Supplemental Information

FFAR1/GPR40 targeting fluorescent probe for beta cell imaging

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Supplemental figures and experimental procedures

Supplementary Figure S1 related to Figure 3: Probe **14**, **15** and **16** with negatively charged fluorophores show no unspecific binding on hFFAR1-HEK293 overexpressing cells.

Supplementary Figure S2 related to Figure 4: Probe **16** binding to hFFAR1 is concentration dependent.

Supplementary Figure S3: Cytoplasmic mean fluorescence of probe **16** (confocal images).

Supplementary Figure S4: Internalization of Probe **16** upon hFFAR1 binding

Supplementary Figure S5 rel. to Figure 6: Cytoplasmic mean fluorescence of probe **16** in picture A-D.

Supplementary Figure S6: Cytoplasmic mean fluorescence of INS1E cells after incubation with **16**

Supplementary Figure S7: FACS-based quantification of probe **16**-fluorescence of GPR40-HEK293 and Min6 cells

Supplementary Figure S8: Summary of GPR40-HEK293 and Min6 cells mean fluorescence

Supplementary Figure S9: Staining of dispersed mouse islets

Supplementary Figure S10: Representative calcium traces for TAK 875 and probe **16**

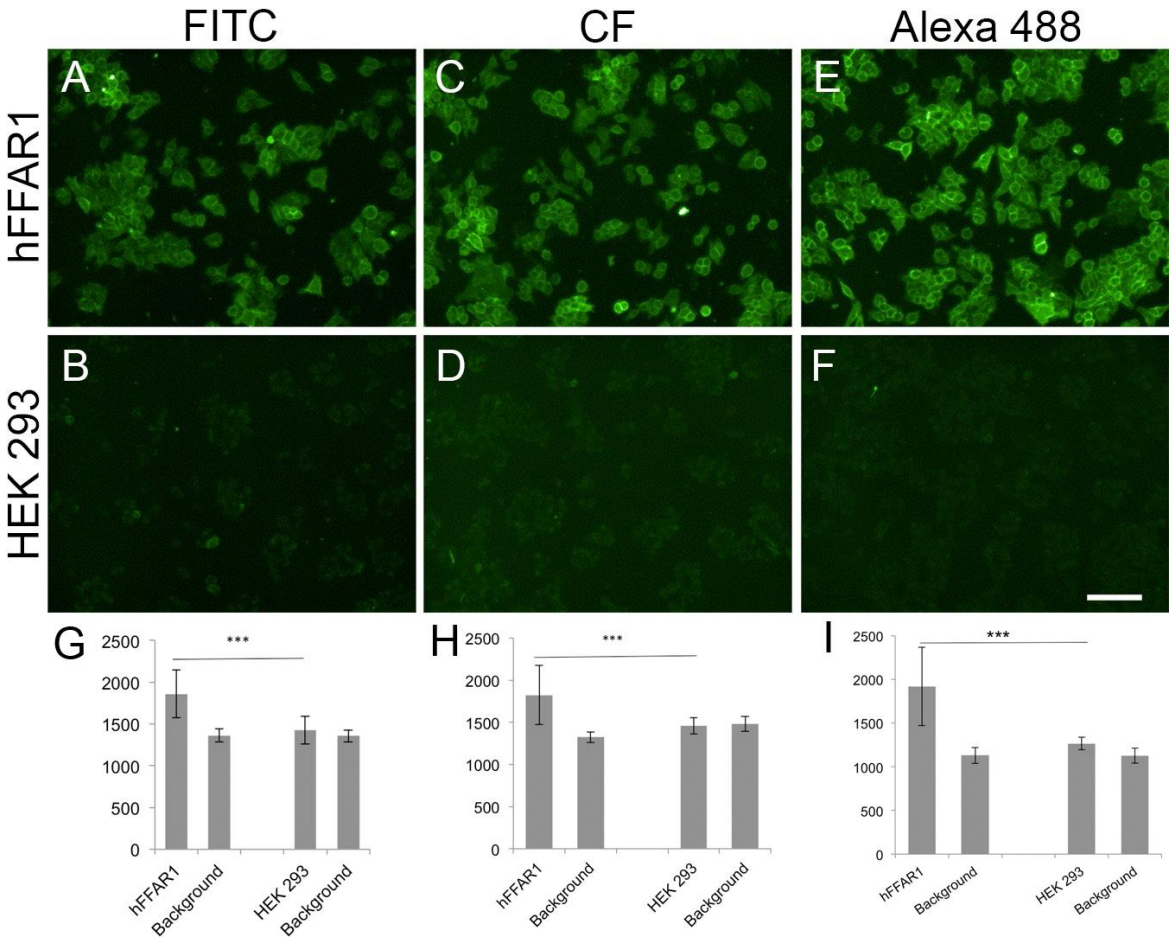
Chemical synthesis of molecules presented in Figure 1

Chemical synthesis of the fluorescent probes 13-19 presented in Figure 2

Sequence Homology of mouse, rat and human FFAR1

Supplemental Figures

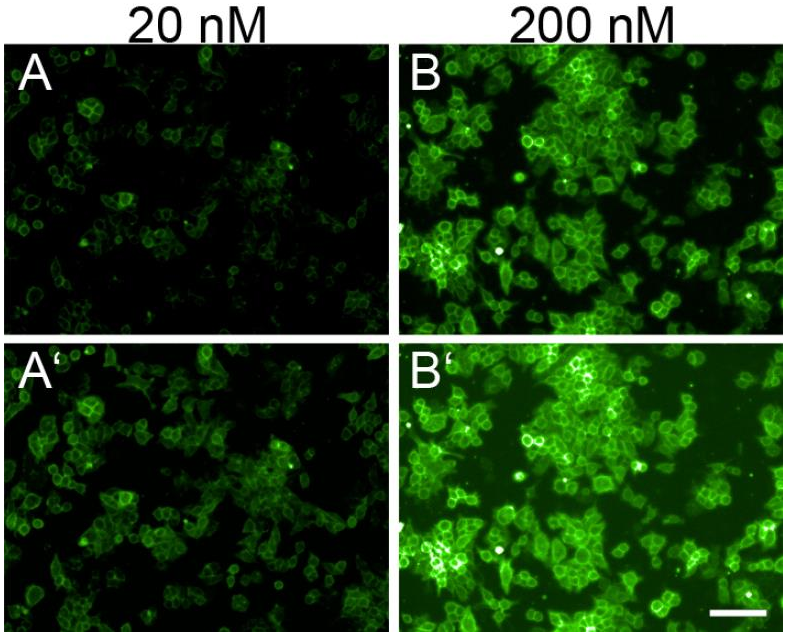
Supplementary Figure S1 related to Figure 3



Probe **14**, **15** and **16** with negatively charged fluorophores show low unspecific binding on hFFAR1-HEK293 overexpressing cells.

hFFAR1-HEK293 overexpressing cells incubated with probe **15** (FITC), **14** (CF) and **16** (Alexa 488) showed a clear signal on the membrane (A,C,E), while HEK293 cells expressing no FFAR1 incubated with the same probes show no fluorescence (B,D,F). Scale bar is 100 µm. (G-I) show the quantification of A-F in mean fluorescence per cell. Student's Test, *P<0.05, **P<0.01, ***P<0.001; error bars indicate SD.

Supplementary Figure S3 related to Figure 4

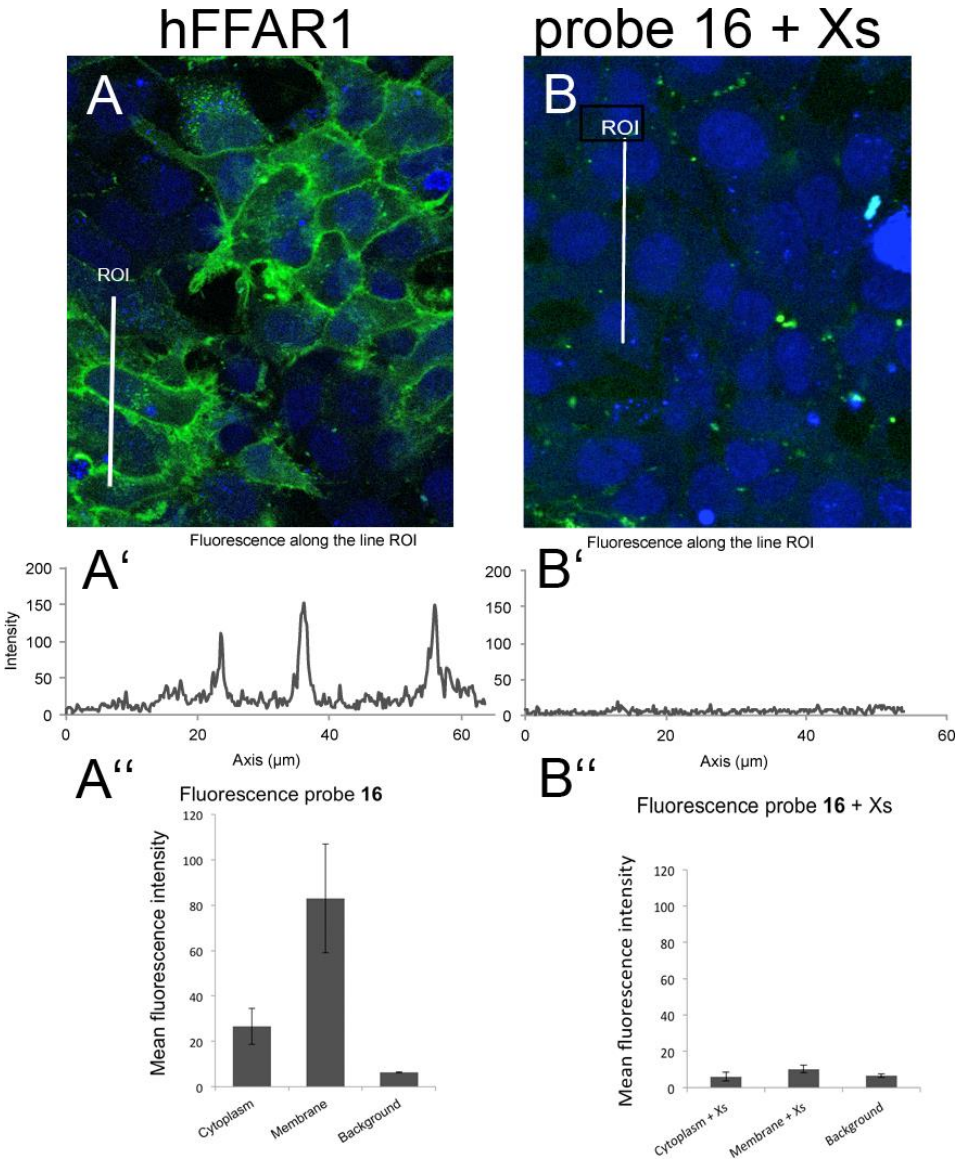


Probe **16** binding to hFFAR1 is concentration dependent.

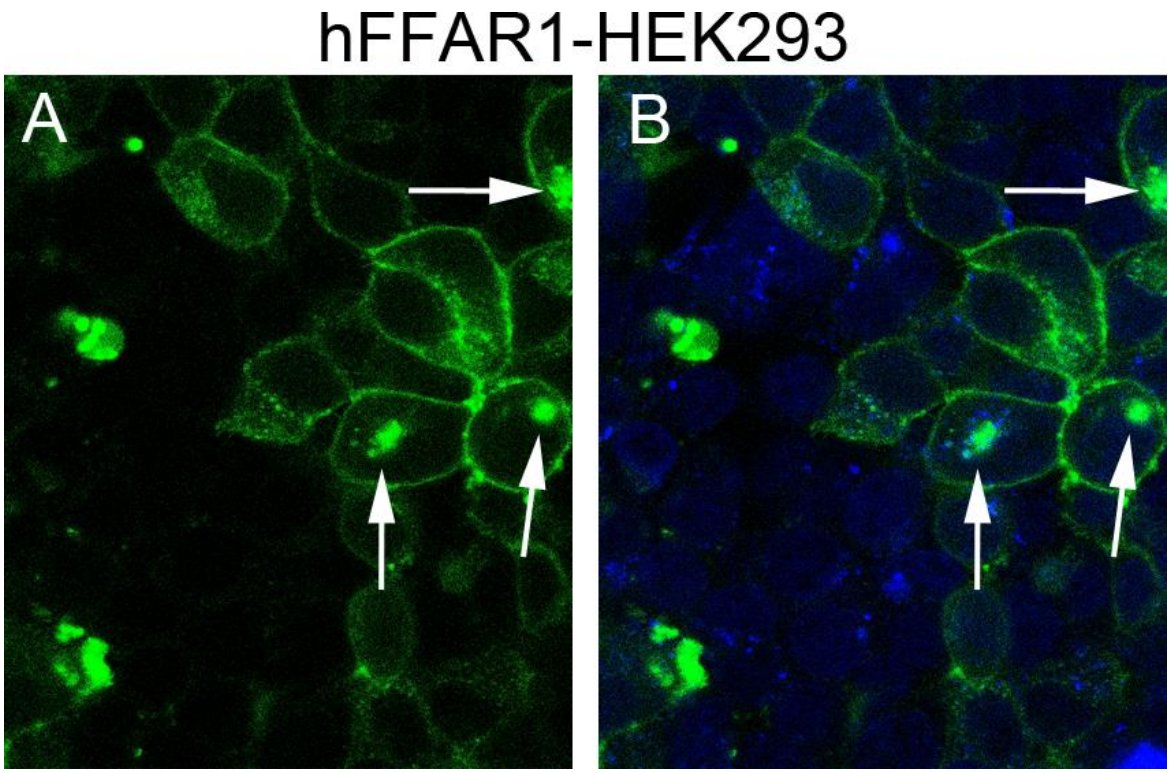
hFFAR1-HEK293 overexpressing cells incubated with probe **16** at a concentration of 200 nM (B, B') showed a more intense fluorescent signal than the same probe at a concentration of 20 nM (A, A'). A and A' show the same image with different intensities to allow improved optical comparison, the intensity was adjusted for optimal signal intensity at 200 nM in the top lane (A and B), while it was adjusted to optimal signal intensity for cells treated with 20 nM in the lower lane (A' and B'). Scale bar is 100 µm.

Supplementary Figure S3: Probe 16 specifically binds the hFFAR1 – confocal microscopy

Confocal microscopy high-resolution pictures of hFFAR1-HEK293 overexpressing cells labeled with probe **16** at a concentration of 2 µM (A) or cells treated with a tenfold excess of unlabeled TAK-875 (Xs) prior to incubation with probe **16** at a concentration of 2 µM (B). Fluorescence along an exemplary linear region of interest (ROI) shows a clear membrane labeling on cells (A'). This effect was no longer visible in the blocking experiment (B). Quantification of fluorescence for cells treated with probe **16** (A'') or cells treated with probe **16** and a tenfold excess of unlabeled TAK-875 (B'') (complete analysis). Green: Alexa488. Blue: DRAQ5 (nuclei). Error bars indicate SD.

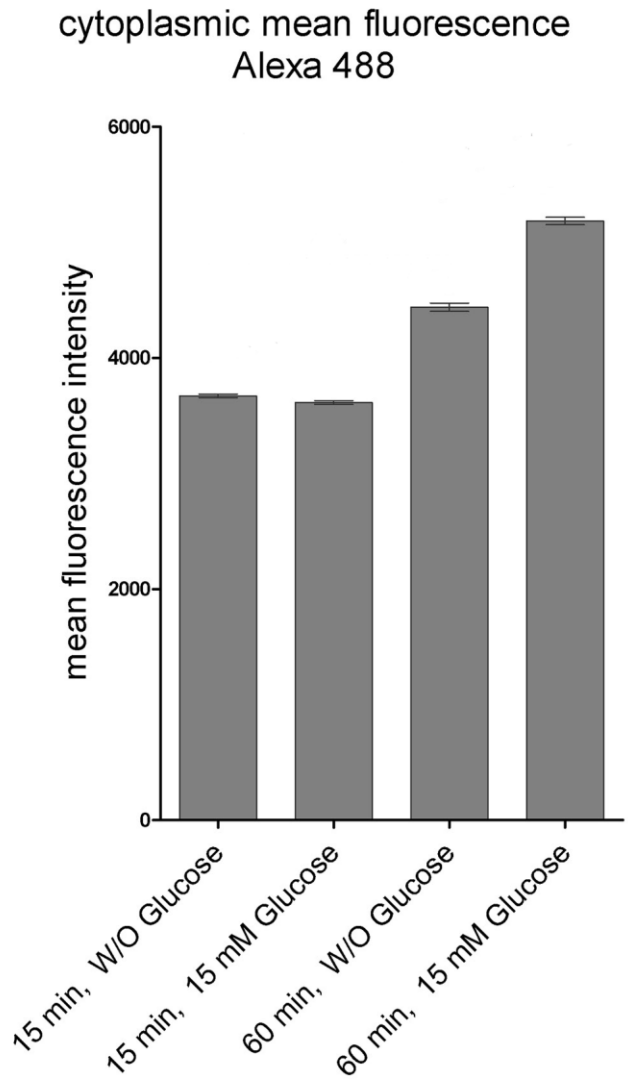


Supplementary Figure S4: Internalization of Probe 16 upon hFFAR1 binding



Confocal microscopy high-resolution pictures of hFFAR1-HEK293 overexpressing cells labeled with probe **16** at a concentration of 2 µM show internalization of probe 16 in vesicles clustered in the perinuclear region indicated by white arrows. (A) Green: Alexa488. (B) Merged Green: Alexa488 and Blue: DRAQ5 (nuclei).

