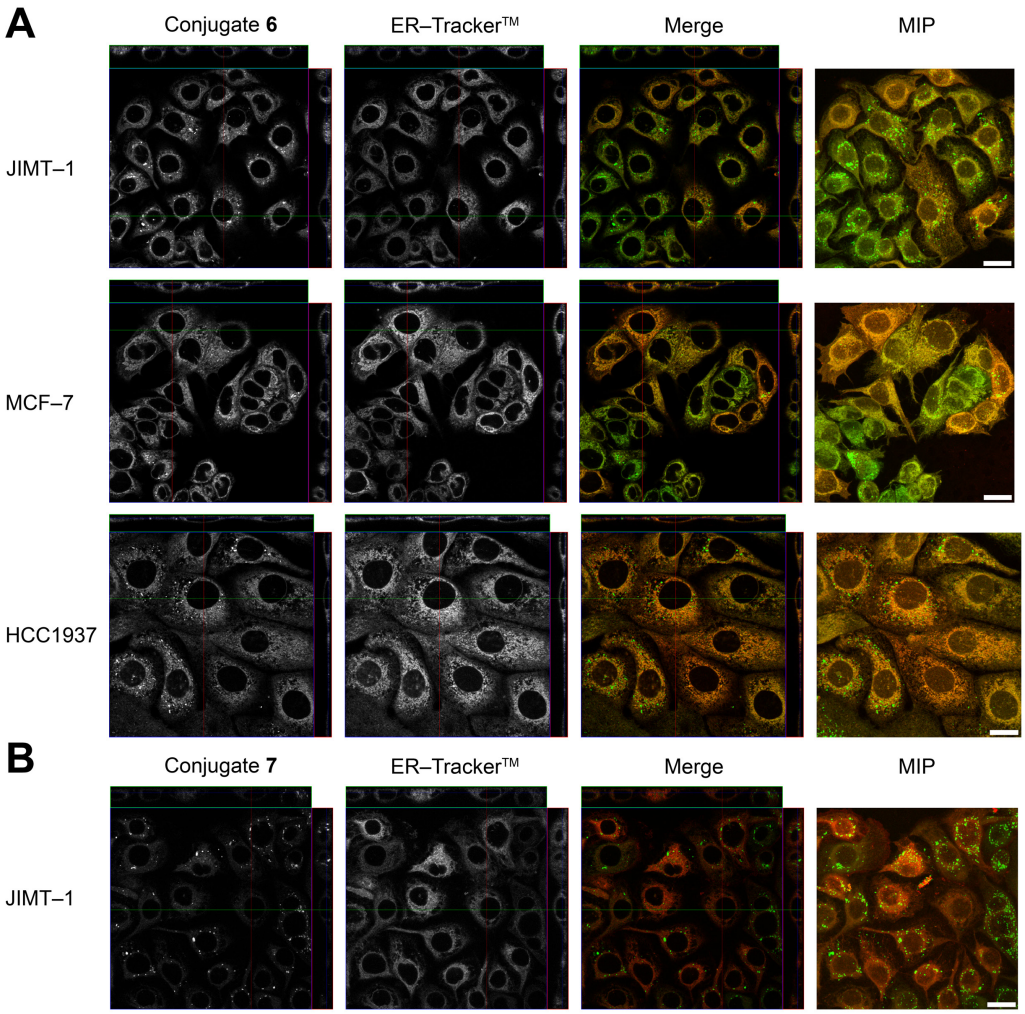


Figure S5. Confocal imaging of fluorescent conjugates in the JIMT-1, MCF-7, or HCC1937 breast cancer cell lines. (*A*) Co-localization between conjugate **6** and ER-Tracker™ Red. (*B*) Co-localization between conjugate **7** and ER-Tracker™ Red. Signals from the individual channels can be seen in black and white but are additionally merged for clarity (green color = conjugate **6** or **7** and red color = ER Tracker™ Red, where yellow/orange color indicates co-

localization). Maximum intensity projections (MIP) are also included to better display the signal from the whole cell volume. Scale bars = 10 μm.

