Electronic Supplementary Information

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General Remarks

All solvents and reagents were purchased from Sigma Aldrich, Merck, Alfa Aesar or Acros and used as supplied without further purification. All reactions using anhydrous conditions were performed under an argon atmosphere in oven-dried glassware.

Reactions were followed by thin layer chromatography (TLC) using coated silica gel plates (Merck, aluminum sheets, silica gel 60 coated with fluorescent indicator F254) and visualized by UV light, potassium permanganate and ninhydrin staining. Flash column chromatography was performed using Merck silica gel 60 (230-400 mesh ASTM) eluting with various solvent mixtures

NMR spectra were obtained on a Bruker Fourier 300 (300 MHz) using deuterated chloroform, methanol or dimethylsulfoxide as solvents and $(CH_3)_4Si$ (^1H) as internal standard; chemical shifts, δ, are expressed in ppm, and coupling constants, J, are expressed in Hz. Multiplicities are given as: bs (broad singlet), s (singlet), d (doublet), dd (double doublet), dt (double triplet), t (triplet), td (triple doublet), tt (triple triplet), q (quartet), quint (quintuplet) and m (multiplet).

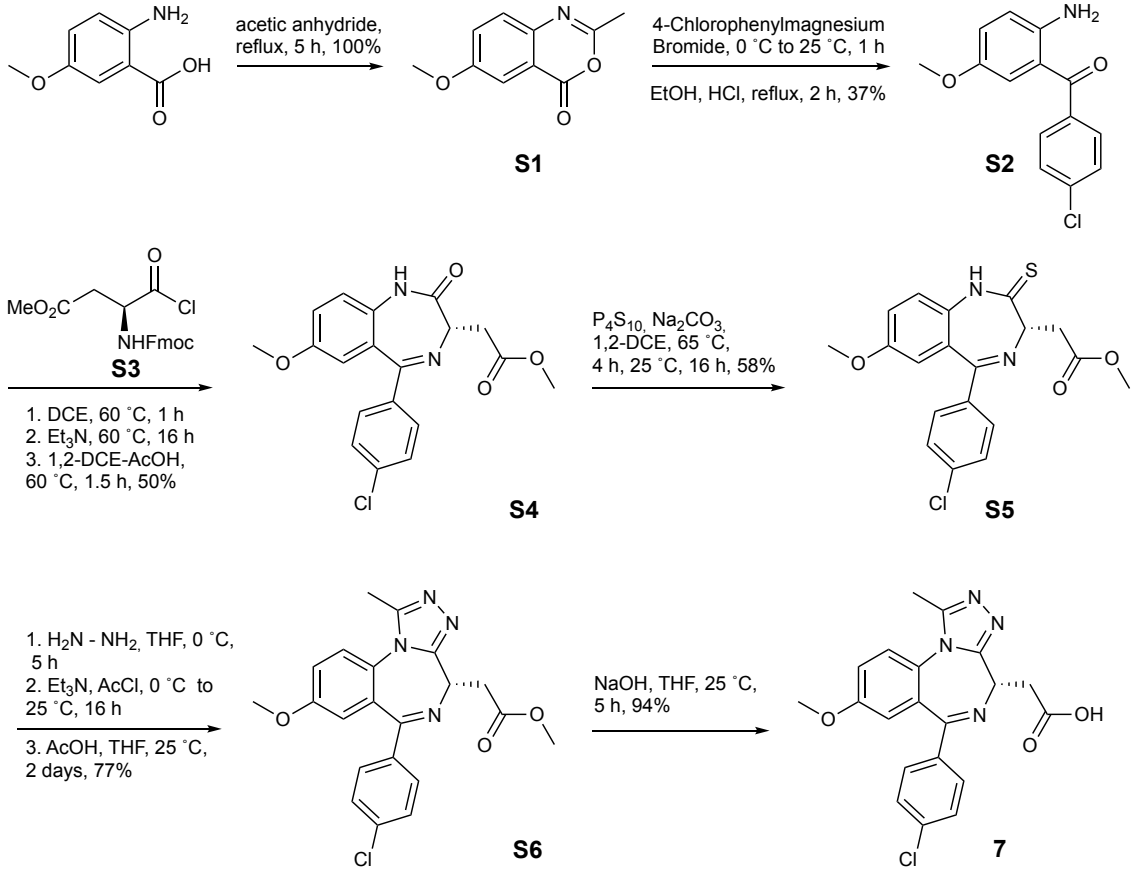
Low-resolution mass spectrometry (LRMS) analysis was recorded on a Waters Quattro micro API and High-Resolution mass spectrometry (HRMS) on a Bruker Daltonics microTOF ESI-TOF mass spectrometer. Calculated and exact m/z values are indicated in Daltons.

For the synthesis of I-BET762 derivatives, their precursors and all fluorescent derivatives, analytical reversed- phase high pressure liquid chromatography (HPLC) was performed on a Waters Separations Module with a Dual Absorbance Detector using a VDspher 100 C18 5 µm, 250 x 4.6 mm column at a flow rate of 1 mL/min with linear gradients of 97 % A/ 3 % B to 100 % B over 15 min (A = Millipore water, B = ACN).

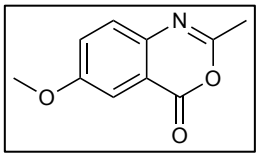
Chemical Synthesis

Synthesis and characterization of I-BET762 carboxylic acid derivative

Literature basis[1].



1. Synthesis of 2-methyl-6-(methyloxy)-4H-3,1-benzoaxazin-4-one (**S1**)

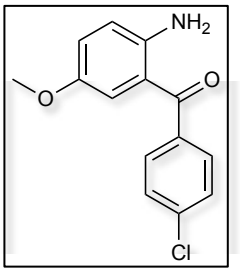


A solution of 2-amino-5-methoxybenzoic acid (2 g, 12 mmol) was refluxed in acetic anhydride (11 mL) for 5 h. The reaction mixture was then concentrated twice in the presence of toluene. The crude compound was washed with diethyl ether to afford the product **S1** as a brown solid (2.32 g, 100%).

^{1}H-NMR (300 MHz, Chloroform-*d*) δ 7.56 (d, *J* = 2.9 Hz, 1H), 7.48 (d, *J* = 8.9 Hz, 1H), 7.35 (dd, *J* = 8.9, 2.8 Hz, 1H), 3.90 (s, 3H), 2.45 (s, 3H).

NMR is in accordance with the literature's report.[1]

2. Synthesis of 2-amino-5-(methyloxy)phenyl](4-chlorophenyl)methanone (**S2**)

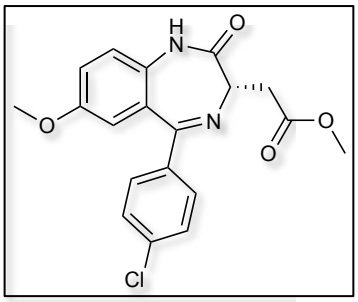


To a solution of **S1** (2.32 g, 12 mmol, 1.24 eq) in a toluene/ether mixture (2/1, 45 mL anhydrous) at -80 ºC was added dropwise a solution of 4-chlorophenylmagnesium bromide (9.7 mL, 9.7 mmol of 1 M solution in Et_2O, 1 eq). The reaction mixture was left at -80 ºC for 30 min. and then allowed to warm up to room temperature. The mixture was stirred at room temperature for 1 h before being quenched with 1 N HCl (12 mL). The aqueous layer was extracted with EtOAc (3 times) and the combined organics were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure to afford the intermediate amide. The crude compound was then dissolved in ethanol (25 mL) and 6 N HCl (9 mL) was added. The mixture was refluxed for 2 h before being concentrated under reduced pressure. EtOAc was added and the mixture neutralized with 1 N NaOH. The aqueous layer was extracted with EtOAc (3x) and the combined organics washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude mixture was purified by chromatography on silica gel with a gradient of hexane:EtOAc (20:80 to 100 % EtOAc) to afford compound **S2** as yellow solid (1.16 g, 37%).