Supplementary Figure S5 related to Figure 6

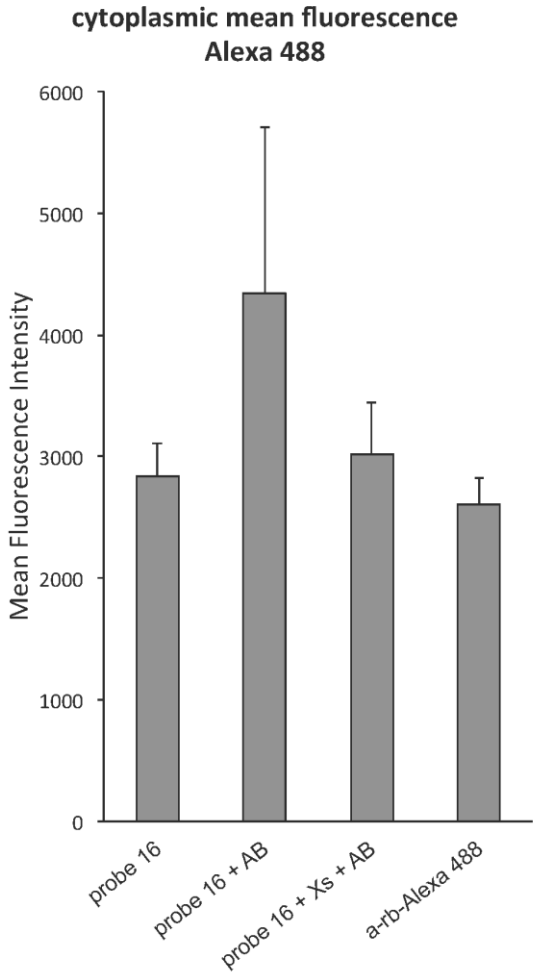


Measurements of cytoplasmic mean fluorescence of probe **16** in picture A-D of Figure 6.

The mean intensity of Alexa488 was measured in all MIN6 cells. This analysis showed the increase in fluorescence with longer incubation time but only after 60 min incubation a glucose-dependent increase in fluorescence can be shown. Error bars indicate s.e.m.

Supplementary Figure S6:

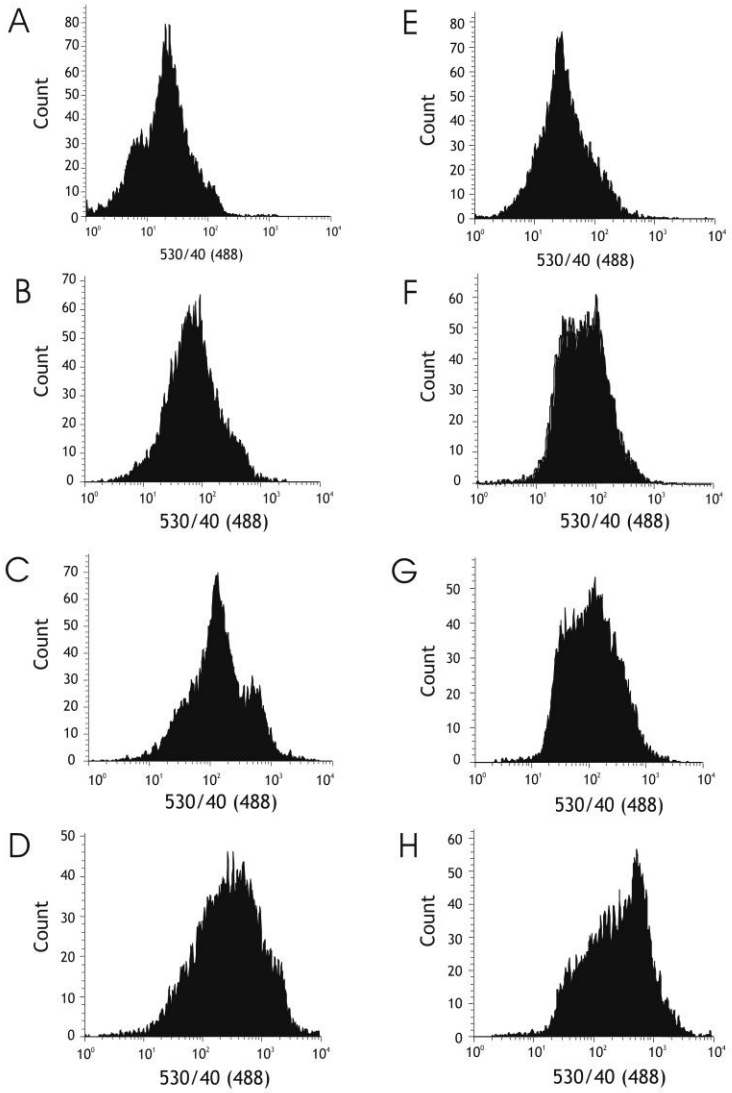
Cytoplasmic mean fluorescence of INS1E cells after incubation with probe 16



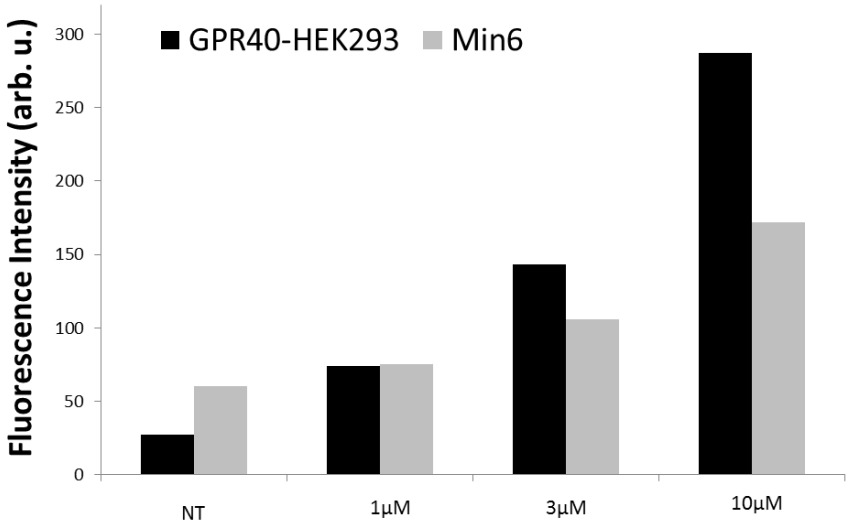
Measurements of cytoplasmic mean fluorescence of probe **16** of Ins1E cells analogous to Figure 6 for MIN6. The mean intensity of Alexa488 was measured in all INS1E cells; error bars indicate SD.

Supplementary Figure S7: FACS-based quantification of probe 16-fluorescence of GPR40-HEK293 and Min6 cells

The cells were stained with probe **16** at different concentrations: A,E: no treatment, B,F: 1µM, C,G: 3µM, and D,H: 10µM of probe **16**. To distinguish non-viable cells or debris from viable cells, cells have been additionally incubated with propidium iodide at 1µM and have been excluded form the analysis by appropriate gating. Mean of fluorescence increased upon increasing concentration of probe **16**. GPR-HEK293 overexpressing cells showed stronger fluorescence intensity in comparison to Min6.

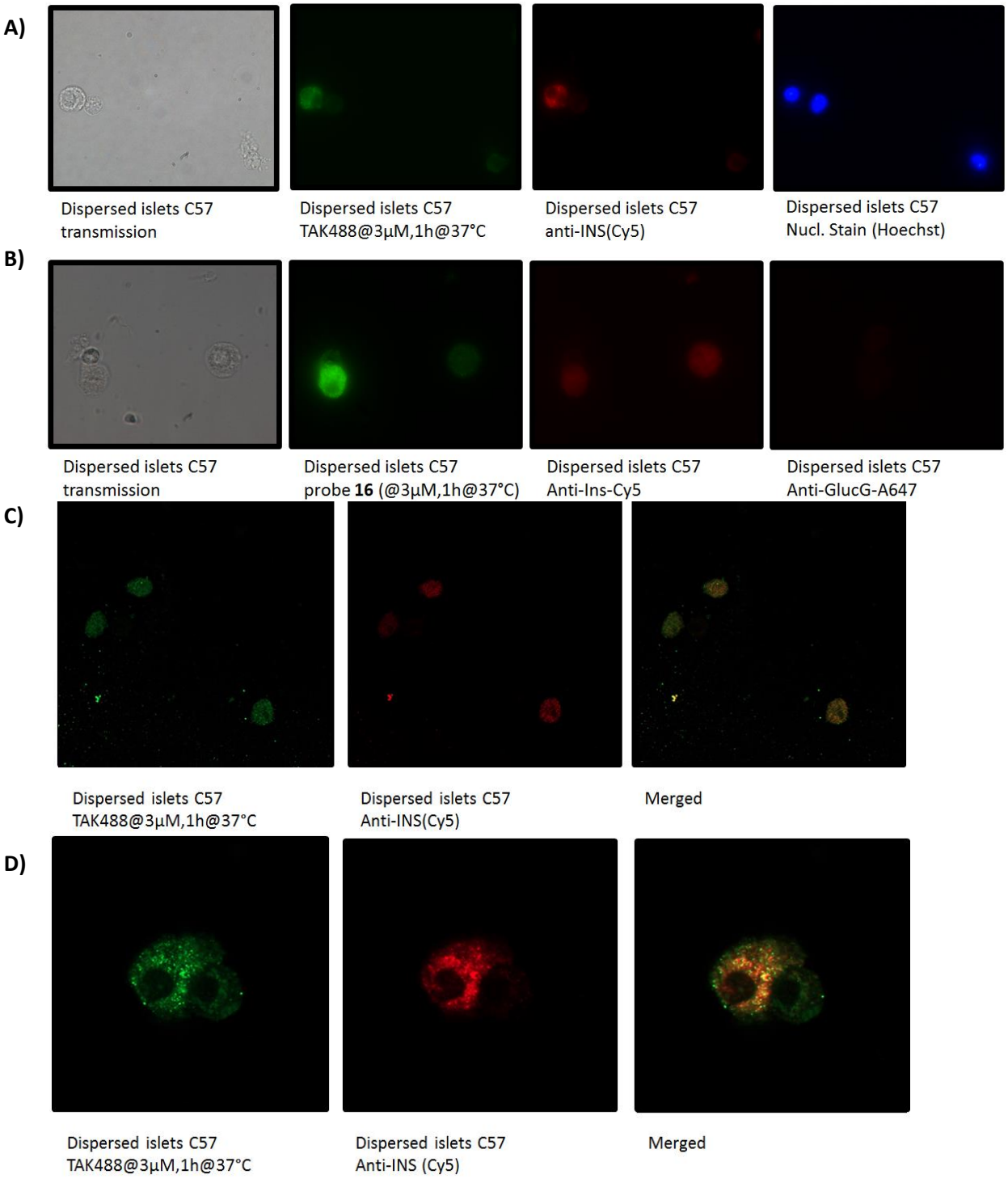


Supplementary Figure S8: Summary of GPR40-HEK293 and Min6 cells mean fluorescence



FACS analysis of GPR40-HEK293 (A-D) and Min6 (E-H) cells using probe **16** at different concentrations (A,E: no treatment, B,F: 1µM, C,G: 3µM, and D,H: 10µM of probe **16**. A dose dependent increase of fluorescence has been observed for both cell lines.

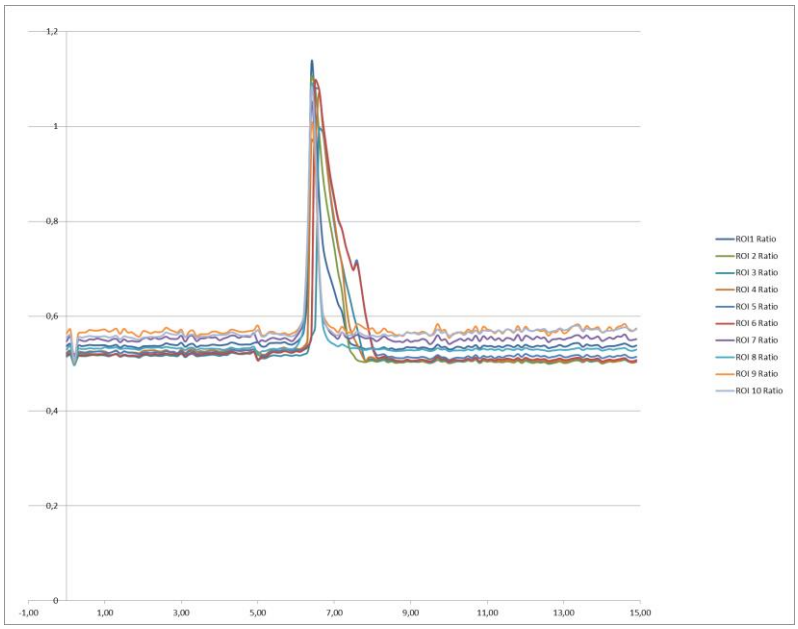
Supplementary Figure S9: Staining of dispersed mouse islets



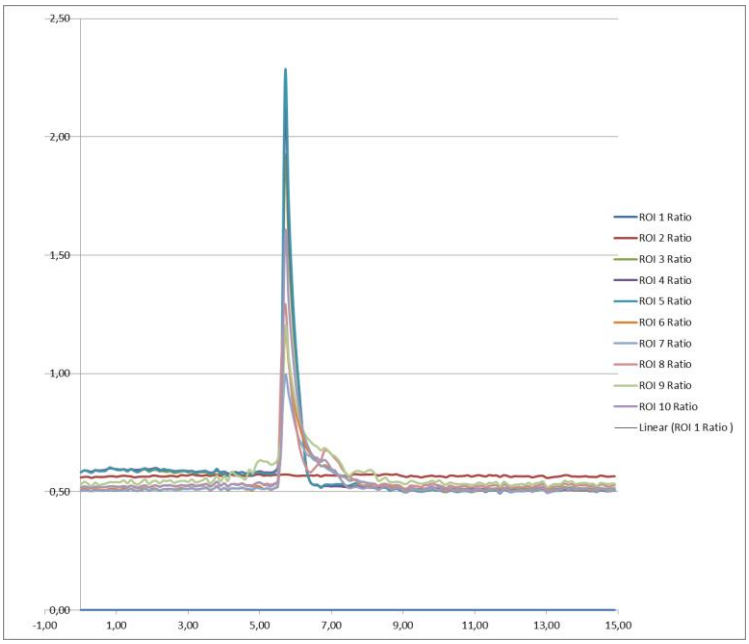
Dispersed islets of C57 mice, stained with anti-INS (labelled with Cy5) and compound **16** (Alexa488) (A: with DAPI control for detection of nuclei, B with glucagon staining as control against alpha cells), C and D confocal images with merged staining of insulin and **16**

Supplementary Figure S10: Representative calcium traces for TAK 875 and probe 6

A) Probe **16** (1µM) (ratio 340 nm/380) = 1.07 ± 0.01)



B) TAK 875 (10 nM) (ratio 340 nm/380) = 1.45 ± 0.14)



Supplemental experimental procedures

General methods

Unless otherwise noted, all reagents were purchased from commercial suppliers (Sigma Aldrich, Fisher) and used without further purification. Alexa 488 and Bodipy 650 were purchased from Life Technologies as NHS ester, Fluorescein isothiocyanate (FITC), carboxyfluorescein (CF) NHS ester, carboxytetramethylrhodamine (TAMRA) NHS ester and Coumarin 343 were purchased from Sigma Aldrich, Cy5.5 NHS ester was purchased from InterChim. Boc-NH-PEG_{63}-NHS ester was purchased from Rapp Polymer GmbH. N_3-PEG_4-NHS ester and N_3-PEG_8-NHS ester were purchased from Iris Biotech GmbH. All solvents used were of HPLC grade. Microwave assisted reactions were performed with a Biotage Initiator device. Reactions were monitored by LC-MS or by thin-layer chromatography on Merck 50x100 mm silica gel 60 aluminum sheets with fluorescent indicator. LC-MS data were acquired using the HP-Agilent 1100 MSD system with a Phenomenex Luna column Luna (C-18, 100 Å pore size, 3 µm particle size, 10x2.0 mm) (METHOD 1: 0 min - 93%H_2O (0.05%TFA) to 1.2min - 95%ACN; 95%ACN until 1.4min; 7%ACN 1.50min; flow: 1 1ml/min; MS: 110-1000MW. METHOD 2: 0 min 80%H_2O (0.05%TFA) to 0.8min-95%ACN; 95%ACN until 1.4min; 20%ACN 1.45min; flow: 1.1ml/min; MS: 110-1000MW). Final fluorescent probes were analyzed with a LC-MS Agilent Technologies 1200 Series system equipped with a Waters Xbridge (C-18, 130 Å pore size, 2.5 µm particle size, 50x2.1 mm) (METHOD 3: 0 min 85%H2O(0.05%TFA) to 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; flow: 0.6 ml/min; MS: 400-1500MW). Silica-gel column chromatography was carried out on a CombiFlash Rf - Isco Teledyne. Reverse-phase preparative HPLC was performed on the HP-Agilent 1100 with either a column from Agilent Zorbax Rx C-18 250x9.4mm, 5 µm (4 mL/min) or with a column from Waters Xbridge OBD C-18-250x19mm, 5 µm (16 mL/min) (Gradient: 0 min – 5 min 25% ACN/H_2O(0.1%TFA) to 30 min - 95%ACN; 95%ACN until 35 min). ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 or 600 systems in d_6-DMSO or $CDCl_3$. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard.

Abbreviations are used as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, dd = doublets of doublet, br = broad. Coupling constants (*J* values) are given in hertz (Hz). Absorption and Emission spectra were acquired with a Thermo Varioskan using the SkanIt 2.4.3 software.

Abbreviations

ACN = acetonitrile

ADDP = 1,1'-(azodicarbonyl)dipiperidine

Boc = *tert*-butyloxycarbonyl

DCM = dichloromethane

DIEA = diisopropylethyl amine

DMF = dimethylformamide

ESI-TOF = electrospray ionization mass spectrometry – time of flight

EtOAc = ethylacetate

HATU = 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

Hept = heptane

HPLC = high performance liquid chromatography

HRMS = high resolution mass spectrometry

LC-MS = liquid chromatography - mass spectrometry

MeOH = methanol

NMR = nuclear magnetic resonance

TBAF = Tetra-*n*-butylammonium fluoride

TFA = trifluoroacetic acid

THF = tetrahydrofuran

TIS = triisopropylsilane

TLC = thin layer chromatography

TBDMS-Cl = tert-butyldimethylsilyl chloride

Supplemental experimental procedure Figure 1 – molecules measured by FLIPR Ca2+ assay

Synthesis of compound **1**

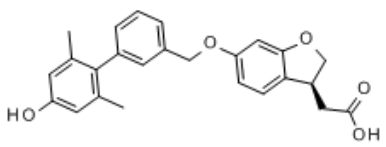
Compound **12** methyl 2-[(3S)-6-[[3-(4-hydroxy-2,6-dimethyl-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate was dissolved in 80 µL of THF and 40 µL of MeOH before 80 µL of 1N aqueous NaOH (800 µmol) were added. The mixture was allowed to stir at 50°C for 45 minutes. The mixture was then concentrated, diluted with water, acidified with 1M HCl aqueous solution and extracted with EtOAc. The organic layer was washed with brine and evaporated under reduced pressure. The crude was purified via silica gel chromatography (0-65% EtOAc/Hept) to afford desired product **1** 2-[(3S)-6-[[3-(4-hydroxy-2,6-dimethyl-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as a white powder (m = 44 mg, 109 µmol, yield 73%).