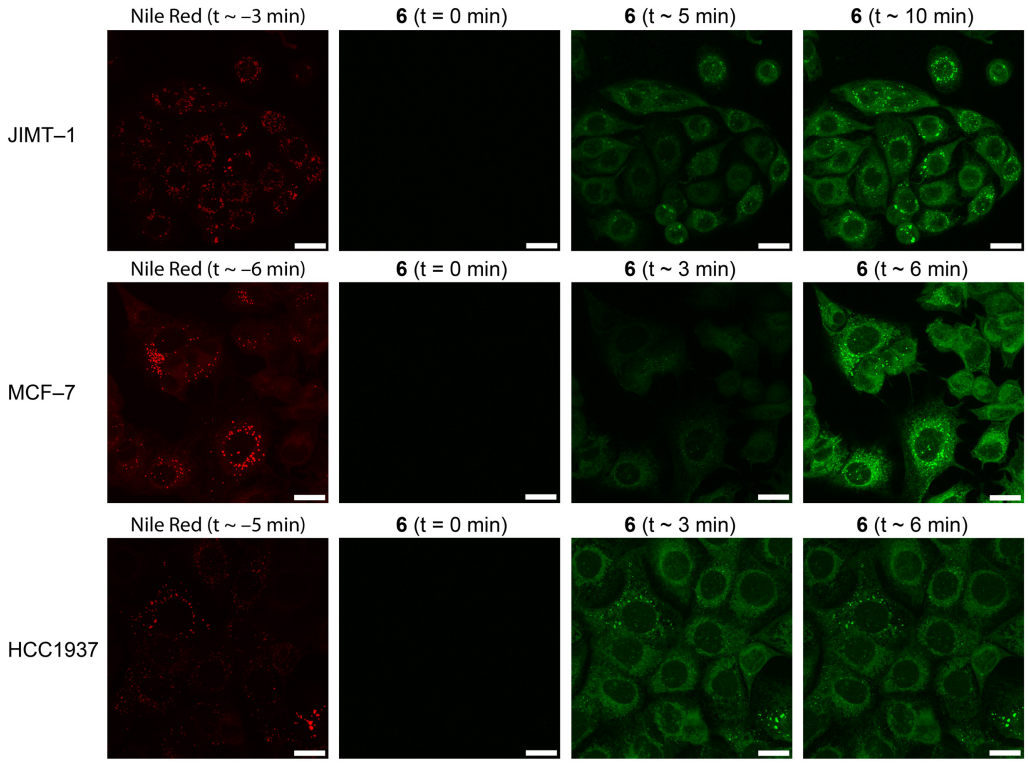


Figure S6. Confocal imaging of fluorescent conjugate **6** in three breast cancer cell lines, JIMT-1, MCF-7, and HCC1937. Co-localization with Nile Red as a marker for lipid droplets. The cells were incubated with Nile Red and images captured. The Nile Red was then bleached until no fluorescence was detected, whereupon conjugate **6** was added to the medium of the cells (t = 0) and images captured at the indicated time points. Scale bars = 10 μm.