^{1}H-NMR (300 MHz, DMSO-*d6*) δ 7.66 – 7.54 (m, 4H), 7.05 (dd, *J* = 9.0, 3.0 Hz, 1H), 6.84 (d, *J* = 9.0 Hz, 1H), 6.72 (d, *J* = 2.9 Hz, 1H), 3.56 (s, 3H). **LRMS**: (m/z) $[M+H]^+$ calcd for $C_{14}H_{12}ClNO_2Na$, 284.0; found 284.0.

NMR is in accordance with the literature's report.[1]

3. Synthesis of Methyl[5-(4-chlorophenyl)-7-methyloxy)-2-oxo-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl]acetate (**S4**)

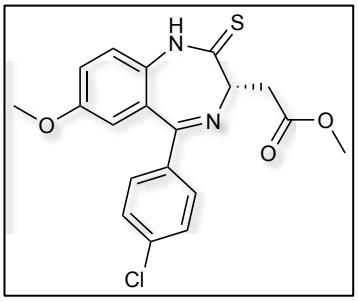


N-Fmoc-L-aspartic acid 4-methyl ester (1.63 g, 4.5 mmol, 1.02 eq) was dissolved in anhydrous DCM (30 mL) and $SOCl_2$ (4.8 mL, 66 mmol, 15 eq) was added to the reaction mixture dropwise at 0 ºC. The mixture was refluxed for 2 h before being concentrated under reduced pressure to afford intermediate **S3**. The crude compound was dissolved in anhydrous 1,2-DCE (20 mL) and **S3** (1.16 g, 4.4 mmol, 1 eq) was added. The resulting mixture was refluxed for 2 h and cooled to room temperature before adding Et_3N (2.45 mL, 17.6 mmol, 4 eq). The reaction was refluxed for 16 h. The resulting mixture was concentrated to dryness and the crude dissolved in 1,2-DCE (50 mL). AcOH (2.8 mL, 48.4 mmol, 11 eq) was added carefully and the reaction stirred for 2 h at 60 ºC before being concentrated in vacuum and dissolved in DCM. The organic layer was washed with 1 N HCl and the aqueous layer extracted with DCM (3 times). The combined organic layers were washed with water, brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude was recrystallized in CH_3CN to afford **S4** as an off-white colored solid (0.83 g, 50%).

^{1}H-NMR (300 MHz, DMSO-*d6*) δ 10.52 (s, 1H), 7.50 (s, 4H), 7.23 (s, 2H), 6.75 (d, *J* = 2.4 Hz, 1H), 3.92 (d, *J* = 7.1 Hz, 1H), 3.70 (s, 3H), 3.61 (s, 3H), 3.27 – 3.14 (m, 1H), 3.03 (dd, *J* = 16.6, 6.6 Hz, 1H). **LRMS**: (m/z) $[M+H]^+$ calcd for $C_{19}H_{18}ClN_2O_4$, 373.1; found 373.0

NMR is in accordance with the literature's report.[1]

4. Synthesis of Methyl-[5-(4-chlorophenyl)-7-(methyloxy)-2-oxo-2,3-dihydro-1*H*-benzodiazepin- 3-yl]acetate (**S5**)

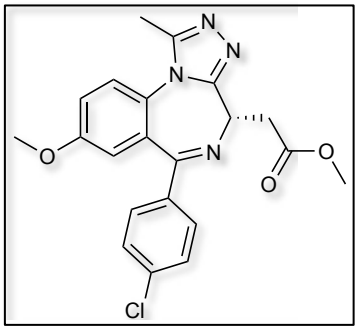


P_4S_{10} (3 g, 13.7 mmol, 1.8 eq) and Na_2CO_3 (1.5 g, 13.7 mmol, 1.8 eq) were dissolved in 1,2-DCE (50 mL) and stirred at room temperature for 2 h. The amide **S4** (2.83 g, 7.6 mmol, 1.0 eq) was dissolved in 1,2-DCE (50 mL) and added to the P_4S_{10} and Na_2CO_3 suspension. The resulting reaction was stirred for 4 h at 65 ºC followed by 16 h at room temperature.
The obtained residue was taken in a saturated $NaHCO_3$ solution and extracted with DCM (3 times). The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude compound was then recrystallized with DCM/*i*-Pr_2O for 12 h to yield compound **S5** as a yellow solid (1.7 g, 58%).

^{1}H-NMR (300 MHz, Chloroform-*d*) δ 7.49 (d, *J* = 8.6 Hz, 2H), 7.36 (d, *J* = 2.0 Hz, 2H), 7.20 (d, *J* = 8.9 Hz, 1H), 7.12 (dd, *J* = 8.8, 2.8 Hz, 1H), 6.77 (d, *J* = 2.8 Hz, 1H), 4.38 (t, *J* = 6.8 Hz, 1H), 3.75 (s, 3H), 3.73 (s, 3H), 3.63 (dd, *J* = 16.8, 6.5 Hz, 1H), 3.38 (dd, *J* = 16.9, 7.1 Hz, 1H). **LRMS**: (m/z) $[M+H]^+$ calcd for $C_{19}H_{18}ClN_2O_3S$, 389.1; found 388.8.

NMR is in accordance with the literature's report.[1]

5. Synthesis of Methyl-2-((4S)-6-(4-Chlorophenyl)-8-methoxy-1-methyl-4H-benzol[f][1,2,4]triazolo[4,3-a][1,4]diazepin-4-yl)acetate (**S6**)

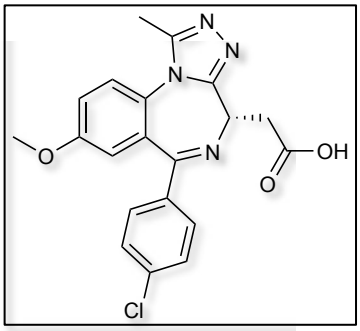


The thioamide **S5** (1.7 g, 4.4 mmol, 1 eq) was suspended in THF (65 mL) and hydrazine monohydrate added (0.64 mL, 13.2 mmol, 3 eq) at 0 ºC dropwise. The mixture was stirred for 5 h at 0 ºC followed by the addition of Et_3N (1.84 mL, 13.2 mmol, 3 eq) and AcCl (0.94 mL, 13.2 mmol, 3 eq) dropwise. The reaction mixture was then allowed to warm to room temperature and stirred for 16 h before being concentrated under reduced pressure. The crude compound was dissolved in DCM and washed with water. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the intermediate hydrazone. The intermediate was dissolved in THF (65 mL) and AcOH (10 mL) was added. The reaction mixture stirred for 2 days before being concentrated under reduced pressure. The crude solid was triturated in *i*-Pr_2O and decanted to give compound **S6** (1.4 g, 77%).