^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.94 (s, 6H), 2.61 (dd, 1H, J = 16.6, 9.0 Hz), 2.82 (dd, 1H, J = 16.6, 5.6 Hz), 3.79 (m, 1H, 9.1, 9.0, 6.4, 5.6 Hz), 4.28 (dd, 1H, J = 9.1, 6.4 Hz), 4.74 (t, 1H, J = 9.1 Hz), 5.05 (s, 2H), 6.45 (s, 1H), 6.48 (d, 1H, J_{ortho} = 8.0 Hz), 6.58 (s, 2 H), 7.01 (d, 1H, J_{ortho} = 8.0 Hz), 7.06 (d, 1H, J_{ortho} = 7.5 Hz), 7.15 (s, 1H), 7.38 (d, 1H, J_{ortho} = 7.5 Hz), 7.42 (t, 1H, J_{ortho} = 7.5 Hz).

^{13}C NMR (150 MHz, $CDCl_3$) δ: 20.9, 37.5, 39.1, 70.3, 97.5, 107.4, 114.2, 121.2, 124.3, 125.5, 128.6, 128.7, 129.3, 134.4, 137.1, 137.7, 141.0, 154.2, 160.4, 161.1, 175.9

Retention time R_t = 0.93 min (METHOD 1)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{25}H_{23}O_5$: 403.1551; Found: 403.1586



Synthesis of compound **2** methyl ester

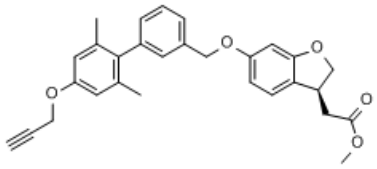
Compound **12** methyl 2-[(3S)-6-[[3-(4-hydroxy-2,6-dimethyl-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate (250 mg, 597 µmol), propargyl bromide - 80% in toluene (650 µL, 5.97 mmol), potassium carbonate (115 mg, 836 µmol) and potassium iodide (19.8 mg, 0.2 eq) were dissolved in 10 mL of DMF, and the mixture was allowed to stir under argon at 60°C for 24 hours. The volatiles were removed under reduced pressure and the crude was purified through silica gel chromatography (0-20% EtOAc-Hept) to afford methyl 2-[(3S)-6-[[3-(2,6-dimethyl-4-prop-2-ynoxy-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate as a colorless oil (m = 241 mg, 528 µmol, yield 88%).

^{1}H NMR (600 MHz, $CDCl_3$) δ: 2.01 (s, 6H), 2.55 (t, 1H, J = 2,0 Hz), 2.57 (dd, 1H, J = 16.6,9.2 Hz), 2.74 (dd, 1H, J = 16.6, 5.4 Hz), 3.73 (s, 3H), 3.81 (m, 1H, 9.2, 9.1, 6.2, 5.4 Hz), 4.27 (dd, 1H, J = 9.1, 6.2 Hz), 4.71 (d, 2H, J = 2.0 Hz), 4.76 (t, 1H, J = 9.1 Hz), 5.07 (s, 2H), 6.47 (d, 1H, J_{meta} = 2.0 Hz), 6.48 (dd, 1H, J_{ortho} = 8.2 Hz, J_{meta} = 2.2 Hz), 6.74 (s, 2 H), 7.02 (d, 1H, J_{ortho} = 8.2 Hz), 7.08 (d, 1H, J_{ortho} = 7.5 Hz), 7.18 (s, 1H), 7.38 (d, 1H, J_{ortho} = 7.5 Hz), 7.43 (t, 1H, J_{ortho} = 7.5 Hz).

^{13}C NMR (150 MHz, $CDCl_3$) δ: 21.2, 37.8, 39.5, 51.8, 55.7, 70.3, 75.4, 78.8, 97.5, 107.3, 113.5, 121.5, 124.3, 125.6, 128.58, 128.65, 129.1, 135.1, 137.1, 137.6, 140.9, 156.3, 159.9, 161.1, 172.3

Retention time R_t = 0.90 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{29}H_{27}O_5$: 455.1864; Found: 455.1918



Synthesis of compound **2**

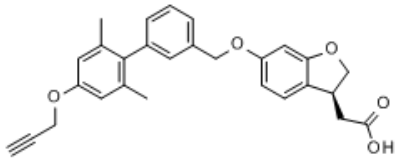
Methyl 2-[(3S)-6-[[3-(2,6-dimethyl-4-prop-2-ynoxy-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate (220 mg, 472 µmol) synthesized previously was dissolved in 1.9 mL of THF and 950 µL of MeOH before 965 µL of 1N aqueous NaOH (965 µmol) were added. The mixture was allowed to stir at room temperature for 30 minutes. The mixture was concentrated, diluted with water, acidified with 1M HCl aqueous solution and extracted with EtOAc. The organic layer was washed with brine and evaporated under reduced pressure to afford compound **2** 2-[(3S)-6-[[3-(2,6-dimethyl-4-prop-2-ynoxy-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as a white powder (m = 201 mg, 454 µmol, yield 96%).

^{1}H NMR (600 MHz, $CDCl_3$) δ: 2.02 (s, 6H), 2.55 (t, 1H, J = 2,4 Hz), 2.63 (dd, 1H, J = 16.8, 9.2 Hz), 2.82 (dd, 1H, J = 16.8, 5.4 Hz), 3.83 (m, 1H, 9.2, 9.1, 6.2, 5.4 Hz), 4.30 (dd, 1H, J = 9.1, 6.2 Hz), 4.72 (d, 2H, J = 2.4 Hz), 4.78 (t, 1H, J = 9.1 Hz), 5.08 (s, 2H), 6.48 (d, 1H, J_{meta} = 2.2 Hz), 6.52 (dd, 1H, J_{ortho} = 8.2 Hz, J_{meta} = 2.2 Hz), 6.75 (s, 2 H), 7.06 (d, 1H, J_{ortho} = 8.2 Hz), 7.10 (d, 1H, J_{ortho} = 7.5 Hz), 7.19 (s, 1H), 7.39 (d, 1H, J_{ortho} = 7.5 Hz), 7.42 (t, 1H, J_{ortho} = 7.5 Hz).

^{13}C NMR (150 MHz, $CDCl_3$) δ: 20.6, 37.1, 38.7, 55.2, 69.9, 74.8, 78.4, 97.1, 107.0, 113.1, 120.7, 123.8, 125.1, 128.08, 128.14, 128.7, 134.6, 136.6, 137.0, 140.5, 155.9, 159.6, 160.6, 176.1

Retention time R_t = 0.83 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{28}H_{25}O_5$: 441.1707; Found: 441.1738



Synthesis of compound **3** methyl ester

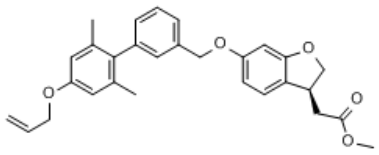
Compound **12** methyl 2-[(3S)-6-[[3-(4-hydroxy-2,6-dimethyl-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate (762 mg, 1.82 mmol) and cesium carbonate (705 mg, 2.16 mmol) were dissolved in 9 mL of ACN under argon. Allyl bromide (740 µL, 8.42 mmol) was added to the solution and the mixture was allowed to stir under microwave heating (40 min, 110°C microwave assisted, 30 sec. pre-stirring). Insolubles were removed by filtration and the mixture was evaporated under reduced pressure. The crude was then diluted in Et_2O and filtered again to remove the last traces of cesium carbonate and evaporated to afford methyl 2-[(3S)-6-[[3-(4-allyloxy-2,6-dimethyl-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate as a yellowish oil (m = 765 mg, 1.67 mmol, yield 92 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.99 (s, 6H), 2.55 (dd, 1H, J = 16.6, 9.0 Hz), 2.75 (dd, 1H, J = 16.6, 5.6 Hz), 3.71 (s, 3H), 3.79 (m, 1H, 9.4, 9.0, 6.1, 5.6 Hz), 4.25 (dd, 1H, J = 9.4, 6.1 Hz), 4.54 (dd, 2H, J = 5.2 Hz), 4.74 (t, 1H, J = 9.0 Hz), 5.05 (s, 2H), 5.28 (dd, 1H, J_{cis} = 10.4 Hz and J_{gem} = 1.4 Hz), 5.41 (dd, 1H, J_{trans} = 17.3 Hz and j_{gem} = 1.4 Hz), 6.07 (ddd, 1H, J_{trans} = 17.3, J_{cis} = 10.4, J = 5.2 Hz), 6.46 (d, 1H, J_{meta} = 2.2 Hz), 6.49 (dd, 1H, J_{ortho} = 8.2 Hz, J_{meta} = 2.2 Hz), 6.67 (s, 2 H), 7.02 (d, 1H, J_{ortho} = 8.2 Hz), 7.09 (d, 1H, J_{ortho} = 7.4 Hz), 7.17 (s, 1H), 7.38 (d, 1H, J_{ortho} = 7.7 Hz), 7.41 (t, 1H, J_{ortho} = 7.4 Hz).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.1, 37.8, 39.5, 51.8, 68.7, 70.3, 97.5, 107.3, 113.4, 117.5, 121.5, 124.3, 125.5, 128.6, 128.7, 129.2, 133.6, 134.4, 137.1, 137.4, 141.1, 157.4, 159.4, 161.1, 172.4

Retention time R_t = 0.99 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{29}H_{29}O_5$: 457.2020; Found: 457.2056



Synthesis of compound **3**

Methyl 2-[(3S)-6-[[3-(4-allyloxy-2,6-dimethyl-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate previously synthesized (15 mg, 32.7 µmol, 1eq) was dissolved in 250 µL of THF and 100 µL of MeOH before 65 µL of 1N aqueous NaOH (65 µmol) were added. The mixture was allowed to stir at room temperature for 45 minutes.

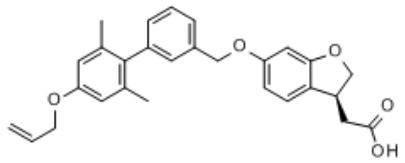
The mixture was concentrated, diluted with water, acidified with 1M HCl aqueous solution down to pH = 2 and extracted with EtOAc three times. The organic layer was evaporated under reduced pressure to afford compound **3** 2-[(3S)-6-[[3-(4-allyloxy-2,6-dimethyl-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as a white powder (m = 11.8 mg, 26.5 µmol, yield 81%).

^{1}H NMR (600 MHz, $CDCl_3$) δ: 2.01 (s, 6H), 2.63 (dd, 1H, J = 16.8, 9.2 Hz), 2.82 (dd, 1H, J = 16.8, 5.3 Hz), 3.83 (m, 1H, 9.2, 9.0, 6.2, 5.3 Hz), 4.30 (dd, 1H, J = 9.0, 6.2 Hz), 4.56 (d, 2H, J = 5.2 Hz), 4.78 (t, 1H, J = 9.0 Hz), 5.08 (s, 2H), 5.30 (dd, 1H, J_{cis} = 10.4 Hz and J_{gem} = 1.0 Hz), 5.44 (dd, 1H, J_{trans} = 17.3 Hz and j_{gem} = 1.0 Hz), 6.10 (ddd, 1H, J_{trans} = 17.3, J_{cis} = 10.4, J = 5.2 Hz), 6.48 (s, 1H), 6.52 (dd, 1H, J_{ortho} = 8.2 Hz), 6.70 (s, 2 H), 7.06 (d, 1H, J_{ortho} = 8.2 Hz), 7.10 (d, 1H, J_{ortho} = 7.4 Hz), 7.19 (s, 1H), 7.39 (d, 1H, J_{ortho} = 7.4 Hz), 7.43 (t, 1H, J_{ortho} = 7.4 Hz).

^{13}C NMR (150 MHz, $CDCl_3$) δ: 20.6, 37.1, 38.6, 68.3, 69.9, 97.1, 107.0, 113.0, 116.9, 120.7, 123.8, 125.0, 128.1, 128.2, 128.7, 133.1, 133.9, 136.6, 136.9, 140.7, 156.9, 159.6, 160.6, 175.5

Retention time R_t = 0.87 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{28}H_{27}O_5$: 443.1863; Found: 443.1935



Synthesis of compound **4**

See synthesis below in section "Supplemental experimental procedure Figure 2"

Synthesis of compound **5**

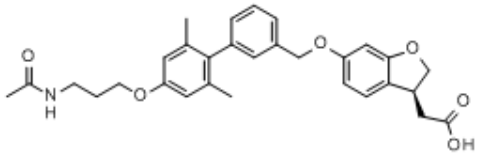
Compound **4** 2-[(3S)-6-[[3-[4-(3-aminopropoxy)-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid (2.9 mg, 6.28 µmol) was dissolved in 250 µL of DMF, before DIEA (3 µl, 17.18 µmol) and acetic anhydride (0.650 µl, 6.92 µmol) were added to the reaction mixture. The reaction was allowed to stir at room temperature for 15 minutes. Then 50 µL of a 1M NaOH aqueous solution (50 µl, 50.00 µmol) were added to the mixture. The crude was directly submitted to preparative HPLC (25% to 95% ACN/water) to afford, after lyophilization, compound **5** 2-[(3S)-6-[[3-[4-(3-acetamidopropoxy)-2,6-dimethyl-phenyl]phenyl] methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as a lyophilized powder (m = 3.1 mg, 6.16 µmol, yield 98%).

^{1}H NMR (500 MHz, d_6-DMSO) δ: 1.81 (s, 3H), 1.84 (p, 2H, J = 6.2 Hz), 1.92 (s, 6H), 2,47 (dd, 1H, J = 16.8, 9.0 Hz), 2,68 (dd, 1H, J = 16.8, 5.6 Hz), 3.19 (q, 2H, J = 6.2 Hz), 3.68 (m, 1H), 3.98 (t, 2H, J = 6.2 Hz), 4.19 (dd, 1H, J = 9.0, 6.9 Hz), 4.68 (t, 1H, J = 9.0 Hz), 5.10 (s, 2H), 6.46 (s, 1H), 6.49 (d, 1H, J_{ortho} = 8.1 Hz), 6.69 (s, 2 H), 7.05 (d, 1H, J_{ortho} = 7.5 Hz), 7.09 (d, 1H, J_{ortho} = 8.1 Hz), 7.14 (s, 1H), 7.39 (d, 1H, J_{ortho} = 7.5 Hz), 7.45 (t, 1H, J_{ortho} = 7.5 Hz), 7.92 (s, 1H), 12.32 (s, 1H)

^{13}C NMR (125 MHz, d_6-DMSO) δ: 20.7, 22.6, 28.9, 35.5, 37.1, 64.9, 69.3, 77.1, 97.0, 107.0, 113.3, 122.0, 124.5, 125.8, 128.6, 128.8, 128.8, 133.9, 136.5, 137.4, 140.3, 157.0, 159.1, 160.7,169.1, 173.0

Retention time R_t = 0.88 min (METHOD 1)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{30}H_{32}NO_6$: 502.2235; Found: 502.2295



Synthesis of compound **6**

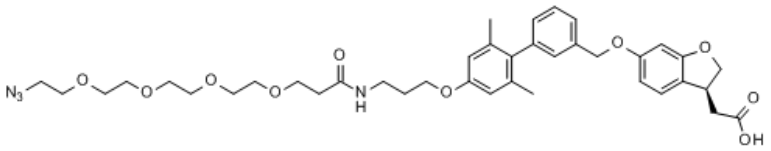
Compound **4** 2-[(3S)-6-[[3-[4-(3-aminopropoxy)-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid previously synthesized (5.0 mg, 10.83 µmol) was dissolved in 400 µL of DMF before DIEA (5.68 µl, 32.50 µmol) was added followed by N_3-PEG_4-NHS ester (2,5-dioxopyrrolidin-1-yl 1-azido-3,6,9,12-tetraoxapentadecan-15-oate, 5 mg, 12.87 µmol). The reaction was allowed to stir at room temperature for 15 minutes. The crude was directly purified through preparative HPLC (25% to 95% ACN/water) to afford, after lyophilization, compound **6** 2-[(3S)-6-[[3-[4-[3-[3-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethoxy]propanoylamino]propoxy]-2,6-dimethyl-phenyl]phenyl] methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as a lyophilized powder (m = 3.7 mg, 5.04 µmol, 46% yield).

^{1}H NMR (500 MHz, d_6-DMSO) δ: 1.85 (p, 2H, J = 6.2 Hz), 1.92 (s, 6H), 2.32 (t, 2H, J = 6.4 Hz), 2.48 (dd, 1H, J = 16.8, 9.0 Hz), 2,68 (dd, 1H, J = 16.5, 5.6 Hz), 3.21 (q, 2H, J = 6.0 Hz), 3.39 (t, 2H, J = 5.2 Hz), 3.48-3.62 (m, 16H, PEG), 3.68 (m, 1H), 3.98 (t, 2H, J = 6.2 Hz), 4.18 (dd, 1H, J = 9.0, 6.9 Hz), 4.68 (t, 1H, J = 9.0 Hz), 5.10 (s, 2H), 6.46 (s, 1H), 6.49 (d, 1H, J_{ortho} = 8.0 Hz), 6.69 (s, 2 H), 7.05 (d, 1H, J_{ortho} = 7.5 Hz), 7.09 (d, 1H, J_{ortho} = 8.0 Hz), 7.14 (s, 1H), 7.39 (d, 1H, J_{ortho} = 7.5 Hz), 7.45 (t, 1H, J_{ortho} = 7.5 Hz), 7.92 (t, 1H, J = 5.4 Hz), 12.32 (s, 1H)

^{13}C NMR (125 MHz, d_6-DMSO) δ: 20.7, 28.9, 35.5, 36.2, 37.1, 49.9, 64.9, 66.9, 69.2, 69.3, 69.5, 69.7, 69.7, 69.8, 69.8, 77.1, 96.9, 106.9, 113.2, 121.9, 124.5, 125.8, 128.6, 128.8, 128.8, 133.7, 136.5, 137.4, 140.3, 157.2, 159.1, 160.7, 170.0, 173.0

Retention time R_t = 0.93 min (METHOD 1)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{39}H_{49}N_4O_{10}$: 733.3454; Found: 733.3558



Synthesis of compound **7**

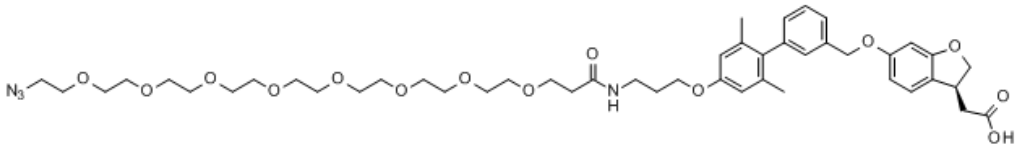
Compound **4** 2-[(3S)-6-[[3-[4-(3-aminopropoxy)-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid previously synthesized (4.4 mg, 9.53 µmol) was dissolved in 300 µL of DMF before DIEA (5.0 µl, 28.63 µmol) was added followed by N_3-PEG_8-NHS ester (2,5-dioxopyrrolidin-1-yl 1-azido-3,6,9,12,15,18,21,24-octaoxaheptacosan-27-oate, 5.92 mg, 10.49 µmol). The reaction was allowed to stir at room temperature for 15 minutes. The crude was directly purified through preparative HPLC (25% to 95% ACN/water) to afford, after lyophilization, compound **7** 2-[(3S)-6-[[3-[4-[3-[3-[2-[2-[2-[2-[2-[2-[2-(2- azidoethoxy) ethoxy]ethoxy]ethoxy]ethoxy]ethoxy] ethoxy]ethoxy] propanoylamino]propoxy]-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as a lyophilized powder (m = 4.1 mg, 4.50 µmol, 47% yield).