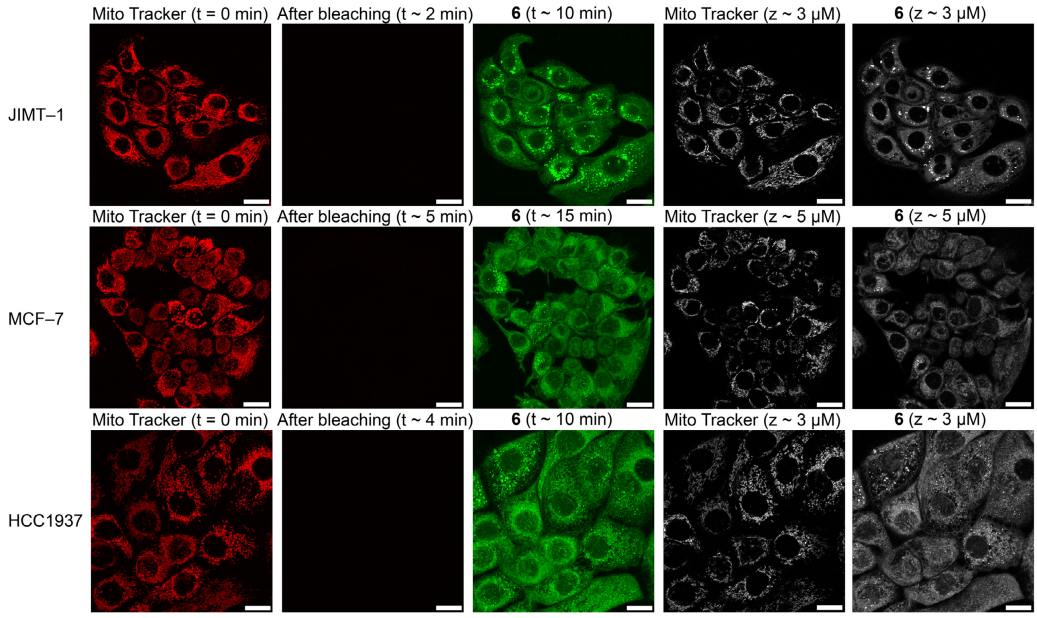


Figure S7. Confocal imaging of fluorescent conjugate **6** in three breast cancer cell lines, JIMT-1, MCF-7, and HCC1937. Absence of co-localization with Mito Tracker as a marker for mitochondria. The cells were incubated with Mito Tracker and images were captured (t = 0). The Mito Tracker was then bleached until no fluorescence was detected. Conjugate **6** was added to the medium of the cells and images captured after ~ 10–15 min. Scale bars = 10 µm. z = position of focal plane.

Epi-fluorescence microscopy. Cells were seeded in glass bottom Petri dishes (MatTek Corporation) and allowed to attach for at least 24 h before the experiments were performed. The cell layer was washed with warm HBSS and then HBSS was added to Petri dish. The fluorescent compounds were added to a final concentration of 2 µM. After 5 and 30 min incubation with conjugate **6** and **7**, respectively, the cells were viewed in a epi-fluoresence microscope (Carl Zeiss AB, Sweden) and imaged. First an image was taken with DIC and then images were taken in fluorescence mode of the same cells. Fluorescence was elicited with the excitation wavelength at 420 nm and the emission was registered at 540 nm. The images in Figures S8 and S9 were analyzed in Volocity software.

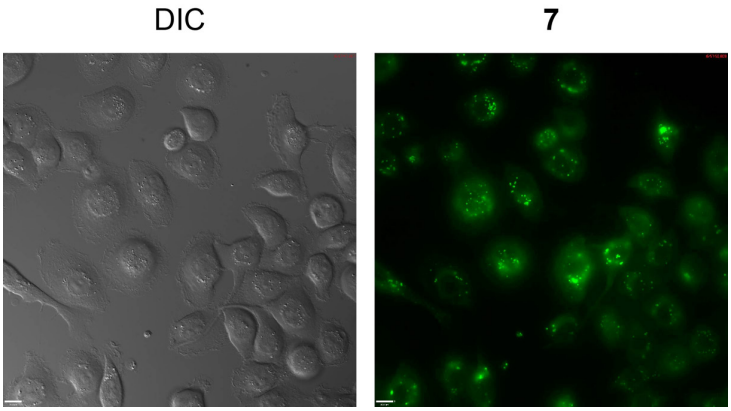


Figure S8. Subcellular distribution of the fluorescent salinomycin conjugate **7** in breast cancer cell line JIMT-1 cells. The cells were treated for 30 min with 2 μM of **7**. The cells were imaged in a Zeiss epi-fluorescence microscope. Scale bars = 10 μm. DIC: Differential interference contrast.