^{1}H-NMR (300 MHz, Chloroform-*d*) δ 7.53 – 7.45 (m, 2H), 7.39 (d, *J* = 9.0 Hz, 1H), 7.36 – 7.29 (m, 2H), 7.20 (dd, *J* = 8.9, 3.0 Hz, 1H), 6.88 (d, *J* = 2.9 Hz, 1H), 4.59 (dd, *J* = 7.7, 6.4 Hz, 1H), 3.80 (s, 3H), 3.62 (d, *J* = 2.8 Hz, 1H), 3.60 (d, *J* = 1.6 Hz, 1H), 2.60 (s, 3H). **LRMS**: (m/z) $[M+H]^+$ calcd for $C_{21}H_{20}ClN_4O_3$, 411.1; found 411.0

NMR is in accordance with the literature's report.[1]

6. Synthesis of 6-(4-Chlorophenyl)-1-methyl-8-(methyloxy)-4*H*-[1,2,4]triazolo[4,3-a)[1,4]benzodiazepin-4-yl]acetic acid (**7**)



Compound **S6** (1.4 g, 3.4 mmol) was dissolved in THF (40 mL) and a solution of 1 N NaOH (7 mL) was added. The reaction mixture was stirred for 5 h at room temperature before being quenched with 0.5 N HCl (14.3 mL) and concentrated under reduced pressure. Water was added to the residue and the aqueous layer was extracted with DCM (3 times). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford compound **7** as a yellow solid (1.3 g, 94%).

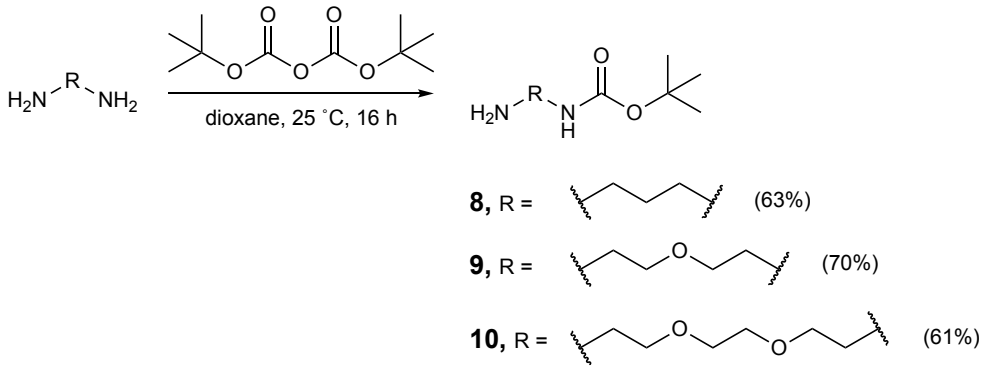
^{1}H-NMR (300 MHz, Chloroform-*d*) δ 7.51 (d, *J* = 8.6 Hz, 2H), 7.44 (d, *J* = 8.9 Hz, 1H), 7.33 (d, *J* = 8.6 Hz, 2H), 7.24 – 7.20 (m, 1H), 6.89 (d, *J* = 2.8 Hz, 1H), 4.58 (t, *J* = 6.9 Hz, 1H), 3.81 (s, 3H), 3.63 (dd, *J* = 9.9, 7.0 Hz, 2H), 2.64 (s, 3H). **LRMS**: (m/z) $[M+H]^+$ calcd for $C_{20}H_{18}ClN_4O_3$, 396.8; found 396.9.

NMR is in accordance with the literature's report.[1]

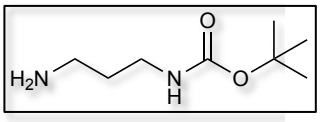
Synthesis and characterization of fluorescein-labelled I-BET762

Diamine protection

Literature basis[2].



1. Synthesis of *tert*-butyl-(3-aminopropyl)carbamate (**8**)

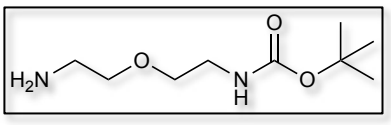


1,3-diaminopropane (1.14 mL, 13.7 mmol) was dissolved in dry dioxane (3 mL) and stirred at room temperature. Di-*tert*-butyl dicarbonate (0.52 mL, 2.3 mmol) was added in dioxane (2 mL) dropwise to the reaction mixture. This was stirred for 12 h and concentrated under reduced pressure. The residue was dissolved in water and rinsed with DCM (3 times). The combined organics were rinsed with brine, dried over $MgSO_4$, filtered and concentrated in vacuum to afford compound **8** as clear oil (251.4 mg, 63%).

^{1}H-NMR (300 MHz, Chloroform-*d*) δ 4.90 (s, 1H), 3.29 – 3.07 (m, 3H), 2.77 (t, *J* = 6.6 Hz, 1H), 1.91 (bs, 2H), 1.61 (q, *J* = 6.8 Hz, 2H), 1.43 (s, 9H). **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_8H_{19}N_2O_2$,175.1441; found 175.1449.

NMR is in accordance with the literature's report.[2]

2. Synthesis of *tert*-butyl (2-(2-aminoethoxy)ethyl)carbamate (**9**)

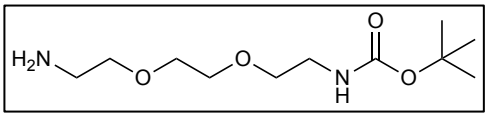


2,2'-oxydiethanamine (0.3 mL, 2.76 mmol) was dissolved in dry dioxane (3 mL) and stirred at room temperature. Di-*tert*-butyl dicarbonate (0.1 mL, 0.46 mmol) was added in dioxane (2 mL) dropwise to the reaction mixture. This was stirred for 12 h and concentrated under reduced pressure. The residue was dissolved in water and rinsed with DCM (3 times). The combined organics were rinsed with brine, dried over $MgSO_4$, filtered and concentrated in vacuum to afford compound **9** as clear oil (66 mg, 70%).

^{1}H-NMR (300 MHz, Chloroform-*d*) δ 4.99 (s, 1H), 3.47 (m, *J* = 10.7, 5.3 Hz, 4H), 3.34 – 3.17 (m, 2H), 2.84 (t, *J* = 5.2 Hz, 2H), 1.69 – 1.46 (m, 2H), 1.41 (d, *J* = 0.9 Hz, 9H); **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_9H_{21}N_2O_3$, 205.1547; found 205.1543.

NMR is in accordance with the literature's report.[2]

3. Synthesis of *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (**10**)



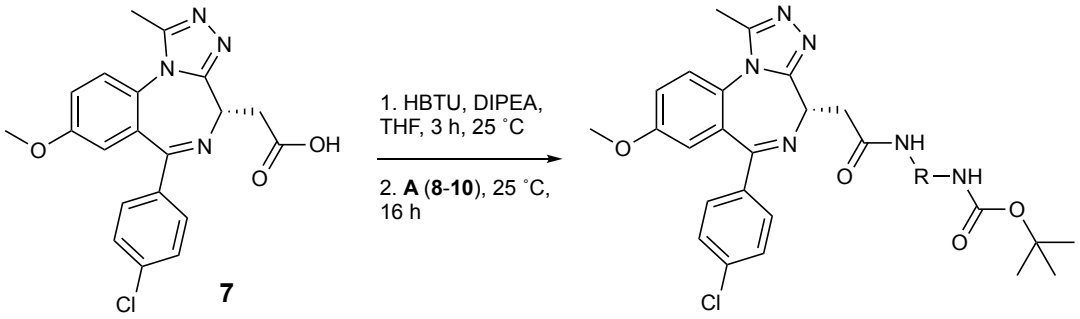
To a solution of 2,2'-(ethylenedioxy)-bis[ethylamine] (5.4 mmol, 5.9 eq), in dioxane (4 mL) was added dropwise at room temperature over 2 h a solution of di-*tert*-butyl dicarbonate (200 mg, 0.92 mmol, 1 eq) in dioxane (1 mL). The mixture was stirred for 12 h and then concentrated under reduced pressure. The residue was dissolved in water and rinsed with DCM (3 times). The combined organic layers were rinsed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo to give **10** (139 mg, 61%) of a pale yellow oil.