^{1}H NMR (500 MHz, d_6-DMSO) δ: 1.85 (p, 2H, J = 6.2 Hz), 1.92 (s, 6H), 2.32 (t, 2H, J = 6.4 Hz), 2.48 (dd, 1H, J = 16.8, 9.0 Hz), 2,68 (dd, 1H, J = 16.5, 5.6 Hz), 3.21 (q, 2H, J = 6.2 Hz), 3.39 (t, 2H, J = 5.2 Hz), 3.48-3.62 (m, 32H, PEG), 3.68 (m, 1H), 3.98 (t, 2H, J = 6.2 Hz), 4.18 (dd, 1H, J = 9.0, 6.9 Hz), 4.68 (t, 1H, J = 9.0 Hz), 5.09 (s, 2H), 6.46 (s, 1H), 6.49 (d, 1H, J_{ortho} = 8.0 Hz), 6.69 (s, 2 H), 7.05 (d, 1H, J_{ortho} = 7.5 Hz), 7.09 (d, 1H, J_{ortho} = 8.0 Hz), 7.14 (s, 1H), 7.39 (d, 1H, J_{ortho} = 7.5 Hz), 7.45 (t, 1H, J_{ortho} = 7.5 Hz), 7.92 (t, 1H, J = 5.2 Hz), 12.32 (s, 1H)

^{13}C NMR (125 MHz, d_6-DMSO) δ: 20.7, 28.9, 35.5, 36.2, 37.1, 49.9, 64.9, 66.9, 69.2, 69.3, 69.5, 69.7, 69.7, 69.8, 69.8, 77.1, 96.9, 106.9, 113.2, 121.9, 124.5, 125.8, 128.6, 128.8, 128.8, 133.7, 136.5, 137.4, 140.3, 157.2, 159.1, 160.7, 170.0, 173.0

Retention time R_t = 0.92 min (METHOD 1)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{47}H_{65}N_4O_{14}$: 909.4503; Found: 909.4621



Synthesis of compound **8**

Compound **4** 2-[(3S)-6-[[3-[4-(3-aminopropoxy)-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid previously synthesized (9.8 mg, 16.99 µmol) was dissolved in 500 µL of DMF before DIEA (6 µl, 34.35 µmol) was added followed by Boc-NH-PEG_{63}-NHS ester (55,4 mg, 15,31 µmol) (5.9 mg, 10.49 µmol). The reaction was allowed to stir at room temperature for 15 minutes, the solvent was evaporated under reduced pressure and the crude was redissolved in 1 mL of DCM. Then 250 µL of TFA/TIS/water (95:2.5:2.5) were added, and the reaction ran for 45 min. The crude was directly purified through preparative HPLC (25% to 95% ACN/water) to afford, after lyophilization, compound **8** 2-[(3S)-6-[[3-[4-[3-[3-(2-aminoethoxy)PEG-63-propanoylamino]propoxy] -2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as a colorless oil (m = 29.7 mg, 8.94 µmol, 53% yield).

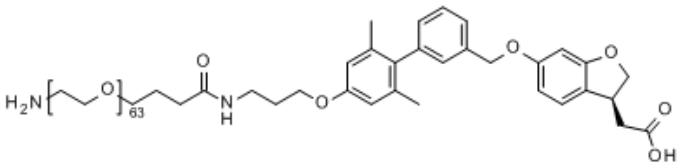
^{1}H NMR (500 MHz, d_6-DMSO) δ: 1.72 (p, 2H, J = 6.4 Hz), 1.85 (p, 2H, J = 6.4 Hz), 1.92 (s, 6H), 2.32 (t, 2H, J = 7.2 Hz), 2.48 (dd, 1H, J = 16.8, 9.0 Hz), 2,68 (dd, 1H, J = 16.5, 5.6 Hz), 2.97 (t, 2H, J = 5.2 Hz), 3.21 (q, 2H, J = 6.2 Hz), 3.36 (t, 2H, J = 6.4 Hz), 3.40-3.75 (m, PEG), 3.98 (t, 2H, J = 6.2 Hz), 4.18 (dd, 1H, J = 9.0, 6.8 Hz), 4.68 (t, 1H, J = 9.0 Hz), 5.09 (s, 2H), 6.46 (s, 1H), 6.49 (d, 1H, J_{ortho} = 8.0 Hz), 6.69 (s, 2 H), 7.05 (d, 1H, J_{ortho} = 7.5 Hz), 7.09 (d, 1H, J_{ortho} = 8.0 Hz), 7.14 (s, 1H), 7.39 (d, 1H, J_{ortho} = 7.5 Hz), 7.45 (t, 1H, J_{ortho} = 7.5 Hz), 7.86 (t, 1H, J = 5.2 Hz)

^{13}C NMR (125 MHz, d_6-DMSO) δ: 20.7, 25.4, 28.9, 32.1, 35.5, 37.1, 38.8, 64.9, 66.9, 69.3, 69.4, 69.6, 69.7, 69.8, 77.1, 96.9, 106.9, 113.2, 121.9, 124.5, 125.8, 128.6, 128.7, 133.7, 136.5, 137.4, 140.3, 157.2, 159.1,

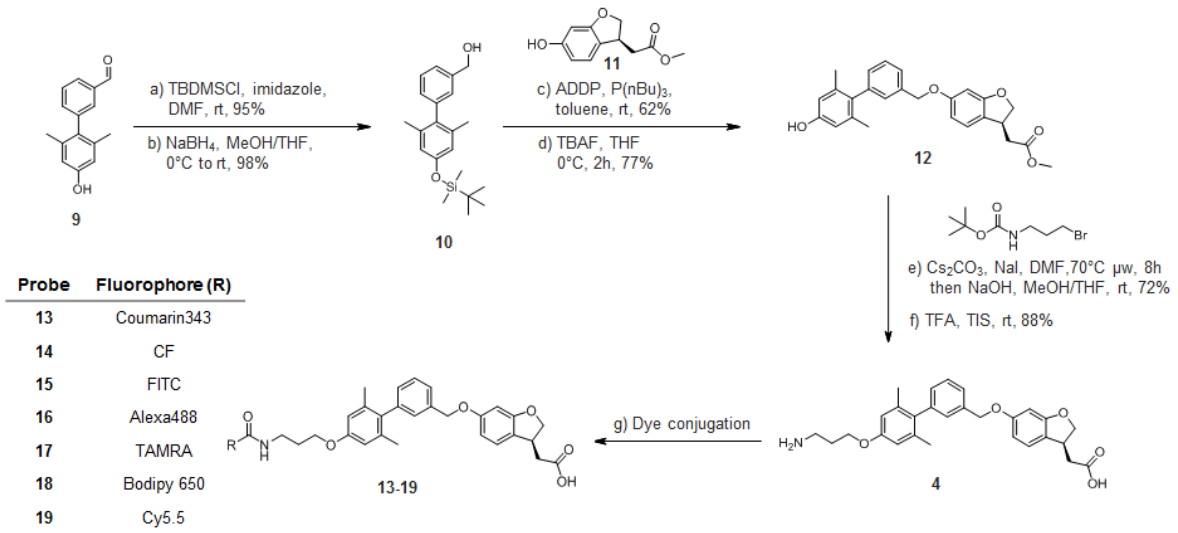
160.7, 171.7, 173.1

Retention time R_t = 0.81 min (METHOD 1)

HRMS (ESI-TOF) m/z: No mass



Supplemental experimental procedure Figure 2 – Syntheses of the fluorescent probes 13-19



Probe	Fluorophore (R)
13	Coumarin343
14	CF
15	FITC
16	Alexa488
17	TAMRA
18	Bodipy 650
19	Cy5.5

Compound **9** and **11** were synthesized as described by Negoro et al., 2012

Synthesis of compound **10** – step (a)

Hydroxy-dimethylbiphenyl carbaldehyde **9** (5.40 g, 23.74 mmol), TBDMS-Cl (4.30 g, 28.50 mmol) and imidazole (4.85 g, 71.20 mmol) were dissolved in 35 mL of DMF. The mixture was allowed to stir at room temperature for 1 hour. The reaction was followed by LCMS and TLC (25% EtOAc/Hept).

After 60 minutes of reaction, the mixture was diluted with 100 mL of EtOAc and 50 mL of brine and the

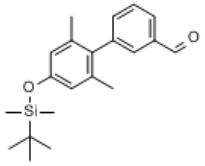
organic phase was washed with brine (3 x 50 mL) and water (1 x 50 mL), before being dried over $MgSO_4$, filtered and evaporated under reduced pressure. Purification was performed via silica gel chromatography (0->5% EtOAc/Hept), affording the desired product 3-[4-[tert-butyl(dimethyl)silyl]oxy-2,6-dimethyl-phenyl]benzaldehyde as an oil (m = 7.68 g, 22.55 mmol, yield 95%).

^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.23 (s, 6H), 1.00 (s, 9H), 1.96 (s, 6H), 6.60 (s, 2H), 7.41 (d, 1H, J_{ortho}= 7.6 Hz), 7.57 (t,1H, J_{ortho}= 7.6 Hz), 7.67 (s, 1H), 7.84 (d, 1H, J_{ortho}= 7.6 Hz), 10.04 (s, 1H).

^{13}C NMR (150 MHz, $CDCl_3$) δ: -4.28, 18.2, 20.9, 25.7, 118.9, 127.8, 129.1, 131.2, 133.5, 136.0, 136.7, 137.1, 142.2, 154.7, 192.4

Retention time Rt = 1.08 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{21}H_{27}O_2Si$: 339.1786; Found: 339.2366



Synthesis of compound **10** – step (b)

3-[4-[tert-butyl(dimethyl)silyl]oxy-2,6-dimethyl-phenyl]benzaldehyde synthesized in the previous step (7.68 g, 22.55 mmol) was dissolved in MeOH(15 mL)/THF(30 mL) at 0°C, and sodium borohydride $NaBH_4$ (0.98 g, 25.93 mmol) was added. The mixture was allowed to stir under argon at 0°C for 1h30. Volatiles were evaporated and the mixture was diluted with a pH=5 water solution (50 mL) and EtOAc (100 mL). The organic phase was washed successively with brine (50 mL) and water (50 mL), dried over $MgSO_4$ and concentrated to give the desired product **10** [3-[4-[tert-butyl(dimethyl)silyl]oxy-2,6-dimethyl-

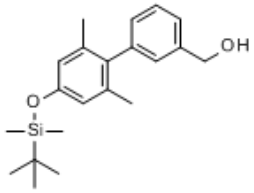
phenyl]phenyl]methanol as a white powder (m = 7.69 g, 22.44 mmol, quant.)

^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.26 (s, 6H), 1.03 (s, 9H), 1.99 (s, 6H), 4.75 (s, 2H), 6.60 (s, 2H), 7.08 (d, 1H, J_{ortho}= 7.6 Hz), 7.15 (s, 1H), 7.35 (d, 1H, J_{ortho}= 7.6 Hz), 7.42 (t, 1H, J_{ortho}= 7.6 Hz).

^{13}C NMR (150 MHz, $CDCl_3$) δ: -4.28, 18.2, 21.0, 25.8, 65.5, 118.6, 125.1, 128.2, 128.6, 129.1, 134.8, 137.1, 140.9, 141.5, 154.3

Retention time R_t = 0.98 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{21}H_{29}O_2Si$: 341.1942; Found: 341.2454



Synthesis of compound **12 –** step (c)

Compound **10** [3-[4-[tert-butyl(dimethyl)silyl]oxy-2,6-dimethyl-phenyl]phenyl]methanol (3 g, 8.32 mmol, 1eq), (S)-methyl-(6-hydroxy-2,3-dihydrobenzofuran-3-yl)acetate **11** (1.77 g, 8.32 mmol) and tributylphosphine $P(nBu)_3$ (3 mL, 11.55 mmol) were dissolved in 150 mL of toluene before a solution of 1,1'-(azodicarbonyl)dipiperidine ADDP (3 g, 11.55 mmol, dissolved in 50 mL of toluene) was added dropwise during 20 minutes under argon. The mixture was then put in an ultrasound bath for 10 minutes at room temperature. The reaction was followed by LCMS which showed appearance of the desired mass and TLC (25% EtOAc/Hept) which indicated total consumption of the phenol starting material. Then 100 mL of hexane were added and the insoluble material was removed by filtration. The filtrate was concentrated and the residue was purified through silica gel chromatography (0->15% EtOAc-Hept) to

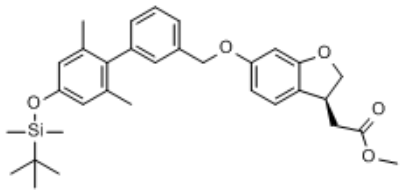
afford the desired product methyl 2-[(3S)-6-[[3-[4-[tert-butyl(dimethyl)silyl]oxy-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate as a pale oil (m= 2.75 g, 5.16 mmol, yield 62%)

^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.22 (s, 6H), 1.00 (s, 9H), 1.95 (s, 6H), 2.55 (dd, 1H, J = 16.5, 9.0 Hz), 2.75 (dd, 1H, J = 16.5, 5.6 Hz), 3.71 (s, 3H), 3.79 (m, 1H, J = 9.1, 9.0, 6.4, 5.6 Hz), 4.25 (dd, 1H, J = 9.1, 6.4 Hz), 4.75 (t, 1H, J = 9.1 Hz), 5.05 (s, 2H), 6.45 (d, 1H, J_{meta} = 2.0 Hz), 6.49 (dd, 1H, J_{ortho} = 8.2 Hz, J_{meta} = 2.0 Hz), 6.58 (s, 2H), 7.00 (d, 1H, J_{ortho} = 8.2 Hz), 7.08 (d, 1H, J_{ortho} = 7.5 Hz), 7.17 (s, 1H), 7.35 (d, 1H, J_{ortho} = 7.5 Hz), 7.40 (t, 1H, J_{ortho} = 7.5 Hz).

^{13}C NMR (150 MHz, $CDCl_3$) δ: -4.28, 18.2, 21.0, 25.8, 37.8, 39.5, 51.8, 70.4, 97.5, 107.4, 118.6, 121.5, 124.3, 125.5, 128.6, 128.7, 129.3, 134.7, 137.1, 137.2, 141.4, 154.3, 160.0, 161.2, 172.

Retention time R_t = 1.18 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{32}H_{39}O_5Si$: 531.25722; Found: 531.2634



Synthesis of compound **12 –** step (d)

Methyl 2-[(3S)-6-[[3-[4-[tert-butyl(dimethyl)silyl]oxy-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate synthesized in the previous step (2.5 g, 4.55 mmol, 1eq) was dissolved in 20 mL of THF and the solution was cooled to 0°C before TBAF (5.5 mL, 5.5 mmol, 1M solution in THF) was

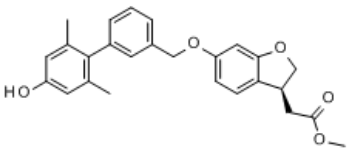
added. The mixture was allowed to stir at 0°C for 1 hour. Solvent was removed by evaporation under reduced pressure. Purification was performed through silica gel chromatography (0-40% EtOAc-Hept) to afford desired product **12** methyl 2-[(3S)-6-[[3-(4-hydroxy-2,6-dimethyl-phenyl)phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetate as a white powder (m = 1.47g, 3.51 mmol, yield 77%)

^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.96 (s, 6H), 2.55 (dd, 1H, J = 16.6, 9.0 Hz), 2.75 (dd, 1H, J = 16.6, 5.6 Hz), 3.71 (s, 3H), 3.79 (m, 1H, 9.1, 9.0, 6.4, 5.6 Hz), 4.25 (dd, 1H, J = 9.1, 6.4 Hz), 4.75 (t, 1H, J = 9.1 Hz), 5.05 (s, 2H), 6.45 (d, 1H, J_{meta} = 2.0 Hz), 6.48 (dd, 1H, J_{ortho} = 8.2 Hz, J_{meta} = 2.0 Hz), 6.58 (s, 2 H), 7.01 (d, 1H, J_{ortho} = 8.2 Hz), 7.06 (d, 1H, J_{ortho} = 7.5 Hz), 7.15 (s, 1H), 7.36 (d, 1H, J_{ortho} = 7.5 Hz), 7.41 (t, 1H, J_{ortho} = 7.5 Hz).