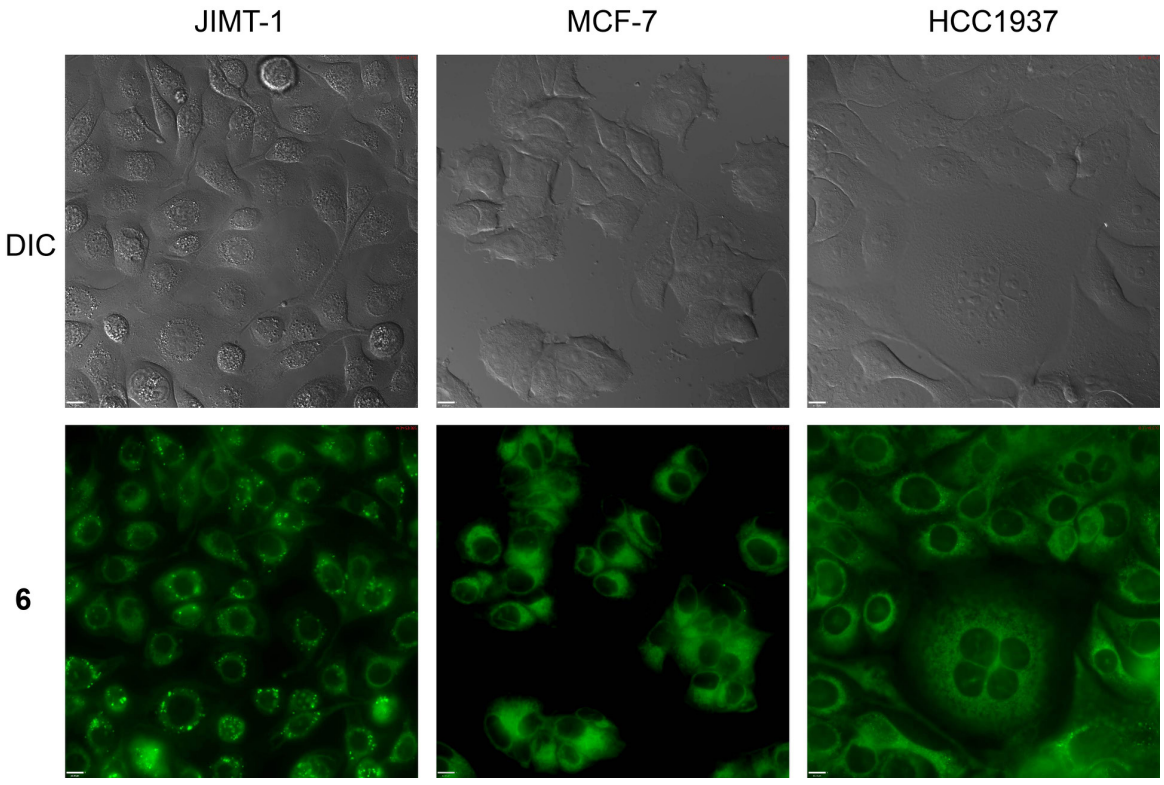


Figure S9. Subcellular distribution of the fluorescent salinomycin conjugate **6** in breast cancer cell lines JIMT-1, MCF-7, and HCC1937. The cells were treated for 5 min with 2 μM of **6**. The cells were imaged in a Zeiss epi-fluoresence microscope. Scale bars = 10 μm. DIC: Differential interference contrast.

VIII. Evaluation of intracellular calcium levels.

Cytoplasmic calcium imaging. The JIMT-1 cells were seeded in glass bottom culture dishes (MatTek Corporation) and 24 to 72 h later, the cells were rinsed with a solution containing 135.5 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11.6 mM HEPES, pH 7.4) (HEPES solution). The cells were then loaded with 250 µl of 4 µM Fluo-4 AM (Life Technologies, F-14201) diluted with 0.02% pluronic F-127 in HEPES solution for 90 min at room temperature. After loading, the cells were rinsed and left in the HEPES solution for 30 min at room temperature to allow de-esterification of intracellular Fluo-4 AM. The Petri dishes were transferred to the microscope and the fluorescence was elicited with the excitation wavelength at 488 nm, using Zeiss LSM 510 laser-scanning confocal microscope with a 40x oil objective. Images were acquired at room temperature with the frequency of one image per second for 700 s and the compounds were added to the HEPES solution at 60 s after start of the time lapse. Nominally calcium-free HEPES solution was used to test the influence of extracellular calcium. To investigate if the cytosolic Ca^{2+} is from ER, the Ca^{2+} release channels in ER membranes were blocked with 100 µM of Ryanodine (HB1320, Hello Bio, Bristol, UK) and 50 µM of 2-aminoethoxydiphenyl borate (2-APB) (D9754-1g, Sigma-Aldrich, Stockholm, Sweden) at 60 s. At 300 s, salinomycin (2 µM) was added and the images were continuously taken until 700 s. The intensity of the Fluo-4 fluorescence was analysed using the Zeiss LSM 5 analysis software.