^{1}H-NMR (300 MHz, Chloroform-*d*) δ 5.20 (s, 1H), 3.58 (s, 4H), 3.49 (dt, *J* = 8.9, 5.2 Hz, 4H), 3.28 (d, *J* = 5.3 Hz, 2H), 2.89 – 2.77 (m, 2H), 1.74 (s, 3H), 1.40 (s, 9H). **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_{11}H_{24}N_2O_4$, 249.1800; found 249.1809.

NMR is in accordance with the literature's report.[2]

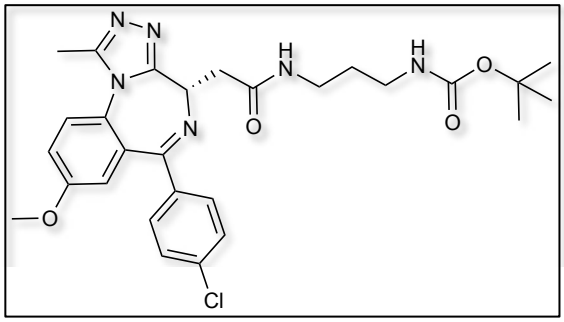
Conjugation of I-BET762 carboxylic derivative with Boc-protected diamine spacer

Literature basis[3].



11, A = compound **8** (45%)
12, A = compound **9** (75%)
13, A = compound **10** (56%)

1. Synthesis of compound **11**

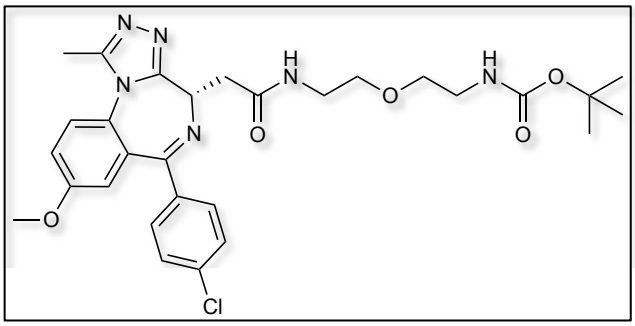


The carboxylic acid **7** (100 mg, 0.26 mmol) was degassed under vacuum and refilled with argon prior adding anhydrous THF (3 mL). To this solution was added DIPEA (87 µL, 0.52 mmol, 2 eq) and HATU (190 mg, 0.52 mmol, 2 eq). The reaction was stirred for 3 h at room temperature, time at which compound **8** (66 mg, 0.38 mmol, 1.5 eq) was added dropwise to the mixture. This was stirred 12-14 h at room temperature before being concentrated under reduced pressure. The crude material was dissolved in DCM and washed with 1N HCl. The aqueous phase was extracted twice with DCM. The combined organics were then washed with 1 N NaOH followed by brine, dried in $MgSO_4$, filtered and concentrated under reduced pressure to afford compound **11** as a yellow solid (64 mg, 45%).

^{1}H-NMR (300 MHz, Chloroform-*d*) δ 7.84 (s, 1H), 7.47 – 7.41 (m, 2H), 7.37 (d, *J* = 8.9 Hz, 1H), 7.31 – 7.25 (m, 2H), 7.17 (dd, *J* = 8.9, 2.9 Hz, 1H), 6.84 (d, *J* = 2.9 Hz, 1H),

5.76 (s, 1H), 4.69 (dd, *J* = 7.9, 6.0 Hz, 1H), 3.77 (s, 3H), 3.45 (dq, *J* = 21.1, 14.1, 7.1 Hz, 3H), 3.15 (d, *J* = 6.0 Hz, 2H), 2.61 (s, 3H), 1.67 (p, *J* = 6.6 Hz, 2H), 1.49 (bs, 2H), 1.40 (s, 9H) signals from the PEG linker predicted to overlap. **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_{28}H_{33}ClN_6NaO_4$, 575.2132; found 575.2144.

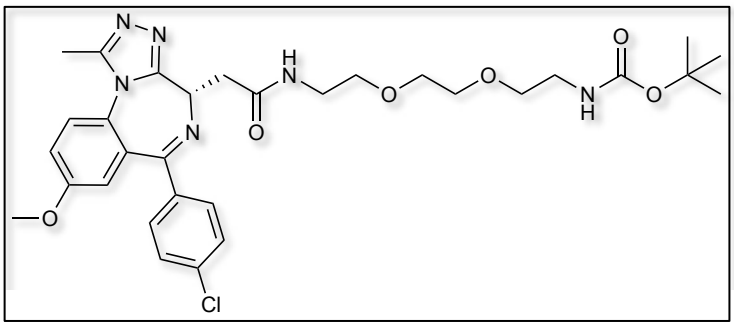
2. Synthesis of compound **12**



The carboxylic acid **7** (52 mg, 0.13 mmol) was degassed under vacuum and refilled with argon prior adding anhydrous THF (6 mL). To this solution was added DIPEA (45 µL, 0.26 mmol) and HBTU (98.6 mg, 0.26 mmol). The reaction was stirred for 3 h at room temperature, time at which compound **9** (40 mg, 0.19 mmol) was added dropwise to the mixture. This was stirred for 12 h before being concentrated under reduced pressure. The crude material was dissolved in ethyl acetate and washed successively with saturated $NaHCO_3$, water and brine, before drying in $MgSO_4$, filtering and concentrating under reduced pressure to afford compound **12** as a yellow solid (64 mg, 75%).

^{1}H-NMR (300 MHz, Chloroform-*d*) δ 7.53 – 7.46 (m, 2H), 7.36 – 7.30 (m, 3H), 7.20 (dd, *J* = 9.0, 2.9 Hz, 1H), 6.86 (d, *J* = 2.9 Hz, 1H), 5.38 (s, 1H), 4.89 (s, 1H), 4.67 – 4.54 (m, 1H), 3.80 (s, 3H), 3.74 – 3.62 (m, 2H), 3.52 (t, *J* = 5.6 Hz, 4H), 3.34 – 3.28 (m, 4H), 3.00 (s, 2H), 2.80 (s, 1H), 2.63 (s, 3H), 1.44 (d, *J* = 1.1 Hz, 9H); signals from the PEG linker predicted to overlap. **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_{29}H_{35}ClN_6NaO_5$, 605.2250; found 605.2244.