^{13}C NMR (150 MHz, $CDCl_3$) δ: 20.4, 37.3, 39.0, 51.3, 69.9, 97.0, 106.9, 113.5, 121.0, 123.8, 125.0, 128.1, 128.2, 128.8, 133.8, 136.6, 137.2, 140.5, 153.8, 159.5, 160.6, 171.9

Retention time R_t = 0.78 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{26}H_{25}O_5$: 417.1707; Found: 417.1750



Synthesis of compound **4** – step (e)

Compound **12** (250 mg, 597.4 µmol), tert-butyl (3-bromopropyl)carbamate (223 mg, 936.5 µmol), potassium carbonate (99.1 mg, 716.9 µmol) and sodium iodide (17.9 mg, 119.5 µmol) were dissolved in 3 mL of DMF under argon. The reaction mixture was microwaved at 70°C for 8 hours. Then, another 1,5

equivalent of bromide and 1,1 equivalent of potassium carbonate were added to the reaction mixture, and a second run of microwave heating (1 hours, 70°C) was needed to reach completion of reaction. The reaction was diluted with 10 mL of brine and 20 mL of EtOAc. The organic phase was then washed with brine (3 x 15 mL) and then with 5% NH_4Cl solution (2 x 15 mL), dried over sodium sulfate, filtered, and evaporated under reduced pressure.

The crude was then dissolved in 2 mL of THF and 0,6 mL of methanol before 800 µL of a 2,5M sodium hydroxide aqueous solution (2 mmol) were added. The mixture was allowed to stir at room temperature for 30 minutes.

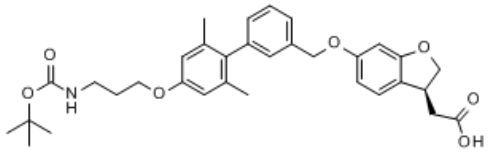
The mixture was concentrated under reduced pressure, diluted with 15 mL of water, acidified with 1 HCl until pH = 2 and extracted with DCM (3 x 15 mL). The organic phase was then dried over sodium sulfate, filtered and evaporated under reduced pressure before being purified via silica gel chromatography (0-10% MeOH/DCM) to afford 2-[(3S)-6-[[3-[4-[3-(tert-butoxycarbonylamino)propoxy]-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as a pale yellow oil (m = 241 mg, 429.1 µmol, 72% yield).

^{1}H NMR (600 MHz, d_6-DMSO) δ: 1.37 (s, 9H), 1.81 (quint, 2H, J = 6.5 Hz), 1.90 (s, 6H), 2.46 (dd, 1H, J = 16.6, 8.9 Hz), 2.68 (dd, 1 H, J = 16.6, 5.6 Hz), 3.08 (q, 2 H, J = 6.5 Hz), 3.66 (m, 1 H, J = 9.1, 8.9, 6.6, 5.6 Hz), 3. 95 (t, 2 H, J = 6.5 Hz), 4.17 (dd, 1 H, J = 9.1, 6.6 Hz), 4.66 (t, 1 H, J = 9.1 Hz), 5.08 (s, 2 H), 6.44 (d, 1H, J_{meta} = 2.1 Hz), 6.46 (dd, 1H, J_{ortho} =8.2 Hz, J_{meta} = 2.1 Hz), 6.66 (s, 2 H), 6.87 (t, 1H, J = 6.5 Hz), 7.04 (d, 1H, J_{ortho} = 7.5 Hz), 7.08 (d, 1H, J_{ortho} = 8.2 Hz), 7.12 (s, 1H), 7.36 (d, 1H, J_{ortho} = 7.5 Hz), 7.43 (t, 1H, J_{ortho} = 7.5 Hz).

^{13}C NMR (150 MHz, d_6-DMSO) δ: 20.7, 28.2, 29.3, 37.0, 37.1, 65.0, 69.3, 77.1, 77.5, 96.9, 107.0, 113.2, 121.9, 124.5, 125.8, 128.5, 128.5, 128.7, 133.6, 136.5, 137.3, 140.3, 155.6, 157.2, 159.0, 160.6, 173.0

Retention time R_t = 0.75 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{33}H_{38}NO_7$: 560.2654; Found: 560.2760



Synthesis of **4** – step (f)

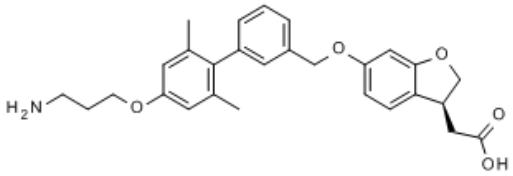
2-[(3S)-6-[[3-[4-[3-(tert-butoxycarbonylamino)propoxy]-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid synthesized in the previous step (120 mg, 213,7 µmol) was dissolved in 2,5 mL of dichloromethane before TFA (500 µl, 6.5 mmol) + 1% triisopropylsilane (25 µl, 213,65 µmol) were added. The mixture was allowed to stir at room temperature for 10 minutes. The reaction mixture was diluted with 3 mL of water. The organic phase was washed with brine (3 x 3 mL). During the last washing step with brine, product precipitated as a slightly brown solid. It was filtered, redissolved in a mixture of acetonitrile and water, and lyophilized to afford desired product **4** 2-[(3S)-6-[[3-[4-(3-aminopropoxy)-2,6-dimethyl-phenyl]phenyl]methoxy]-2,3-dihydrobenzofuran-3-yl]acetic acid as an off-white lyophilized powder (m = 86.6 mg, 187.6 µmol, 88 % yield).

^{1}H NMR (400 MHz, d_6-DMSO) δ: 1.91 (s, 6H), 2.04 (dd, 2H, J = 7.5, 6.2 Hz), 2.46 (dd, 1H, J = 16.4, 9.0 Hz), 2.70 (dd, 1 H, J = 16.4, 5.6 Hz), 2.94 (t, 2 H, J = 7.5 Hz), 3.67 (m, 1 H, 9.1, 9.0, 6.8, 5.6), 4.07 (t, 2 H, J = 6.2 Hz), 4.18 (dd, 1 H, J = 9.1, 6.8 Hz), 4.67 (t, 1H, J = 9.1 Hz), 5.09 (s, 2 H), 6.44 (d, 1H, J_{meta} = 2.3 Hz), 6.46 (dd, 1H, J_{ortho} = 8.0, J_{meta} = 2.3 Hz), 6.71 (s, 2H), 7.04 (d, 1H, J_{ortho} = 7.7 Hz), 7.10 (d, 1H, J_{ortho} = 8.0 Hz), 7.12 (s, 1H), 7.38 (d, 1H, J_{ortho} = 7.7 Hz), 7.44 (t, 1H, J_{ortho} = 7.7 Hz), 8.21 (s, 3H), 12.36 (s, 1H)

^{13}C NMR (150 MHz, d_6-DMSO) δ: 20.7, 26.9, 37.2, 37.1, 64.5, 69.4, 77.1, 97.0, 107.0, 113.3, 122.0, 124.5, 125.8, 128.5, 128.5, 128.7, 133.9, 136.5, 137.4, 140.3, 157.0, 159.1, 160.7, 173.0

Retention time R_t = 0.52 min (METHOD 2)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{28}H_{30}NO_5$: 460.2129; Found: 460.2131



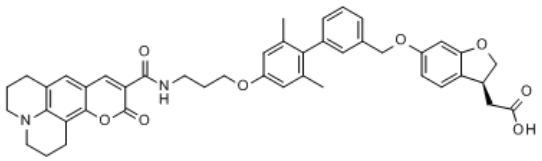
Synthesis of probe **13** (TAK-Coumarin343)

Coumarin 343 (5.2 mg, 18.2 µmol), HATU (6.9 mg, 18.2 µmol) and DIEA (8.4 µL, 48.3 µmol) were dissolved in 200 µL of DMF and 200 µL of DCM in a small Eppendorf vial protected from light with aluminum foil, and were mixed for 5 minutes at room temperature before the addition of compound **4** (8.1 mg, 16.3 µmol). The reaction mixture was allowed to stir for one hour. The crude was directly purified by preparative HPLC (25->95% ACN) to afford, after lyophilization, the desired product **13** TAK-Coumarin343 conjugate as a yellow powder (m = 1.7 mg, 2.3 µmol, yield 14%).

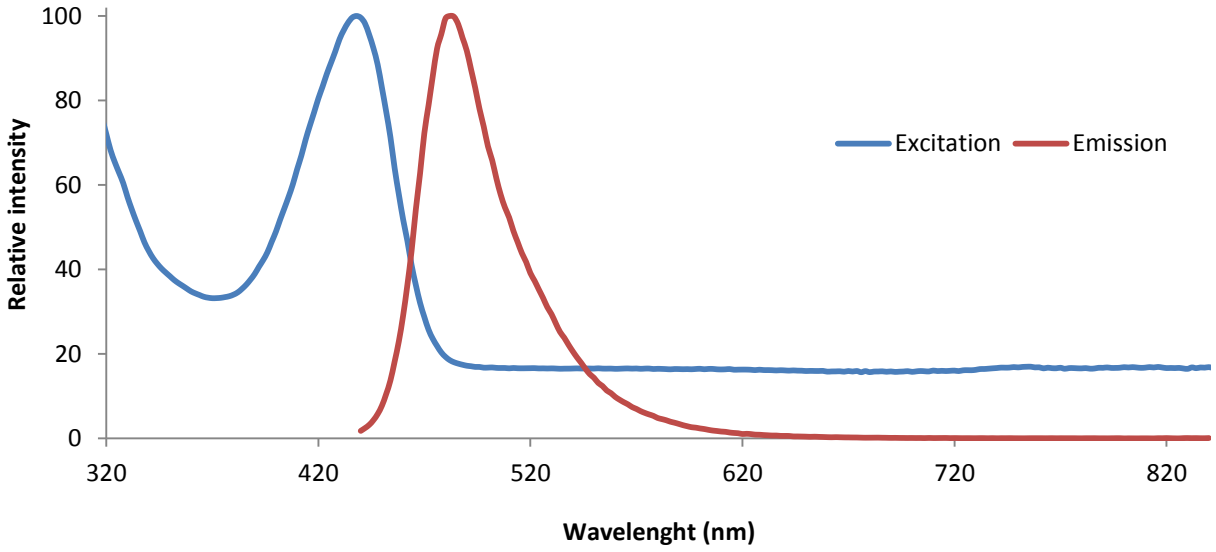
Retention time R_t = 5.172 min (METHOD 3)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{44}H_{43}N_2O_8$: 727.3025; Found: 727.3092

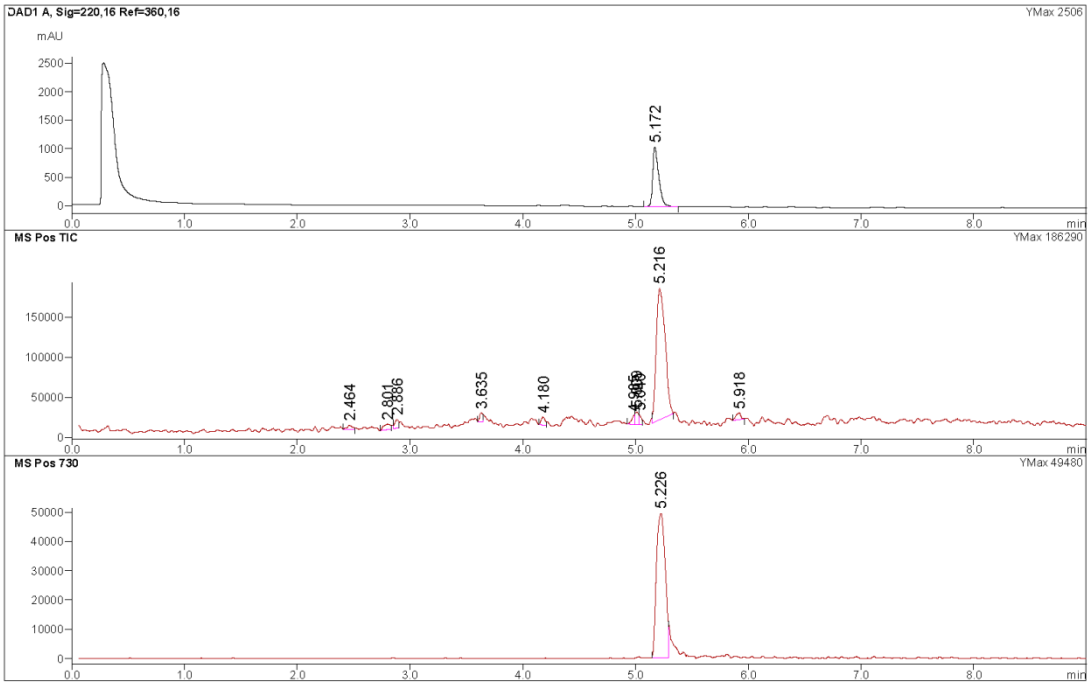
Max Abs/Em 438/482 nm



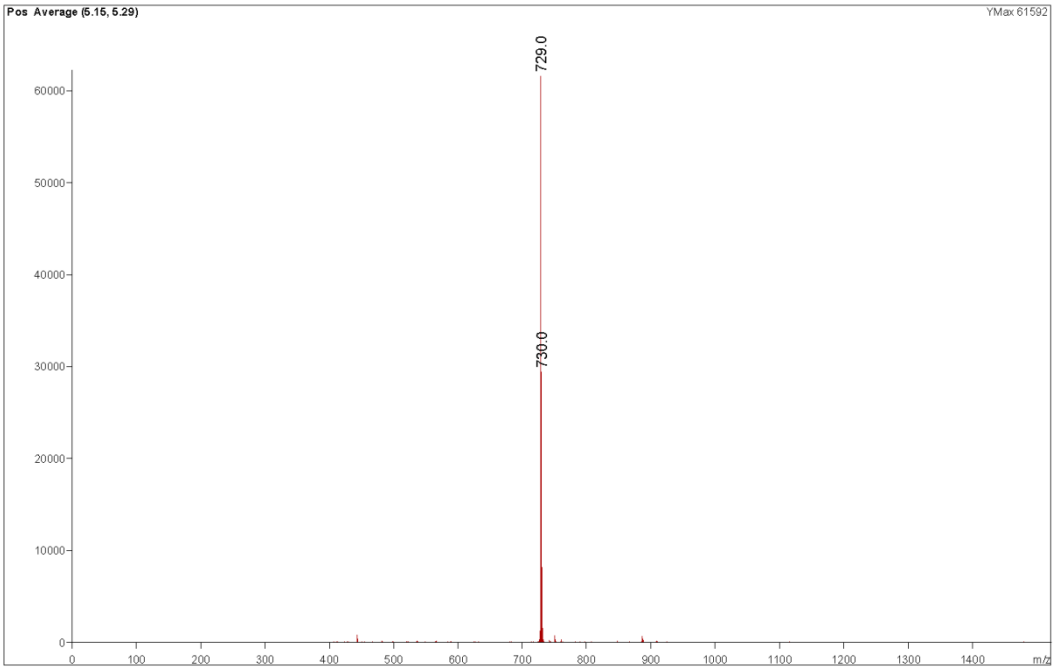
Fluorescence Ex/Em spectra of TAK-Coumarin343 in MeOH



Data File	C:\Daten\2015\NOVEMBER\16NOV0001124.D	Sample Name	RBE-138 TAK-Coumarin		
Injection Date	26-Nov-2015 19:11:26	Vial	5	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	7	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	729				



Data File	C:\Daten\2015\NOVEMBER\16NOV0001124.D	Sample Name	RBE-138 TAK-Coumarin		
Injection Date	26-Nov-2015 19:11:26	Vial	5	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	7	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	729				



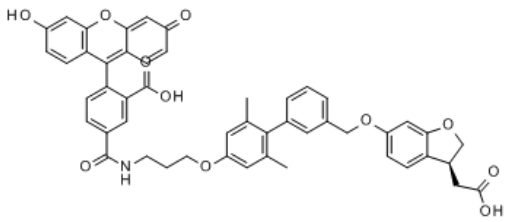
Synthesis of probe **14** (TAK-CF)

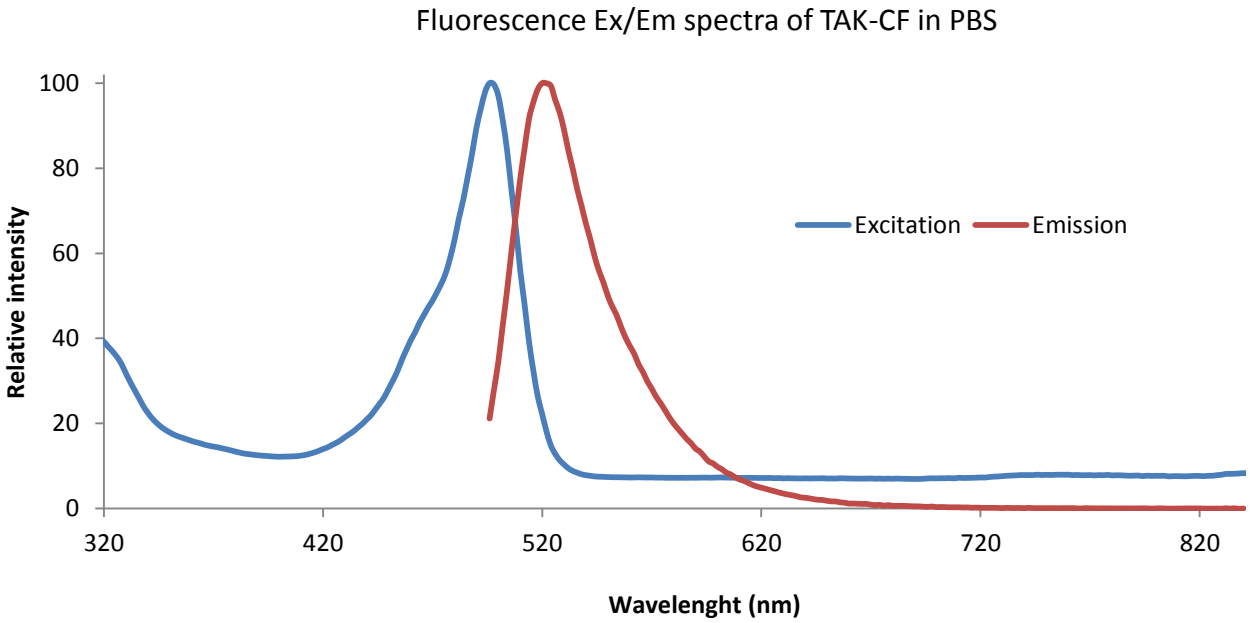
Compound **4** (6.5 mg, 13.1 µmol) was dissolved in 500 µL of DMF in a small Eppendorf vial protected from light with aluminum foil, followed by DIEA (9.1 µL, 52.2 µmol) and carboxyfluorescein NHS ester (5.1 mg, 10.7 µmol). The reaction was allowed to stir for 30 minutes at room temperature, in the dark. The crude was directly purified through preparative HPLC (25->95% ACN) to afford, after lyophilization, the desired product **14** TAK-CF conjugate as a yellow powder (m = 4.7 mg, 5.7 µmol, yield 53%).