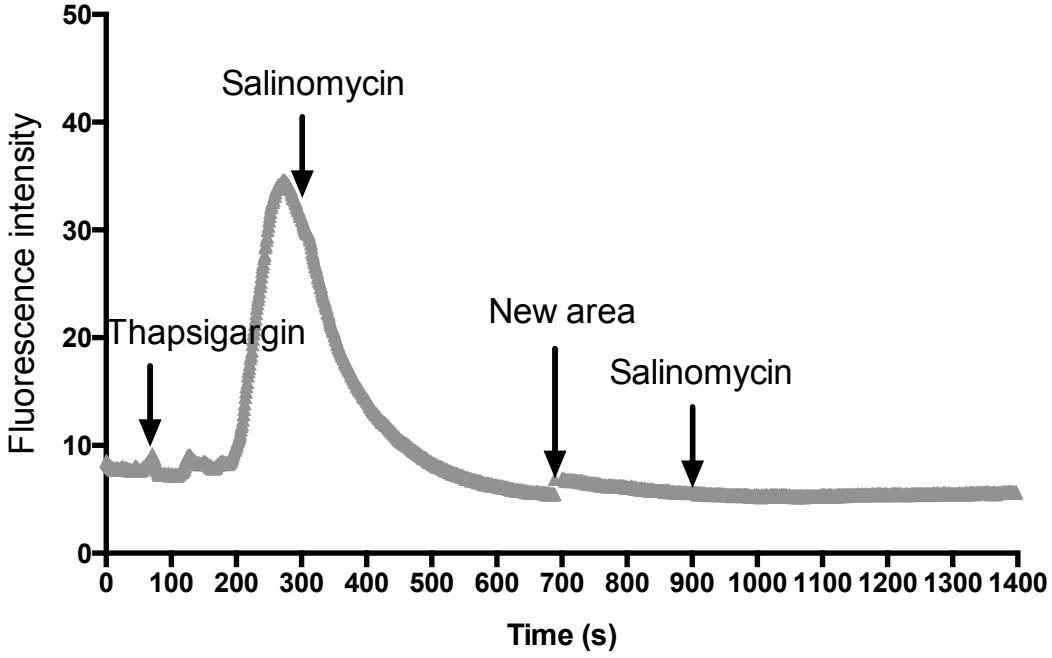


Figure S10. Salinomycin treatment failed to increase cytosolic Ca^{2+} after treatment with the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin. JIMT-1 cells were labelled with Fluo-4 AM and images were captured. At 60 s time point, 1 μM of thapsigargin (Sigma T9033) was added in Ca^{2+} free medium to empty the storage of Ca^{2+} in the ER. At 300 s time point, 2 μM of salinomycin was added to the Ca^{2+} free medium. After 700s, a new area was viewed for capturing of images for another 700 s. At 900 s time point, 2 μM of salinomycin was added again.

Intracellular calcium detection through flow cytometry. The JIMT-1 cells were seeded in Petri dishes (5 cm diameter) and after 24 h of incubation, salinomycin (0.5 μM or 50 nM) or carbonate **3** (50 nM) was added to the cells and the cells were further incubated for 72 h. The JIMT-1 cells were then loaded with 2 mL of a serum-free medium containing 1 μM Fluo-3 AM (Life Technologies, F14218) and 0.02% pluronic F-127 (Sigma-Aldrich, P2443) for 1 h at 37 °C. After loading, the cells were rinsed and left in FBS-free medium containing 1 mM of probenecid (Sigma-Aldrich, P8761) for 30 min at 37 °C to allow de-esterification of intracellular Fluo-3 AM. The cells were then washed with HBSS and Accutase containing 1 mM of probenecid was used to detach the cells (10 min incubation at 37 °C). Four mL cold HBSS supplemented with 1 mM of probenecid and 1% FBS was added to stop Accutase. The cells were kept on ice before analysis in a BD Accuri C6 flow cytometer. CFlow software was used to evaluate the data.