3. Synthesis of compound **13**

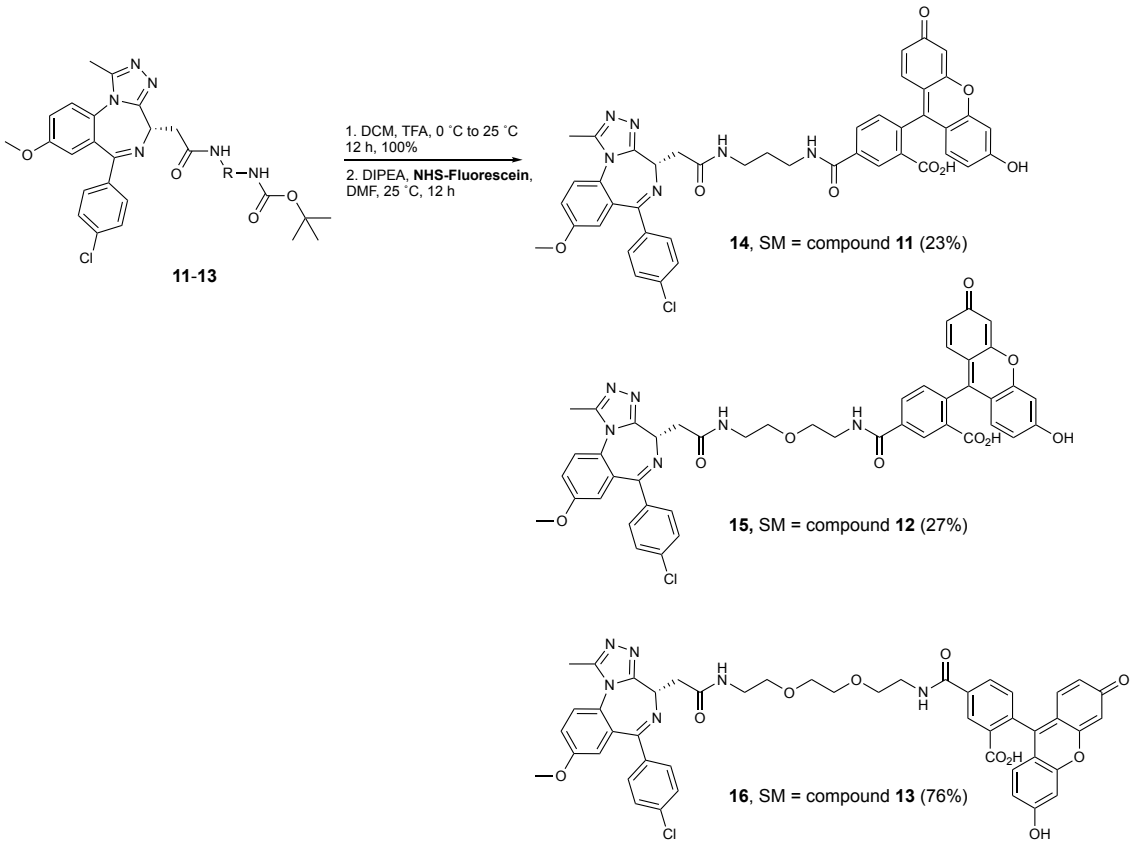


To a solution of acid **7** (100 mg, 0.26 mmol) in THF at room temperature was added DIPEA (90 µL, 0.52 mmol, 2 equiv.) followed by HATU (197.7 mg, 0.52 mmol, 2 equiv.). The reaction mixture was stirred for 3 h at this temperature and compound **10** (0.52 mmol, 2 equiv.) was added dropwise. The mixture was stirred for 12 h before being concentrated under reduced pressure. The crude material was dissolved in DCM and washed with 1 N HCl. The aqueous layer was extracted twice with DCM. The combined organics were washed with 1 N NaOH followed by brine, dried over $MgSO_4$ filtered and concentrated in vacuo. The residue was purified by flash-chromatography on silica using dichloromethane/methanol 95/5 to give compound **13** as a yellow solid (73.6 mg, 56%).

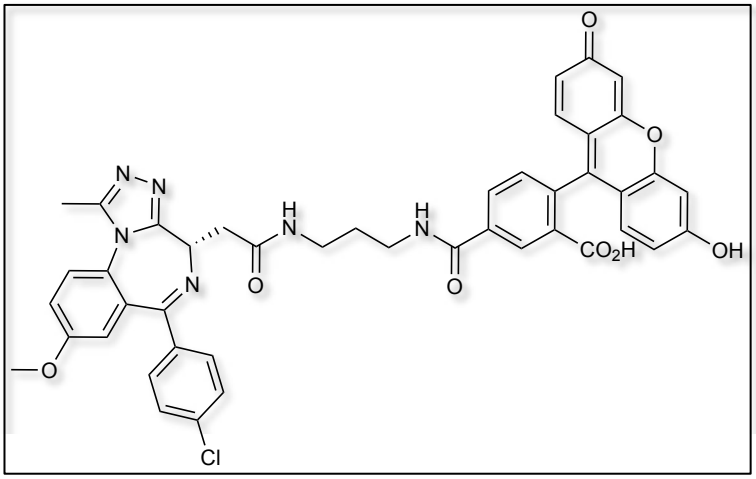
^{1}H-NMR (300 MHz, Chloroform-*d*) δ 7.47 (d, *J* = 8.6 Hz, 2H), 7.36 (d, *J* = 8.9 Hz, 1H), 7.29 (d, *J* = 8.6 Hz, 2H), 7.17 (dd, *J* = 8.9, 2.9 Hz, 1H), 7.02 (s, 1H), 6.82 (d, *J* = 2.8 Hz, 1H), 5.39 – 5.29 (m, 1H), 4.61 (t, *J* = 7.0 Hz, 1H), 3.76 (s, 3H), 3.60 (s, 4H), 3.54 (t, *J* = 5.2 Hz, 4H), 3.51 – 3.44 (m, 3H), 3.33 (dq, *J* = 10.6, 6.3, 5.5 Hz, 3H), 2.58 (s, 3H), 1.39 (s, 9H). **HRMS (ESI)**: m/z calcd $[M+H]^+$ for $C_{31}H_{39}ClN_6O_6$, 627.2692; found 627.2683.

Conjugation of I-BET762-diamine linker with NHS-fluorescein

Literature basis[3].

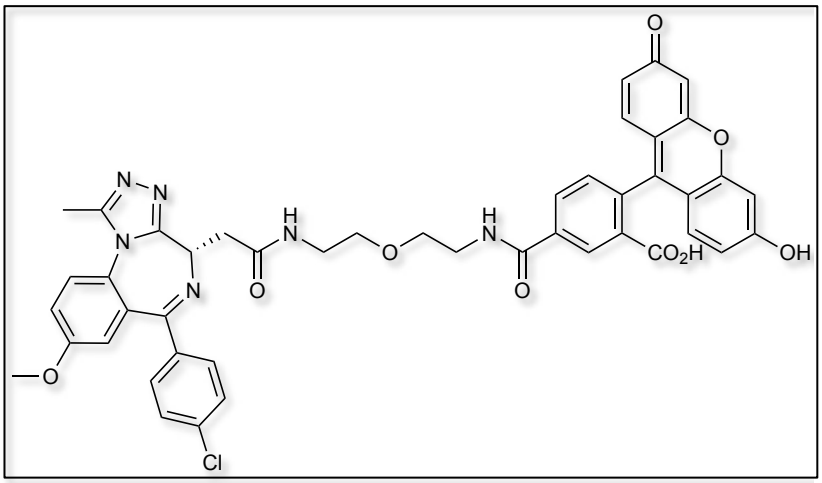


1. Synthesis of compound **14**



Compound **11** (58 mg, 0.1 mmol) was degassed under vacuum and refilled with argon (3 times). This was dissolved in DCM (2 mL) and TFA (1.5 mL, 19.4 mmol) was added at 0 ºC. The reaction was left to warm to room temperature and stirred for 2 h. The reaction mixture was concentrated under reduced pressure and the crude analyzed by ^{1}H-NMR. Proton peak integration revealed that the reaction was complete, since Boc was successfully removed (76.4 mg, 0.14 mmol). The TFA salt was then degassed in vacuum and refilled with argon (3 times). The solid was then dissolved in DMF (6 mL) and DIPEA was added (0.24 mL, 0.14 mmol) followed by NHS fluorescein (65.8 mg, 0.14 mmol). The round bottom flask was covered in foil to protect the reaction mixture from light and the reaction was stirred for 12 h at room temperature before concentrating under reduced pressure. Toluene was added 3 times to aid the removal of DMF. The crude compound was purified by preparative TLC with a gradient of acetone:hexane (7:3, two runs) and acetone (100%). The product was extracted in DCM and concentrated to afford **14** as a yellow solid (26 mg; 23%). **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_{44}H_{36}ClN_6O_8$, 811.2278; found 811.2267.