Retention time R_t = 4.099 min (METHOD 3)

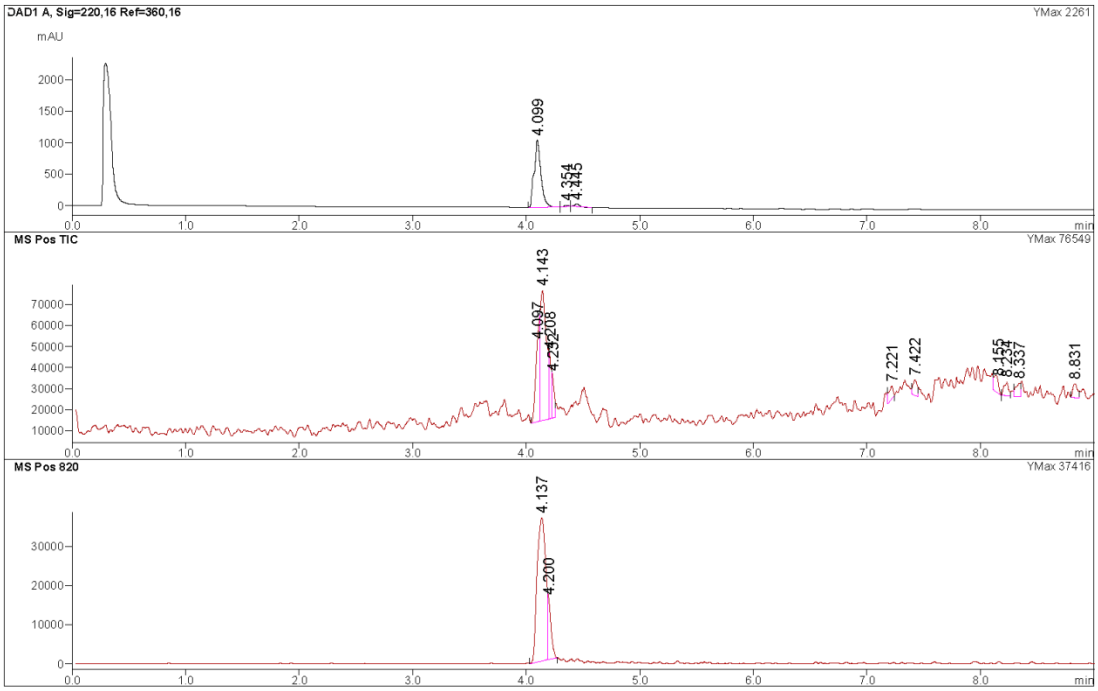
HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{49}H_{40}NO_{11}$: 818.2607; Found: 818.2663

Max Abs/Em 496/520 nm

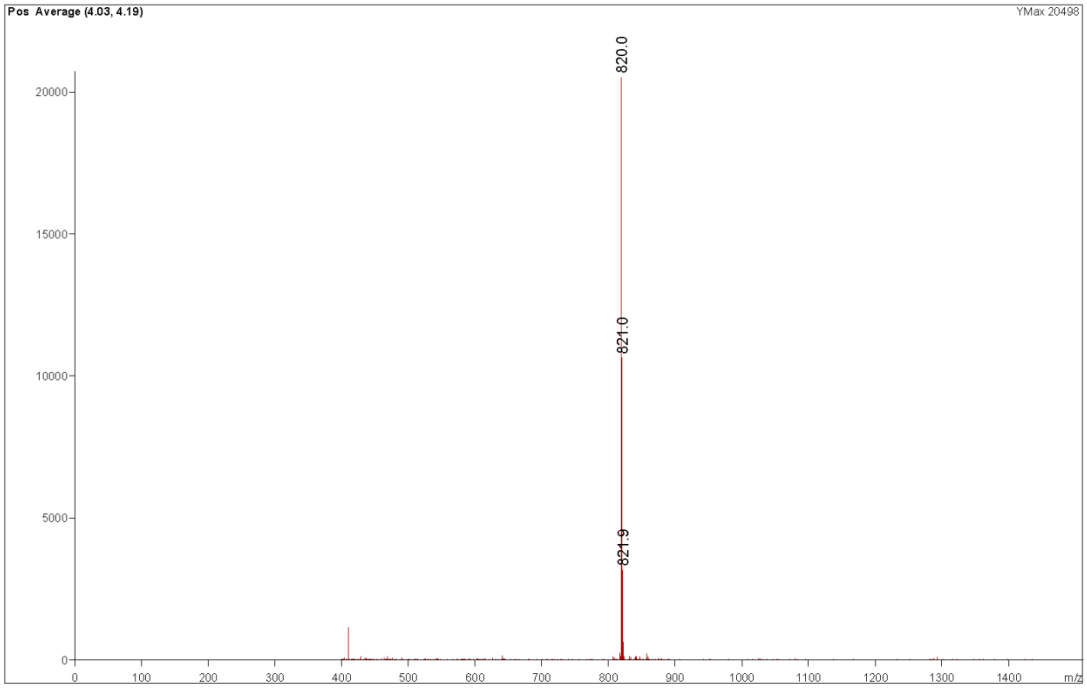



Fluorescence Ex/Em spectra of TAK-CF in PBS
Relative intensity
100
80
60
40
20
0
320
420
520
620
720
820
Wavelenght (nm)
Excitation
Emission

Data File	C:\Daten\2015\NOVEMBER\16NOV0001199.D	Sample Name	RBE-249- TAK-CF		
Injection Date	27-Nov-2015 17:09:28	Vial	13	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	1	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	819				



Data File	C:\Daten\2015\NOVEMBER\16NOV0001199.D	Sample Name	RBE-249- TAK-CF		
Injection Date	27-Nov-2015 17:09:28	Vial	13	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	1	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	819				



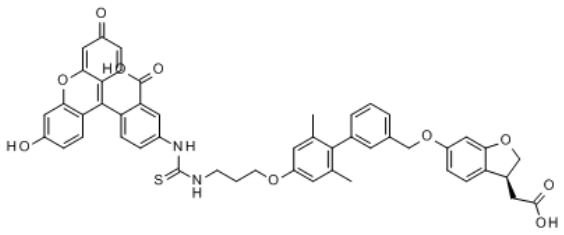
Synthesis of probe **15** (TAK-FITC)

Compound **4** (10.7 mg, 21.53 µmol) was dissolved in 300 µL of DMF in a small Eppendorf vial protected from light with aluminum foil, followed by DIEA (10.3 µL, 59.13 µmol). Then was added fluorescein thioisocyanate (9.8 mg, 25.04 µmol) to the mixture. Reaction was allowed to run for 1 hour in the dark. The crude was directly purified through preparative HPLC (25->95% ACN) to afford, after lyophilization, the desired product **15** TAK-FITC conjugate as a dark orange powder (m = 6.5 mg, 7.62 µmol, 35% yield).

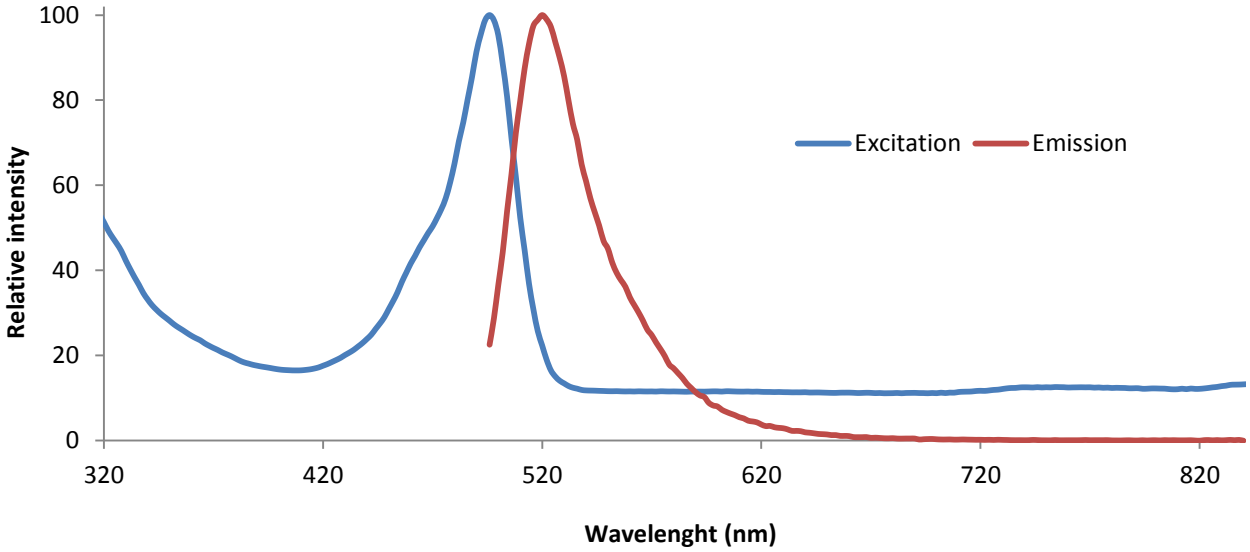
Retention time R_t = 4.172 min (METHOD 3)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{49}H_{41}N_2O_{10}S$: 849.2487; Found: 849.2480

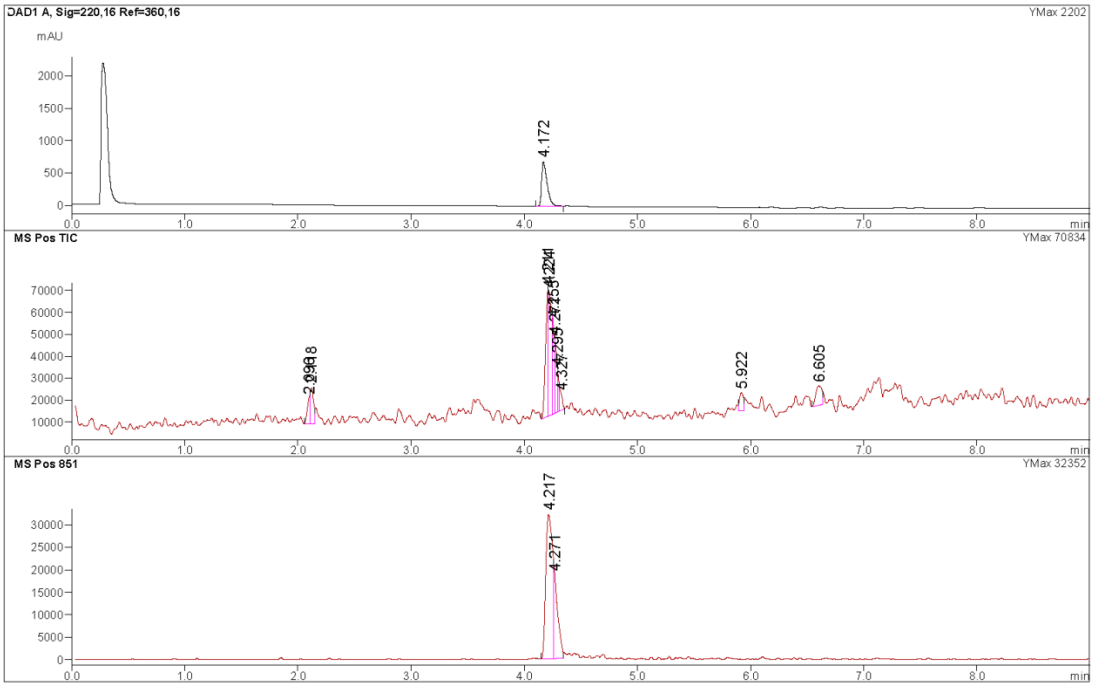
Max Abs/Em 496/520 nm



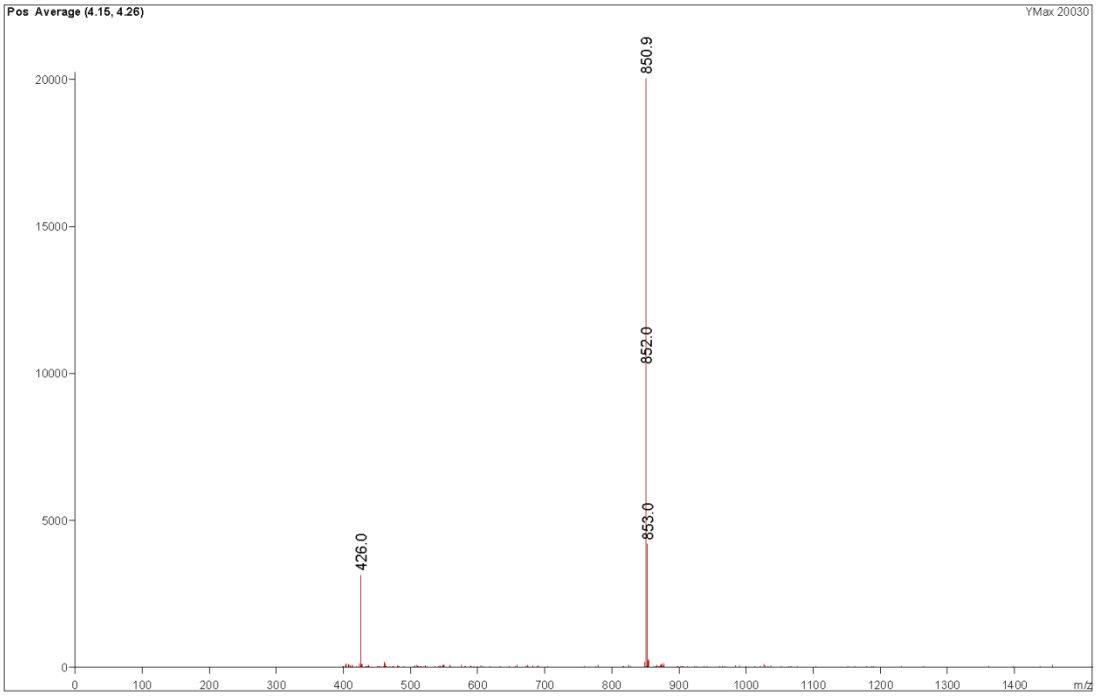
Fluorescence Ex/Em spectra of TAK-FITC in PBS



Data File	C:\Daten\2015\NOVEMBER\16NOV0001122.D	Sample Name	RBE-160 TAK-FITC		
Injection Date	26-Nov-2015 18:49:19	Vial	7	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	5	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	850				



Data File	C:\Daten\2015\NOVEMBER\16NOV0001122.D	Sample Name	RBE-160 TAK-FITC		
Injection Date	26-Nov-2015 18:49:19	Vial	7	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	5	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	850				



Synthesis of probe **16** (TAK-Alexa488)

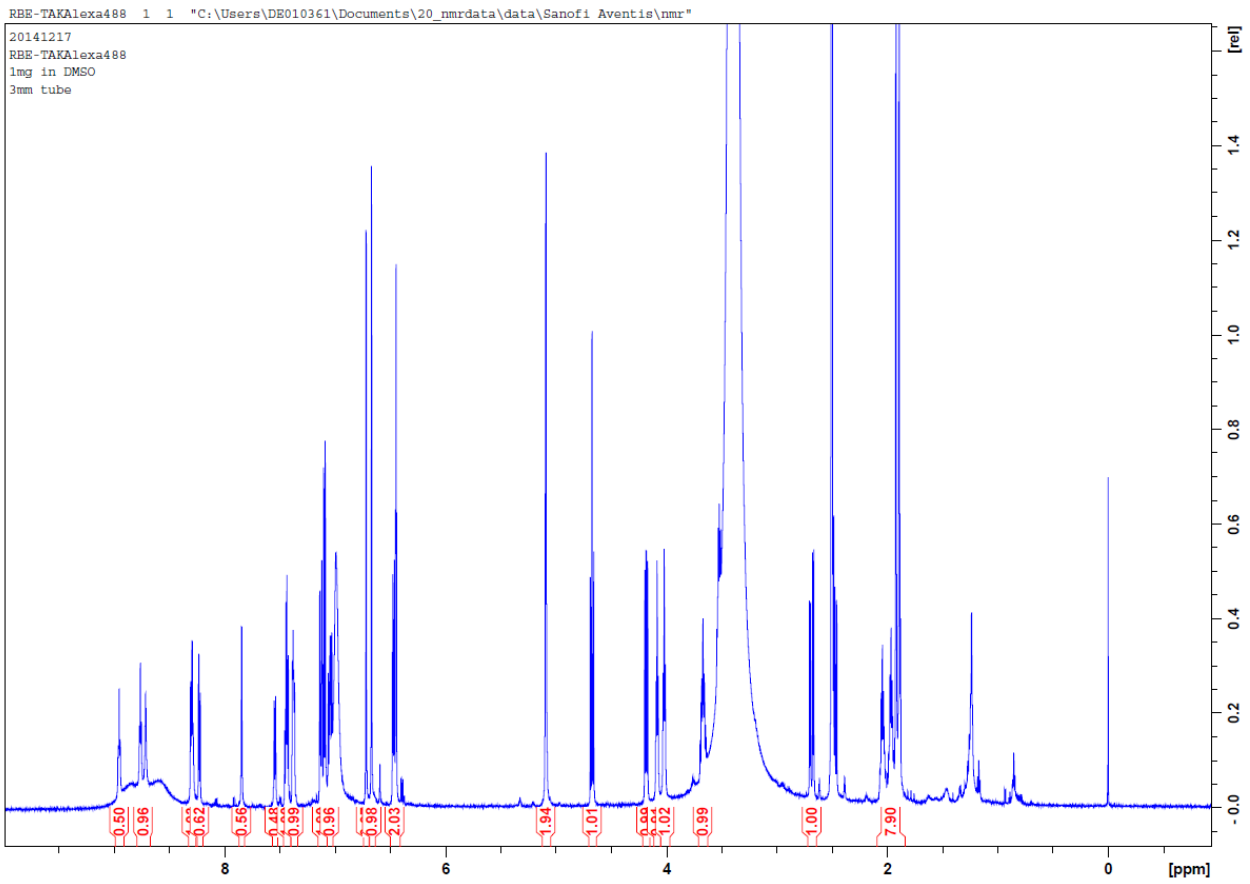
Compound **4** (2.0 mg, 4.33 µmol) was dissolved in 300 µL of water in a small Eppendorf vial protected from light with aluminum foil, followed by DIEA (3 µl, 17.18 µmol). Then was added Alexa-488 NHS ester (3.36 mg, 5.20 µmol) to the mixture and pH was adjusted to pH = 9 with DIEA. The reaction was allowed to run for 10 minutes in the dark.