*IX. Enzyme activity, gene, and protein expression after treatment with salinomycin, carbonate **3**, and NBD conjugates **6** and **7**.*

PKC assay. JIMT-1 cells were seeded and after 24 h of incubation, salinomycin was added to a final concentration of 0.5 μM and the cells were further incubated for 72 h. Cells were lysed in Native lysis Buffer (Abcam) and protein concentrations were determined using the BCA protein assay reagent kit (PIERCE Biotechnology, 23227). The PKC kinase activity was analysed in 2 μg of cell lysate from control or treated cells according to the PKC Kinase Activity Assay Kit (Abcam) as described by the manufacturer.

RNA extraction and gene expression analysis. JIMT-1 cells were seeded and after 24 h of incubation, 50 nM salinomycin or carbonate **3** was added to the medium and the cells were incubated for another 72 h. The medium was removed and the cells were rinsed with PBS. Accutase (Sigma, Stockholm, Sweden) was used to detach the cells (10 min incubation at 37 °C). The cell number was determined using a hemocytometer. Less than 5000000 cells were transferred

to a new centrifuge tube and pelleted at 300*g* for 5 min. After completely aspirating the supernatant, 350 µl of Buffer RLT Plus (RNeasy Plus Mini Kit, 74134, Qaigen, Hilden, Germany) containing 1% β-mercaptoethanol was added to the cells, which were disrupted by pipetting. The samples were stored at -80 °C until further experiments. RNA extraction and gene expression profiling analyses were performed at SCIBLU Genomics, Lund University, Sweden. Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions. The RNA concentration was determined using a ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cDNA synthesis, labelling and subsequent hybridization to the HumanHT-12 v4 Expression BeadChip (Illumina Inc., San Diego, CA, USA) was performed according to the manufacturer's instructions. Microarray data were initially pre-processed and quantile normalized (8) using the GenomeStudio software V2011.1 (Illumina Inc., San Diego, CA, USA). Non-annotated probe sets and probe sets with signal intensities below the median of the negative control intensities in 80% of the samples that did not belong to one condition were excluded. To identify significantly differentially expressed genes between treatment groups, we performed SAM analysis (9) using the TMEV v4.0 software (10). Differentially expressed genes were selected having q-value ≤ 1 and an absolute fold change ≥ 2.0 for further analysis. Supervised Hierarchical Clustering Analysis (HCA) was performed using Pearson correlation, for distance matrix, and group average linkage method in TMEV. GO ontology enrichment analysis was performed using the AmiGO database (11) and Fisher's exact test method. For a list of the differentially expressed genes see Dataset S1.

RNA interference. JIMT-1 cells (75000 cells/cm^2) were seeded in Petri dishes and incubated for 24 h. The cells were then transiently transfected with small interfering RNAs (siRNA) targeting DDIT3 (Ambion, AM16708) and silencer negative control siRNA (Ambion, AM4611) at a final concentration of 20 pM, using Lipofectamine 2000 transfection reagent (Invitrogen, 11668-030) according to the manufacturer's instructions. After 4-6 h of transfection, the medium of the cells was changed and the fresh medium, containing 5 µM salinomycin or 5 µM DMSO, was added. After 72 h of treatment, the cells were harvested using Accutase for the evaluation of proteins by Western blot.