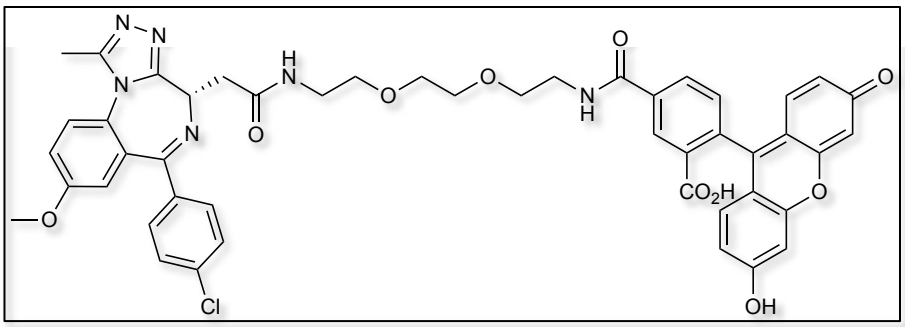
2. Synthesis of compound **15**



Compound **12** (57 mg, 0.098 mmol) was degassed under vacuum and refilled with argon (3 times). This was dissolved in DCM (2 mL) and TFA (1.5 mL, 19.4 mmol) was added at 0 ºC. The reaction was left to warm to room temperature and stirred for 2 h. The reaction mixture was concentrated under reduced pressure and the crude analyzed by ^{1}H-NMR. Proton peak integration revealed that the reaction was incomplete, as Boc was still present in the mixture. The crude was diluted again in DCM (2 mL) and TFA (2 mL, 25.9 mmol) was added at 0 ºC. The reaction was stirred for an additional 5 h at room temperature before being concentrated under reduced pressure. ^{1}H-NMR analysis confirmed that Boc was removed and obtain the intermediate amine in the form of TFA salt, which was used without further purification (62.3 mg, 0.11 mmol). This was then degassed in vacuum and refilled with argon (3 times). The solid was then dissolved in DMF (6 mL) and DIPEA was added (0.19 mL, 0.11 mmol) followed by NHS fluorescein (50 mg, 0.11 mmol). The round bottom flask was covered in foil to protect the reaction mixture from light and the reaction was stirred

for 12 h at room temperature before concentrating under reduced pressure. Toluene was added 3 times to aid the removal of DMF. The crude product was purified by preparative TLC with a gradient of acetone:hexane (7:3, two runs) and acetone (100%). The product was extracted in DCM and concentrated to afford **15** as a yellow solid (25 mg; 27%). **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_{45}H_{38}ClN_6O_9$, 841.2383; found 841.2379.

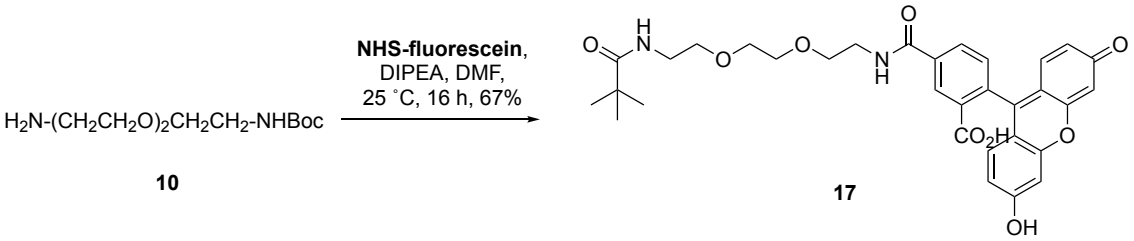
3. Synthesis of compound **16**



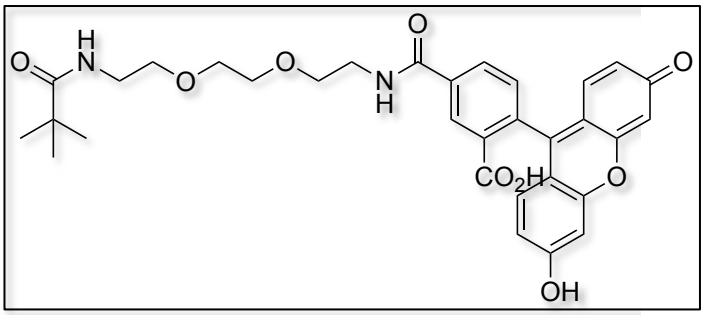
Compound **13** (96.7 mg, 0.15 mmol) was degassed under vacuum, refilled with argon two times and dissolved in DCM (3 mL) Trifluoroacetic acid (TFA) (1 mL, 12 mmol, 80 eq) was added at 0 ºC. The reaction was left to warm to room temperature and stirred for 12 h. The reaction crude was concentrated and Boc deprotection confirmed by ^{1}H-NMR. The deprotected amine was used without further purification. This was dissolved in DMF (4 mL) and DIPEA (296 µL, 1.7 mmol) was added, followed by NHS fluorescein (80.5 mg, 0.17 mmol). The reaction was stirred at room temperature for 12-14 h before being concentrated under reduced pressure. The obtained crude (244 mg) was purified through a column chromatography on silica gel with DCM:MeOH (8:2). The fractions were pooled together and concentrated under reduced pressure to afford the fluorescent (yellow) solid **16** (118 mg, 76%). **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_{47}H_{41}ClN_6O_{10}$, 885.2645; found 885.2635.

Conjugation of Boc-protected diamine linker with NHS-fluorescein

Literature basis[3].



1. Synthesis of compound **17**



Compound **10** (95 mg, 0.38 mmol, 1.6 eq) was degassed under vacuum and refilled with argon (3 times) before dissolving in anhydrous DMF (2 mL). DIPEA (0.33 mL, 1.9 mmol, 5 eq) was added and the mixture stirred for few min. at room temperature. NHS fluorescein (117 mg, 0.24 mmol, 1 eq) was dissolved in the minimum amount of anhydrous DMF possible and added to the reaction vessel under an inert atmosphere. The reaction was stirred for 12 h at room temperature and the crude concentrated under reduced pressure. The crude was purified over a column chromatography on silica gel with DCM:MeOH (85:15). The fractions were pooled and concentrated under reduced pressure to afford compound **17** as a yellow solid (97 mg, 67%).

^{1}H-NMR (300 MHz, Methanol-*d4*) δ 8.43 (d, *J* = 1.5 Hz, 1H), 7.63 (s, 1H), 7.29 (d, *J* = 8.0 Hz, 1H), 6.68 (d, *J* = 2.3 Hz, 2H), 6.62 (d, *J* = 7.4 Hz, 2H), 6.54 (dt, *J* = 8.8, 2.9 Hz, 2H), 3.64 (t, *J* = 3.8 Hz, 4H), 3.53 (ddd, *J* = 14.4, 7.8, 3.8 Hz, 5H), 3.40 (d, *J* = 5.5 Hz, 1H), 3.19 (t, *J* = 5.6 Hz, 1H), 3.11 (t, *J* = 5.6 Hz, 1H), 1.39 (s, 9H). **HRMS (ESI):** m/z calcd $[M+H]^+$ for $C_{32}H_{35}N_2O_{10}$, 607.2286; found 607.2286.