The crude was directly submitted to preparative HPLC (25->95% ACN) to afford, after lyophilization, the desired product **16** TAK-Alexa488 conjugate as a dark orange powder (m = 2.8 mg, 2.86 µmol, 66% yield).

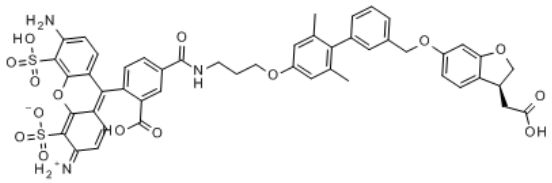
Retention time R_t = 3.185 min (METHOD 3)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{49}H_{43}N_3O_{15}S_2$: 977.2136; Found: 977.2130

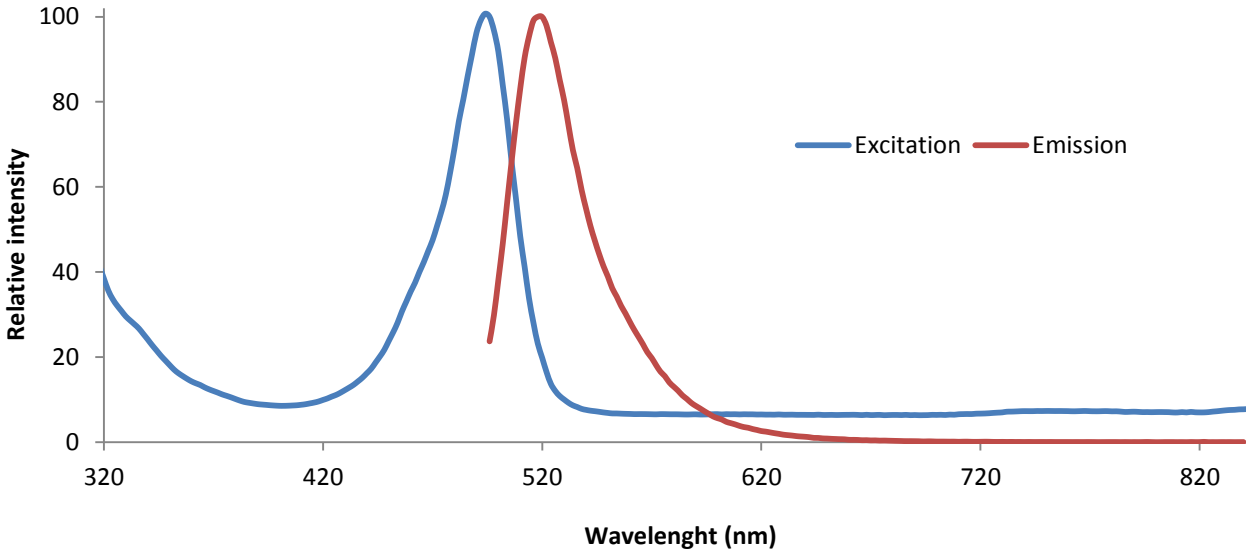
^{1}H NMR (600 MHz, d_6-DMSO) δ: 1.89 (s, 3H), 1.92 (s, 3H), 1.99-1.94 (m, 1H), 2.07-2.01 (m, 1H), 2.46 (t, 1H, *J* = 8.9 Hz), 2.68 (dd, 1H, *J* = 16.7, 5.5 Hz), 3.53-3.50 (m, 2H), 3.70-3.63 (m, 2H), 4.01 (t, 2H, *J* = 6.07 Hz), 4.08 (t, 1H, *J* = 6.07 Hz), 4.18 (dd, 1H, *J* = 8.9, 6.7 Hz), 4.67 (t, 1H, *J* = 9.15 Hz), 5.09 (s, 2H), 6.44 (t, 1H, *J* = 2.0 Hz), 6.46 (dd, 0.5H, *J* = 2.4, 1.4 Hz), 6.48 (dd, 0.5H, *J* = 2.4, 1.4 Hz), 6.67 (s, 1H), 6.72 (s, 1H), 6.99 (sl, 3H), 7.04 (dd, 1,3 Hz, *J* = 11.1, 7.6 Hz), 7.09 (d, 1H, *J* = 8.2 Hz), 7.12 (d, 1H, *J* = 10.4 Hz), 7.39-7.35 (m, 1H), 7.46-7.41 (m, 1H), 7.54 (s, 0.4 H, *J* = 7.8 Hz), 7.85 (s, 0.5H), 8.22 (dd, 0.5H, *J* = 8.2, 1.7 Hz), 8.31-8.27 (m, 1H), 8.71 (s, 0.7H), 8.76 (t, 0.7H, *J* = 5.6 Hz), 8.95 (t, 0.6H, *J* = 5.5 Hz) (mixture of isomers)



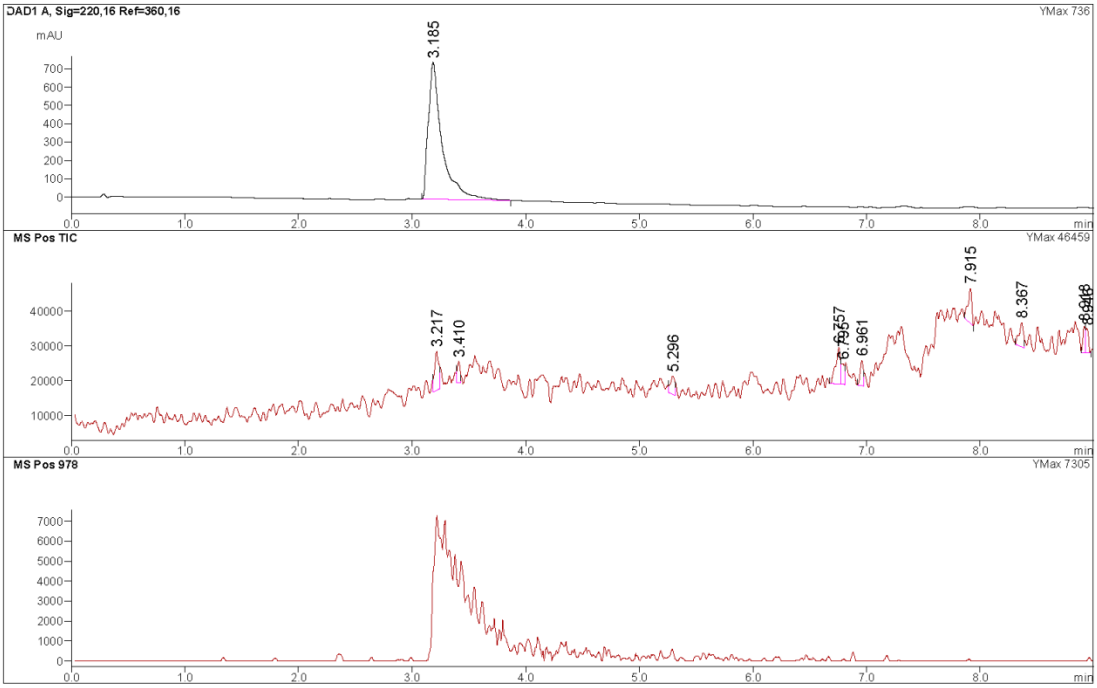
Max Abs/Em 496/518 nm



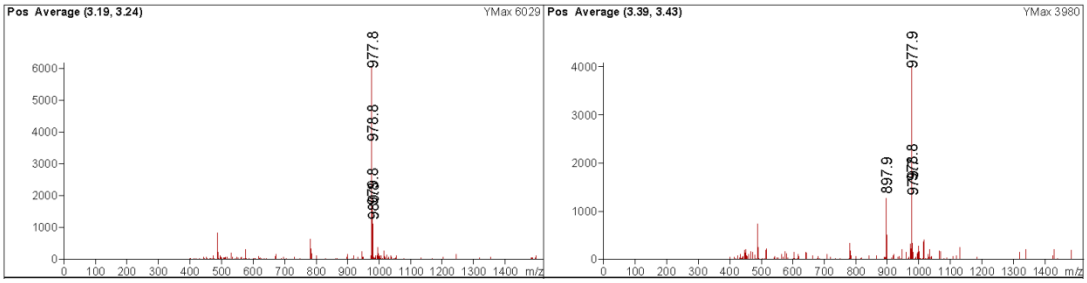
Fluorescence Ex/Em spectra of TAK-Alexa488 in PBS



Data File	C:\Daten\2015\NOVEMBER\16NOV0001200.D	Sample Name	RBE-2-098 TAK-A488		
Injection Date	27-Nov-2015 17:53:52	Vial	12	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	1	Injection Volume	4
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	977				



Data File	C:\Daten\2015\NOVEMBER\16NOV0001200.D	Sample Name	RBE-2-098 TAK-A488		
Injection Date	27-Nov-2015 17:53:52	Vial	12	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	1	Injection Volume	4
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	977				



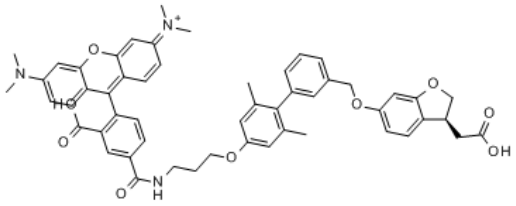
Synthesis of probe **17** (TAK-TAMRA)

Compound **4** (4.8 mg, 9.7 µmol) was dissolved in 400 µL of DMF in a small Eppendorf vial protected from light with aluminum foil, followed by DIEA (3.5 µL, 20.1 µL) and TAMRA NHS-ester (7.4 mg, 9.8 µmol). The reaction was allowed to stir for 30 minutes at room temperature, in the dark. The crude was directly purified through preparative HPLC (25->95% ACN) to afford, after lyophilization, the desired product **17** TAK-TAMRA conjugate as a dark orange powder (m = 5.5 mg, 6.3 µmol, yield 65%).

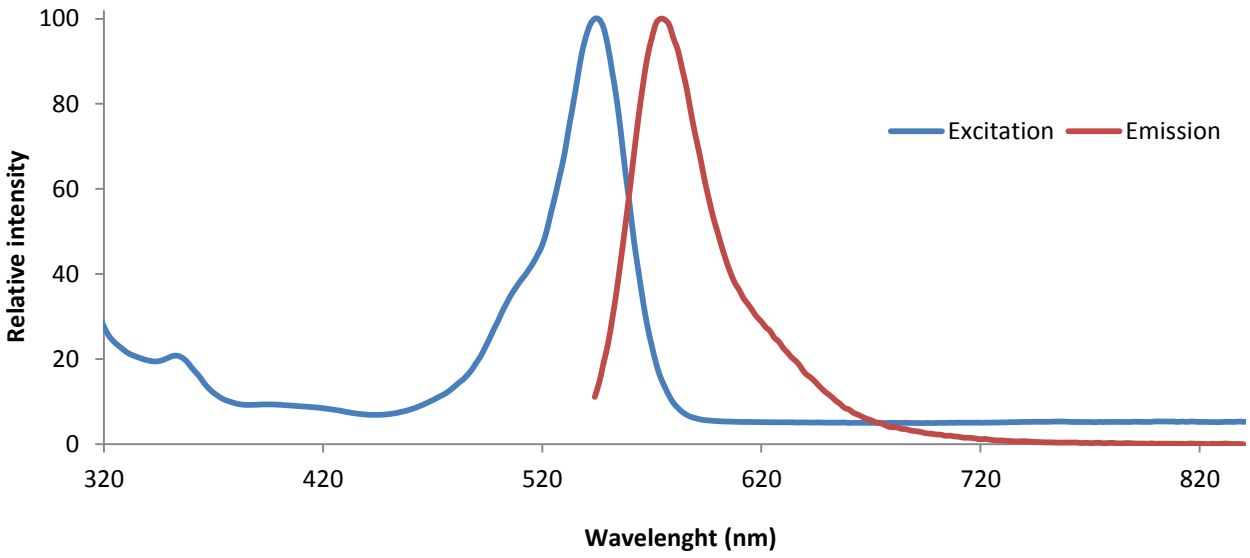
Retention time Rt = 4.172 (METHOD 3)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{53}H_{50}N_3O_9$: 872.3552; Found: 872.3592

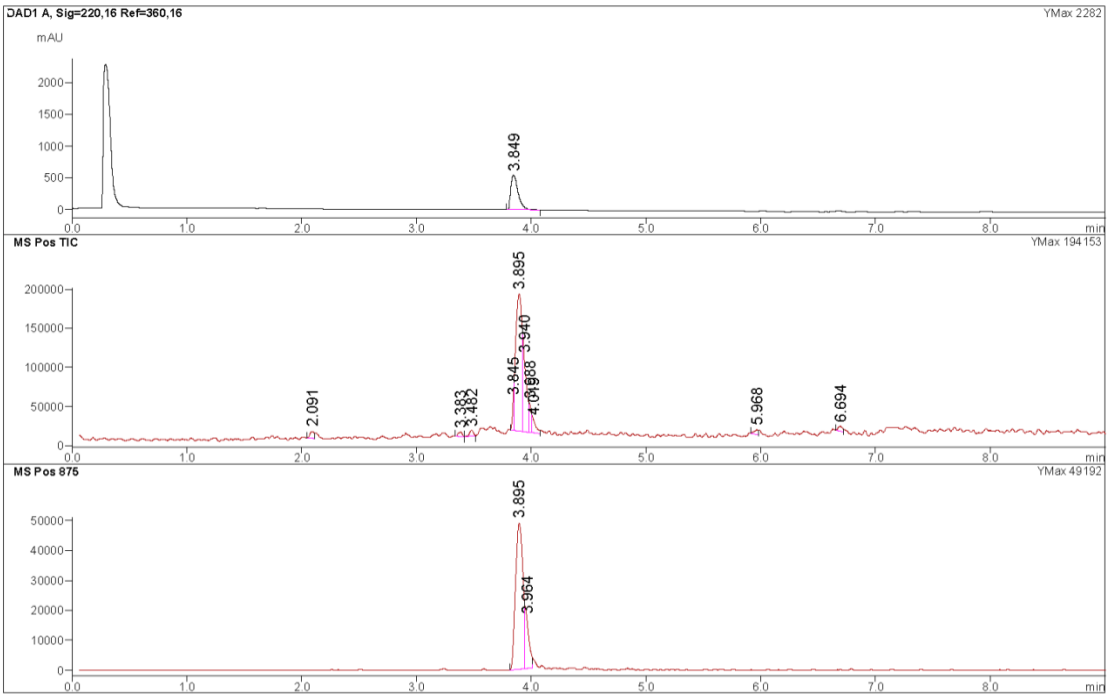
Max Abs/Em 544/574 nm



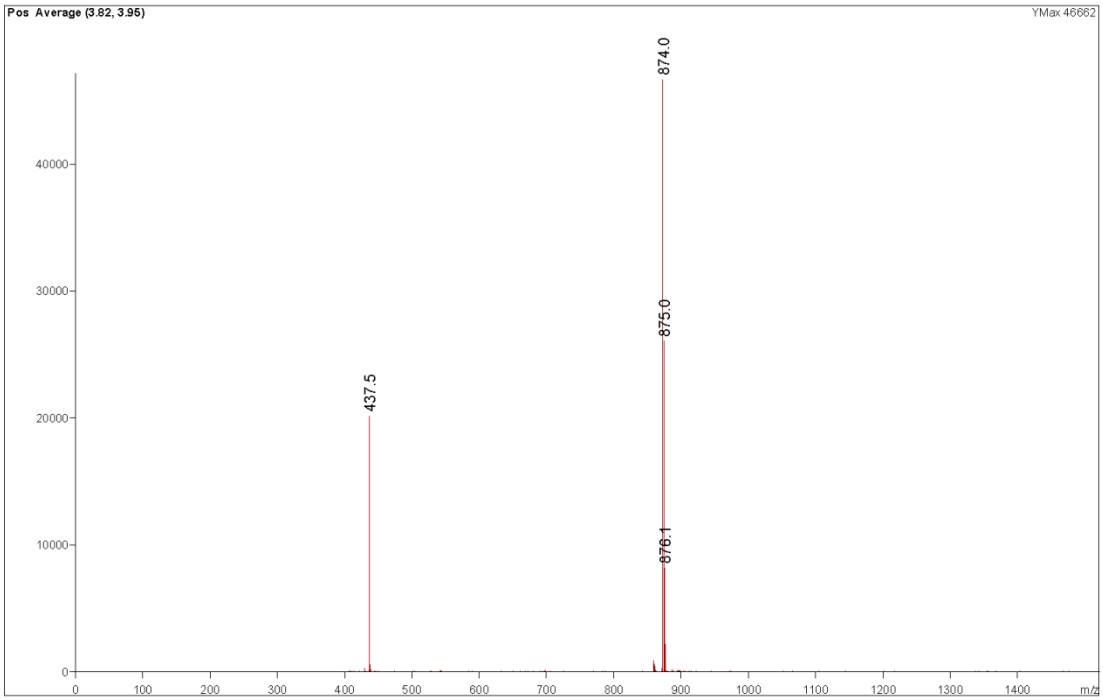
Fluorescence Ex/Em spectra of TAK-TAMRA in MeOH



Data File	C:\Daten\2015\NOVEMBER\16NOV0001126.D	Sample Name	RBE-246 TAK-TAMRA		
Injection Date	26-Nov-2015 19:33:34	Vial	8	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	9	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	874				



Data File	C:\Daten\2015\NOVEMBER\16NOV0001126.D	Sample Name	RBE-246 TAK-TAMRA		
Injection Date	26-Nov-2015 19:33:34	Vial	8	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	9	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	874				



Synthesis of probe **18** (TAK-Bodipy 650)

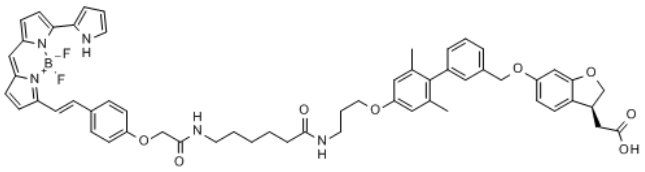
Compound **4** (2.6 mg, 3.92 µmol) was dissolved in 500 µL of DMF in a small Ependorf vial protected from light with aluminum foil, followed by DIEA (2.6 µL, 14.89 µmol). Then Bodipy 650/665-X NHS ester (2.1 mg, 3.26 µmol) was added to the mixture. Reaction was allowed to run overnight in the dark.