Western blot. After 72 h of treatment, cells were harvested using Accutase (Sigma) for 10 minutes at 37 °C, then counted in a hemocytometer, pelleted and stored at -80 °C until further use. Sample buffer (62.5 mM Tris-HCl, pH 6.8, 20 % glycerol, 2 % sodium dodecyl sulphate, 5 % β-mercaptoethanol, 1 % NP-40) was added to the pellets (15 µl per 100000 cells). The samples were sonicated 2 x 20 seconds, boiled for 7 minutes, and stored at -20 °C until further application. Mini-PROTEAN® TGX™ Precast Gels (4-20 % precast polyacrylamide gel, 10-well, 30 µl) (BIO-RAD Laboratories, Inc, USA, #4561093) were loaded with 20 µl of prepared sample per well. The gel electrophoresis was performed in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels system (Bio-Rad Laboratories, Inc, USA, #1658004) at 150 V for 5 minutes and 300 V for 15 minutes. Then the gels were blotted onto nitrocellulose membranes using a Trans-Blot Turbo transfer system (Bio-Rad). The membranes were blocked in PBS containing 5 % bovine serum albumin (BSA) (Sigma-Aldrich, Copenhagen, Denmark) and 0.1 % Tween 20 (Sigma-Aldrich) and then incubated with primary antibodies against CHOP (1:1000), eIF2α (1:1000), p-eIF2α (1:1000), GRP78 (1:1000), PERK (1:1000), IRE1α (1:1000), ATF6□ (1:1000), active β-catenin (1:1000), total β-catenin (1:2000), LRP6 (1:1000), p-LRP6 (1:1000), β-actin (1:5000), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000). All antibodies were diluted in PBS containing 0.1 % Tween 20. The antibodies against CHOP (5554), p-eIF2α (3597), eIF2α (9722), LRP6 (3395), and phospho-LRP6 (Ser1490) (2568) were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies against GRP78 (ab21685), ATF6□ (1-7) (ab122897), β-actin (ab6276), and GAPDH (ab9484) were obtained from Abcam (Cambridge, UK). The antibody against IRE1α (pSer724) peptide (NB100-2323) was bought from Novus Biological (Littleton, CO, USA). The antibodies against active β-catenin (#05-665) and total β-catenin (610154) were purchased from Millipore and BD Transduction Labotatories, respectively. The antibody against p-PERK (sc-32577) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) whereas the antibody against secondary horseradish peroxidase (HRP)-conjugated swine anti-rabbit and HRP-conjugated goat anti-mouse immunoglobulin were obtained from Dako (Glostrup, Denmark). After incubation with HRP-conjugated secondary antibody, the membranes were exposed to enhanced chemiluminescence solution (GE Healthcare, Buckinghamshire, UK) to detect the protein bands using the ChemiDoc XRS system (Bio-Rad Inc., Hercules, CA, USA). Data were collected and analyzed using the Quantity One software (Bio-Rad, Hercules, California, USA).

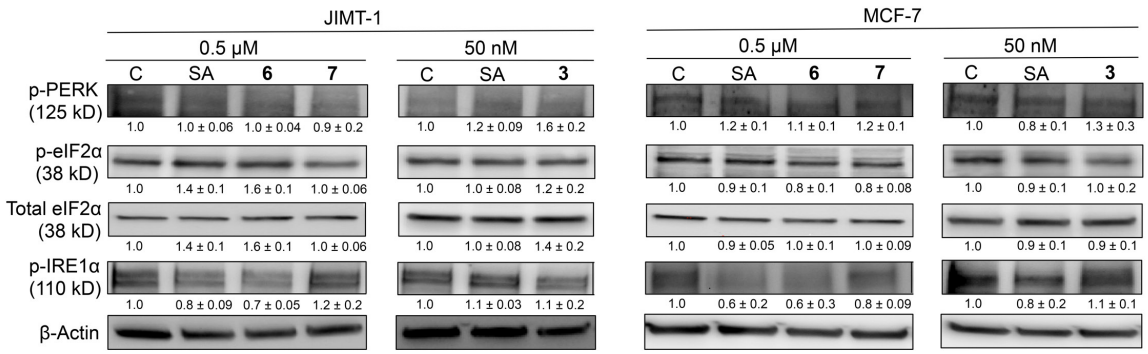


Figure S11. Salinomycin or analog treatment did not activate PERK and IRE1 pathways in JIMT-1 and MCF-7 cells. The cells were treated at the indicated concentrations for 72 h. Representative Western blots (n = 4) used to for densitometric scanning to show the expression of UPR-related proteins. SA, salinomycin; **3**, salinomycin 20-ethyl carbonate; **6**, salinomycin 20-NBD carbonate; **7**, salinomycin 20-NBD carbonate C1-methyl ester.

X. Statistical analysis of biological data.

All data were presented as the mean ± standard error (SE) from at least three experiments. Student's *t*-test was applied to evaluate the difference in two groups. Data from multiple groups were analyzed by one-way ANOVA, followed by Dunnet multiple comparison test. The 95% confidence interval in the multiple comparisons was used. The asterisks indicate statistical significance. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

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