Cellular internalization and trafficking of I-BET-based derivatives

Protein Expression and Purification

Plasmid DNA (BRD4, NCBI accession number NP_490597.1, first bromodomain (BRD4(1)): N44-E168, previously described[4]) was transformed into competent *E. coli* BL21(DE3)-R3-pRARE2 cells (phage-resistant derivative of BL21(DE3) strain), with a pRARE plasmid encoding rare codon tRNAs. Freshly grown colonies were cultured overnight in LB (Luria-Bertani medium) twice concentrated (2X) supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol at 37 ºC. One litre of pre-warmed TB (Terrific Broth medium) was inoculated with 10 mL of the overnight culture and was incubated at 37 ºC. At an OD_{600} nm of 2.5, the culture was cooled down to 18 ºC and expression was induced overnight at 18 ºC with 0.1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG). Cells were then harvested by centrifugation (8,700 x *g*, 15 min, 4 ºC) in a Beckman Coulter Avanti J-20 XP centrifuge, and then re-suspended in lysis buffer (50 mM HEPES, pH 7.5 at 20 °C, 500 mM NaCl, 5% Glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP) and 1:1000 (v/v) Protease Inhibitor Cocktail III (Calbiochem)). Cells were lysed 3 times at 4 °C using a Basic Z Model Cell Disrupter (Constant Systems Ltd, UK) and DNA was removed by precipitation on ice for 30 min with 0.15 % (v/v) of PEI (Polyethyleneimine). Lysates were cleared by centrifugation (16,000 x *g* for 1 h at 4 °C, JA 25.50 rotor, on a Beckman Coulter Avanti J-20 XP centrifuge). Supernatants were applied to nickel-nitrilotriacetic acid agarose columns (Ni-NTA, Qiagen Ltd., 5 mL, equilibrated with 20 mL lysis buffer). The columns were washed once with 30 mL of lysis buffer, then with 20 mL of lysis buffer containing 30 mM Imidazole. Proteins were eluted using a step gradient of Imidazole in lysis buffer (50, 100, 150, 2 x 250 mM Imidazole in 50 mM HEPES, pH 7.5 at 25 ºC, 500 mM NaCl). All fractions were collected and monitored by SDS-polyacrylamide gel electrophoresis (Bio-Rad Criterion™ Precast Gels, 4-12 % Bis-Tris, 1.0 mm, from Bio-Rad, CA.). Half of the eluted proteins was treated overnight at 4 ºC with Tobacco Etch Virus (TEV) protease to remove the hexa-histidine tag (for crystallography and other biophysical experiments). The other half of the proteins was kept with the hexa-histidine tag intact to be used in the SPOT assay experiments. Both tagged and untagged proteins were further purified with size exclusion chromatography on a Superdex 75 16/60 HiLoad gel filtration column (GE/Amersham Biosciences) on an ÄktaPrime™ plus system (GE/Amersham Biosciences). Recombinant BRD4 and BRD9 domains eluted as single symmetrical monomeric peaks. Samples were monitored by SDS-polyacrylamide gel electrophoresis and concentrated to 6-10 mg/mL in the gel filtration buffer, 10 mM HEPES pH 7.5, 500 mM NaCl and 5 % Glycerol using Amicon® Ultra (MILLIPORE) concentrators with a 10 MWCO cut-off. Proteins were aliquoted into 100 µL fractions, flash frozen in liquid nitrogen and stored at -80 ºC until further use. Protein handling was carried out on ice or in a cold room.

Protein Stability Shift Assay (ΔTm)

Thermal melting experiments were carried out using an Mx3005p Real Time PCR machine (Stratagene). Proteins were buffered in 10 mM HEPES pH 7.5, 500 mM NaCl and assayed in a 96 well plate at a final concentration of 2 µM in 20 µL volume. Compounds were added at a final concentration of 10 µM. SYPRO Orange (Molecular Probes) was added as a fluorescence probe at a dilution of 1 in 1000. Excitation and emission filters for the SYPRO-Orange dye were set to 465 nm and 590 nm, respectively.

The temperature was raised with a step of 3 ºC per minute from 25 ºC to 96 ºC and fluorescence readings were taken at each interval. The temperature dependence of the fluorescence during the protein denaturation process was approximated by the equation:

$$y(T) = y_F + \frac{y_U - y_F}{1 + e^{\Delta uG_{(T)}/RT}}$$

where ΔuG is the difference in unfolding free energy between the folded and unfolded state, R is the gas constant and yF and yU are the fluorescence intensity of the probe in the presence of completely folded and unfolded protein respectively. The baselines of the denatured and native state were approximated by a linear fit. The observed temperature shifts, ΔT_m^{obs}, were recorded as the difference between the transition midpoints of sample and reference wells containing protein without ligand in the same plate and determined by non-linear least squares fit.

Table S1. Protein stability shift data for human bromodomains (BET family bromodomains and 4 representatives of other bromodomain containing protein families) in the presence of **11**, I-BET762 and JQ1. Experiments were carried out in 10 mM HEPES pH 7.5 at 25 °C, 500 mM NaCl, and using protein at a final concentration of 2 µM and ligands at 10 µM. SYPRO-Orange was added as a fluorescence probe. Excitation as well as emission was set to 465 nm and 590 nm, respectively. A temperature step of 3 °C per 1 min from 25 °C to 96 °C was applied. The sloping baselines of the initial and final fluorescent species with increasing temperature were approximated by a linear fit.

Protein	11		I-BET762		JQ1	
	ΔTm^{obs}	STD	ΔTm^{obs}	STD	ΔTm^{obs}	STD
BRD2(1)	2.61	0.04	3.33	0.27	6.05	0.02
BRD2(2)	5.37	0.13	7.07	0.17	8.18	0.02
BRD3(1)	4.75	0.16	5.64	0.90	8.55	0.09
BRD3(2)	5.56	0.04	7.05	0.40	8.26	0.01
BRD4(1)	5.39	0.14	7.33	0.76	9.71	0.13
BRD4(2)	5.92	0.33	6.75	0.28	8.31	0.12
BRDT(1)	1.39	0.11	1.96	0.22	4.24	0.07
BRDT(2)	5.72	0.05	8.02	0.66	8.24	0.32
BAZ2B	0.17	0.18	-0.05	0.38	0.21	0.08
CREBBP	-0.59	0.07	0.40	0.24	0.16	0.08
PB1/BD5	-0.06	0.12	-1.18	0.41	0.01	0.09
PCAF	-0.37	0.41	0.62	0.73	0.00	0.25

Isothermal Titration Calorimetry (ITC)

Experiments were carried out on an ITC200 titration microcalorimeter from MicroCal™, LLC (GE Healthcare) equipped with a Washing module, with a cell volume of 0.2003 mL and a 40 µL microsyringe. Experiments were carried out at 15 ˚C while stirring at 1000 rpm, in ITC buffer (20 mM HEPES pH 7.5 (at 25 °C), 200 mM NaCl). The microsyringe was loaded with a solution of protein sample (340 - 400 µM, in ITC buffer) and was carefully inserted into the calorimetric cell which was filled with an amount of the protein (0.2 mL, 27-33 µM in ITC buffer). Following baseline equilibration an additional delay of 60 sec was applied. All titrations were conducted using an initial control injection of 0.3 µL followed by 38 identical injections of 1 µL with a duration of 2 sec (per injection) and a spacing of 120 sec between injections. The titration experiments were designed in such a fashion, as to ensure complete saturation of the proteins before the final injection. The heat of dilution for the proteins were independent of their concentration and

corresponded to the heat observed from the last injection, following saturation of compound binding, thus facilitating the estimation of the baseline of each titration from the last injection. The collected data were corrected for peptide heats of dilution (measured on separate experiments by titrating the proteins into ITC buffer) and deconvoluted using the MicroCal™ Origin software supplied with the instrument to yield enthalpies of binding (ΔH) and binding constants (K_B) in the same fashion to that previously described in detail by Wiseman and co-workers.[5] Thermodynamic parameters were calculated using the basic equation of thermodynamics ($\Delta G = \Delta H - T\Delta S = -RT\ln K_B$, where ΔG, ΔH and ΔS are the changes in free energy, enthalpy and entropy of binding respectively). In all cases a single binding site model was employed, supplied with the MicroCal™ Origin software package. Dissociation constants and thermodynamic parameters are listed on **Table S2**.

Table S2. Isothermal Titration Calorimetry of human BRD4(1) and BRD4(2) with **11**. Titrations were carried out in 50 mM HEPES pH 7.5 (at 25 °C), 150 mM NaCl and 15 °C while stirring at 700 rpm. Proteins were titrated into the compound solution.