The crude was directly purified through preparative HPLC (25->95% ACN) to afford, after lyophilization, the desired product **18** TAK-Bodipy650 conjugate a dark blue powder (m = 2.0 mg, 2.02 µmol, 62% yield).

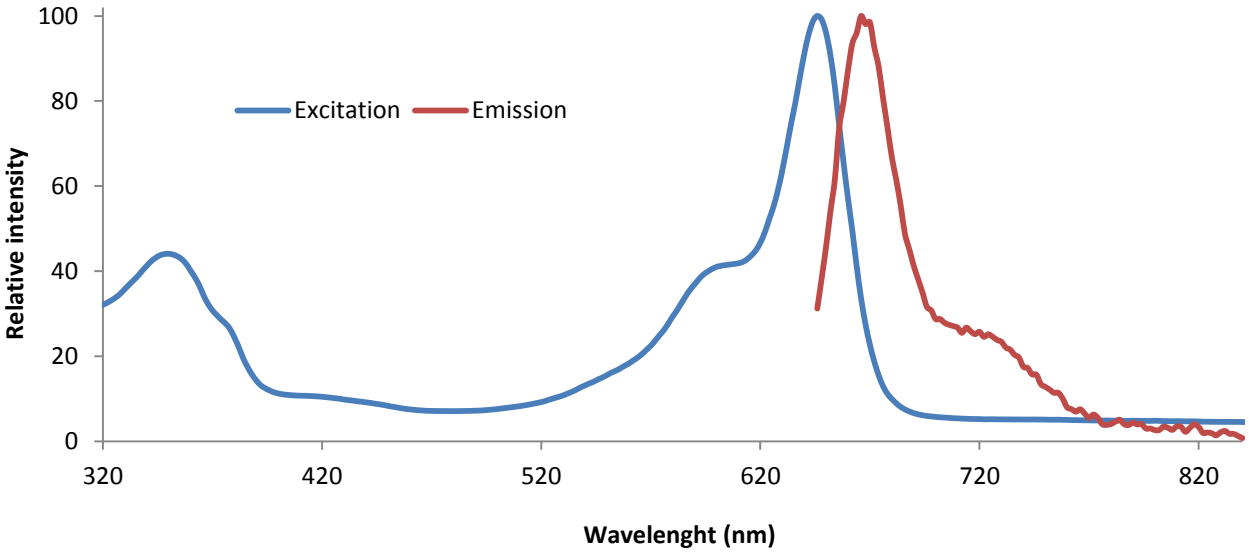
Retention time Rt = 4.925 min (METHOD 3)

HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{57}H_{58}BF_2N_5O_8$: 988.4383; Found: 988.4457

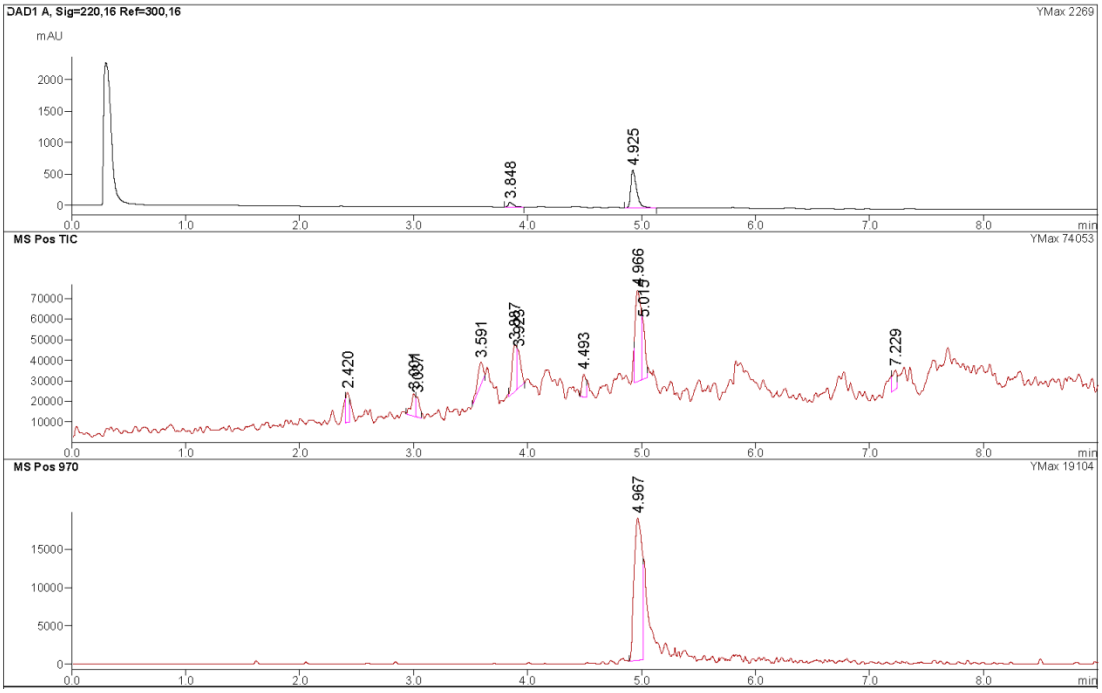
Max Abs/Em 646/666 nm



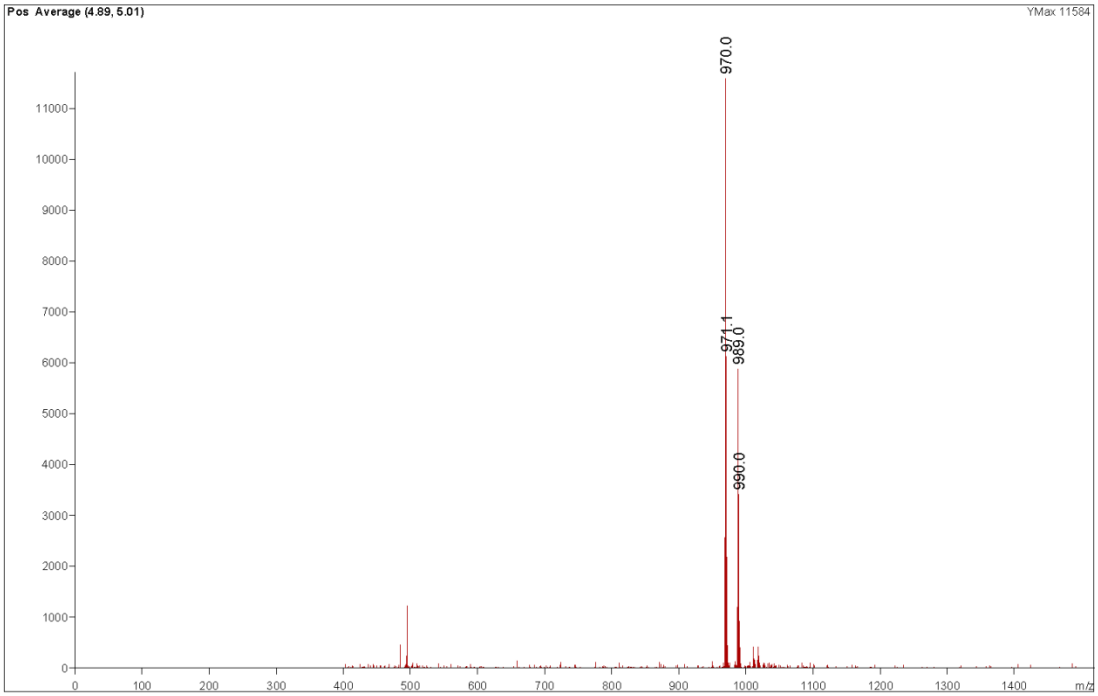
Fluorescence Ex/Em spectra of TAK-Bodipy in MeOH



Data File	C:\Daten\2015\NOVEMBER\16NOV0001191.D	Sample Name	RBE-245- TAK-Bodipy		
Injection Date	27-Nov-2015 14:52:03	Vial	11	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	1	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	969, 989				



Data File	C:\Daten\2015\NOVEMBER\16NOV0001191.D	Sample Name	RBE-245- TAK-Bodipy		
Injection Date	27-Nov-2015 14:52:03	Vial	11	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	1	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	969, 989				



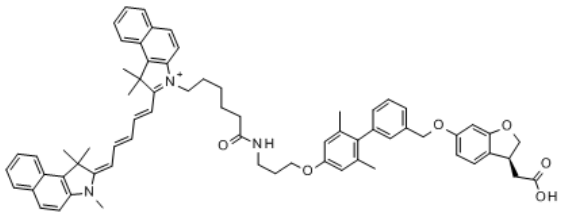
Synthesis of probe **19** (TAK-Cy5.5)

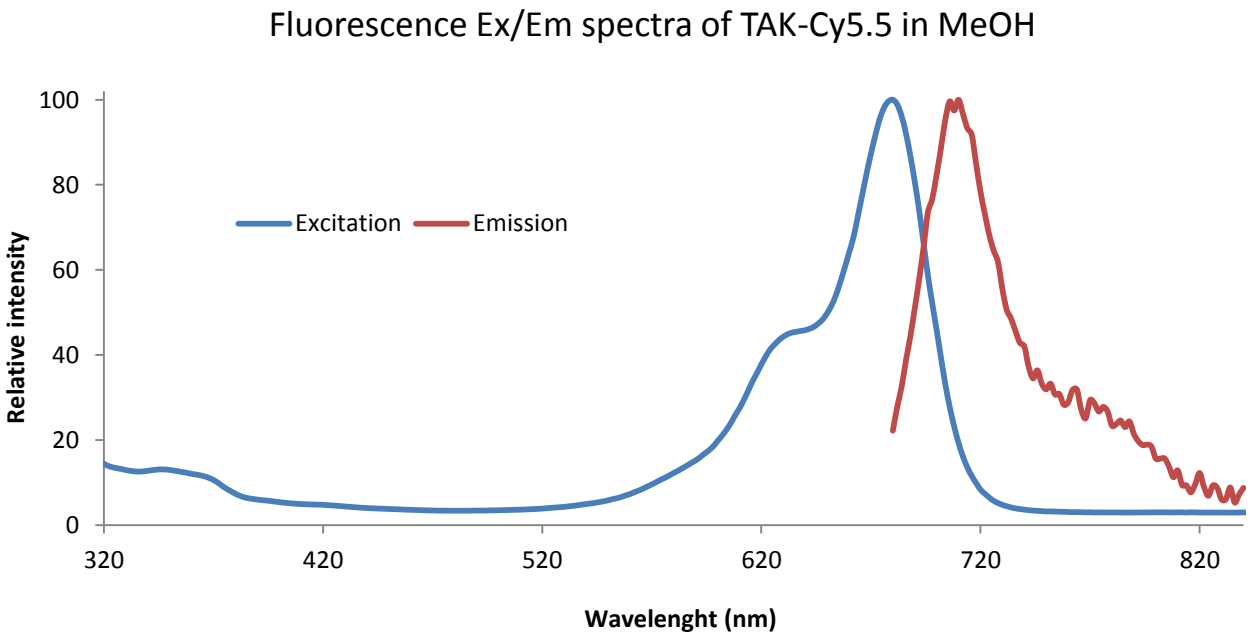
Compound **4** (10.2 mg, 20.52 µmol) was dissolved in 400 µL of DMF in a small Eppendorf vial protected from light with aluminum foil, followed by DIEA (10 µL, 57.41 µmol). Cy5.5 NHS ester (17 mg, 23.72 µmol) was then added to the mixture. Reaction was allowed to run for 10 minutes in the dark. The crude was directly purified through preparative HPLC (25->95% ACN) to afford, after lyophilization, the desired product **19** TAK-Cy5.5 conjugate as a dark blue powder (m = 10.0 mg, 9.73 µmol, 47% yield).

Retention time Rt = 5.096 min (METHOD 3)

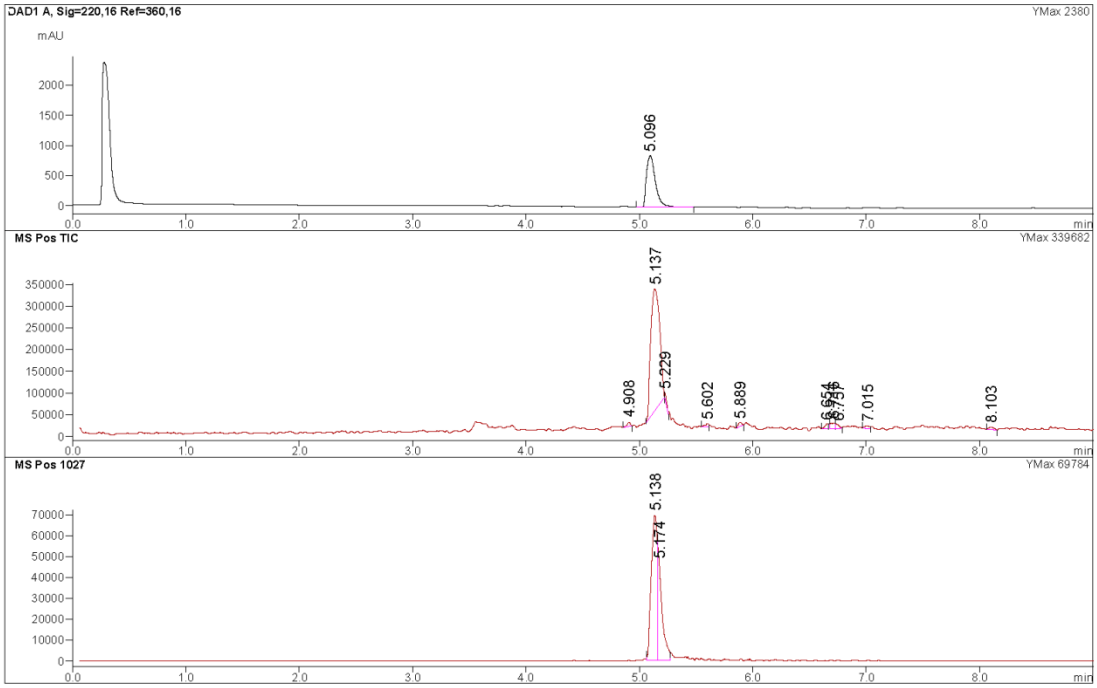
HRMS (ESI-TOF) m/z: $[M-H]^-$: Calcd for $C_{68}H_{72}N_3O_6$: 1026.5421; Found: 1026.5445

Max Abs/Em 680/710 nm

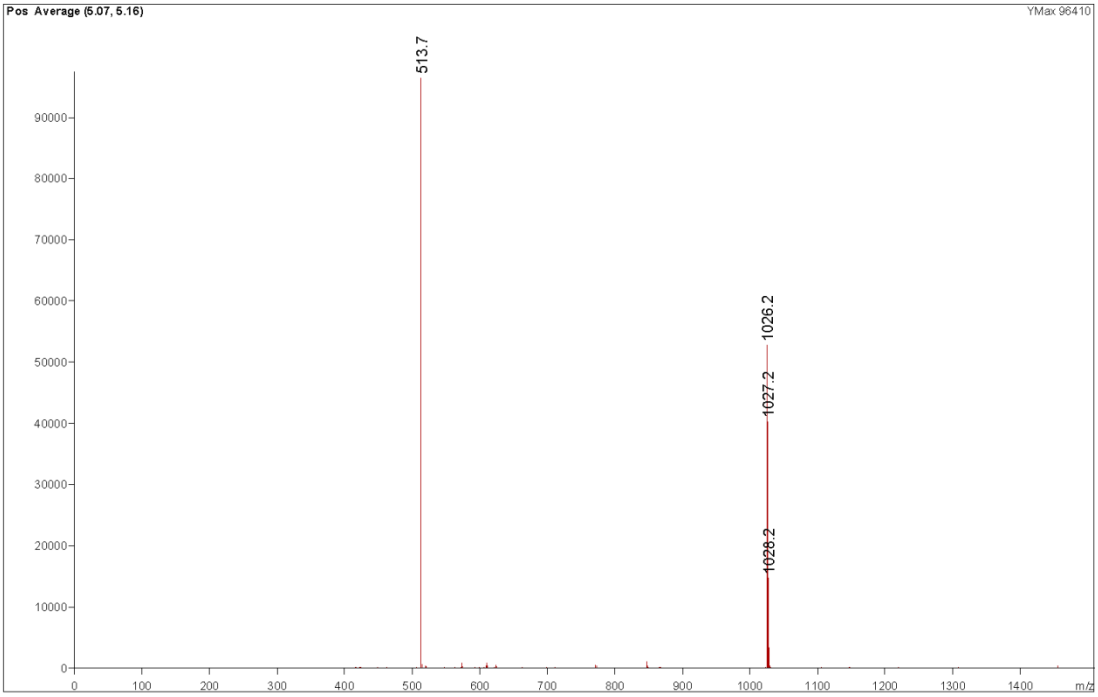




Data File	C:\Daten\2015\NOVEMBER\16NOV0001125.D	Sample Name	RBE-137 TAK-Cy5.5		
Injection Date	26-Nov-2015 19:22:31	Vial	6	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	8	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	1026				



Data File	C:\Daten\2015\NOVEMBER\16NOV0001125.D	Sample Name	RBE-137 TAK-Cy5.5		
Injection Date	26-Nov-2015 19:22:31	Vial	6	Replicate	1
LCMS Method	XBR_SHORT_15_95.M	Sequence Line	8	Injection Volume	2
0 min 85%H2O(0.05%TFA) 6 min-95%ACN; 8.5 min 95%ACN; 10 min 15 %ACN; 0.6 ml/min; 400-1500MW; 2 µL; 50X2.1; 2.5µ; XBridge OST C18; Autosampler					
Sample Info	1026				



Species homology of FFAR1

According to database searches in http://www.expasy.org, the homology of FFAR 1 between the different species is as follows:

Homology FFAR1 Human // FFAR1 Mouse: 83%
Homology FFAR1 Human // FFAR1 Rat: 81.7%
Homology FFAR1 Rat // FFAR1 Mouse: 95.7%

(http://www.uniprot.org/uniprot/O14842)