Protein	[P] (μM)	[Compound] (μM)	K_D (μM)	ΔH^{obs} (kcal/mol)	N	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
BRD4(1)	404	22	0.053 ± 0.003	-11.00 ± 0.028	1.00 ± 0.001	-1.414	-9.59
BRD4(2)	393	22	0.050 ± 0.003	-7.58 ± 0.026	1.03 ± 0.002	2.042	-9.63

AlphaScreen specific binding assay

Bromodomain screening and determination of IC50 values were conducted at Cerep (Celle l'Evescault, France) on a fee-for-service basis. The first domain of Bromodomain 2 (BRD2(1)), the first domain of bromodomain3 (BRD3(1)), and the first and second domain of Bromodomain 4 (BRD4(1) and BRD4(2)) were assayed as described in the **Table S3**. Compounds were tested at several concentrations for IC50 determination. Compound binding was calculated as an inhibition percentage of the binding of a radioactively labelled ligand specific for each target. Results showing an inhibition or simulation higher than 50% were considered to represent significant effects of the test compounds. The IC50 values and Hill coefficients (nH) were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting.

Table S3. BET-bromodomain binding assay protocols.

Assay	Source	Ligand	Incubation	Detection Method
BRD2(1) (h) bromodomain	human recombinant	Biotin-H4 tetra acetyl Lys 5/8/12/16 50 nM	30 min, r.t.	AlphaScreen
BRD3(1) (h) bromodomain	human recombinant (E. coli)	Biotin-H4 tetra acetyl Lys 5/8/12/16 25 nM	30 min, r.t.	AlphaScreen
BRD4(1) (h) bromodomain	human recombinant	Biotin-H4 tetra acetyl Lys 5/8/12/16 50 nM	30 min, r.t.	AlphaScreen
BRD4(2) (h) bromodomain	human recombinant (E. Coli)	Biotin-H4 tetra acetyl Lys 5/8/12/16 50 nM	30 min, r.t.	AlphaScreen

Controls in compound I-BET762 (**6**) study: *BRD2(1) – I-BET151*: 1.5 x 10^{-8} M (nHill = 1.9); *BRD3(1) – I-BET151*: 2.4 x 10^{-8} M (nHill = 2.2); *BRD4(1) – I-BET151*: 1.3 x 10^{-8} M (nHill = 1.3); *BRD4(2) – PFI-1*: 1.1 x 10^{-6} M (nHill = 1.3).
Controls in compounds **14**–**16** study: *BRD2(1) – JQ1*: 3.7 x 10^{-7} M (nHill = 2.1); *BRD3(1) – JQ1*: 7.9 x 10^{-7} M (nHill > 3); *BRD4(1) – JQ1*: 7.9 x 10^{-7} M (nHill = 3); *BRD4(2) – PFI-1*: 3.5 x 10^{-6} M (nHill > 3).

Crystallization

Aliquots of BRD4(1) were set up for crystallization in complex with **11** using a mosquito® crystallization robot (TTP Labtech, Royston UK). Coarse screens were typically setup onto Greiner 3-well plates using three different drop ratios of precipitant to protein per condition (100+50 nL, 75+75 nL and 50+100 nL). Initial hits were optimized further using Greiner 1-well plates and scaling up the drop sizes in steps. Crystallizations were carried out using the sitting drop vapour diffusion method at 4 °C. Crystals of BRD4/BD1 in complex with **11** (1 mM final concentration) were grown by mixing 200 nL of protein (8.3 mg/mL in 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % Glycerol) with 100 nL of reservoir solution containing 20 % PEG6000, 10 % ethylene glycol and 0.2 M LiCl. Crystals formed within a few days from sitting drop plates at 4 ˚C. Prior to data collection, crystals were transferred to a solution consisting of the precipitation buffer supplemented with ethylene glycol and subsequently flash frozen in liquid nitrogen.

Data Collection and Structure Determination

Prior to data collection, all crystals were transferred to a solution consisting of the precipitation buffer supplemented with ethylene glycol and subsequently flash frozen in liquid nitrogen. Data were collected at Diamond Lightsource on beamline I03 at a wavelength of 0.9762 Å. Data were integrated with XDS[6] and scaled with SCALA.[7] Initial phases were calculated by molecular replacement with PHASER[8] using an ensemble of known bromodomain models. Automated model building with ARP/wARP[9] resulted in a more than 90 % complete models. Refinement was carried out with REFMAC[10] after several rounds of manual rebuilding with COOT.[11] The quality of the final models was validated with MOLPROBITY.[12] Hydrogen atoms were included in late refinement cycles. Data collection and refinement statistics can be found in **Table S4**. The model and structure factors have been deposited with PDB accession codes: **6S25** (BRD4(1) with **11**).

Table S4. Data collection and refinement statistics.

Data Collection	
PDB ID	**6S25**
Protein/Ligand	BRD4/BD1 **11**
Space group	$P2_12_12_1$
Cell dimensions: a, b, c (Å) α, β, γ (deg)	37.25 44.23 78.51 90.00 90.00 90.00
Resolution* (Å)	1.10 (1.16-1.10)
Unique observations*	53306 (7537)
Completeness* (%)	99.6 (97.7)
Redundancy*	5.8 (3.6)
Rmerge*	0.064 (0.421)
I/ σI*	13.9 (3.2)
Refinement	
Resolution (Å)	1.10
R_{work} / R_{free} (%)	12.3/14.5
Number of atoms (protein/other/water)	1086/61/207
B-factors (Å^2) (protein/other/water)21.85	11.34/15.70/26.07
r.m.s.d bonds (Å) r.m.s.d angles (°)	0.016 1.981
Ramachadran Favoured (%) Allowed (%) Disallowed (%)	98.33 1.67 0.00

* Values in parentheses correspond to the highest resolution shell.

Fluorescence Recovery after Photo-bleaching (FRAP)

FRAP experiments were performed using a protocol modified from previous studies.[13] Briefly, 30,000 U2OS Flp-In/T-REx cells were plated onto an 8-well glass imaging chamber (Thermo Fisher Scientific, cat. #155411PK) in 300 µL DMEM media (Thermo Fisher Scientific, cat. #31966021) supplemented with 10% fetal bovine serum (v/v, Biosera, cat. #FB-1001/500) and antibiotics (1% Pencillin Streptomycin (Thermo Fisher Scientific, cat. #15070063), 1 µg/mL puromycin (Toku-e, cat. #P025, CAS number 58-58-2). 24 h after seeding cells were treated with 100 nM doxycycline to induce BRD4L-GFP expression. 24 h later, cells were treated with 2.5 µM SAHA. 1 h before imaging, cells were treated with 1 µM I-BET762, **11** or DMSO. The FRAP and imaging system consisted of a Zeiss LSM 710 scan-head (Zeiss GmbH, Jena, Germany) coupled to an inverted Zeiss Axio Observer Z1 microscope equipped with a high-numerical-aperture (N. A. 1.40) 63x oil immersion objective (Zeiss GmbH, Jena, Germany) equipped with an incubator XLmulti S1 set to 37 °C and 5% CO2. GFP fluorescence imaging was carried out with an argon-ion laser (λ = 488 nm) and with a piezomultiplier tube (PMT) detector set to detect fluorescence between 493-555 nm. A region of the nucleus was selected (12.5 µm^2) and after 2 pre-scans, the region was bleached (100 % laser power of 30 mW 488 nm argon-ion laser; 50.42 µsec pixel dwell; 4 iterations). A time-lapse series was taken to record GFP recovery using 0.7-1.0 % of the power with an interval time of 1.0 sec. The image datasets and fluorescence recovery data were exported from the control software of the microscope (ZEN v.2.1) into the EasyFRAP-web server[14] for analysis, where the average intensity of pre-scans was normalized to 1. Data visualization was performed in Origin v.7.

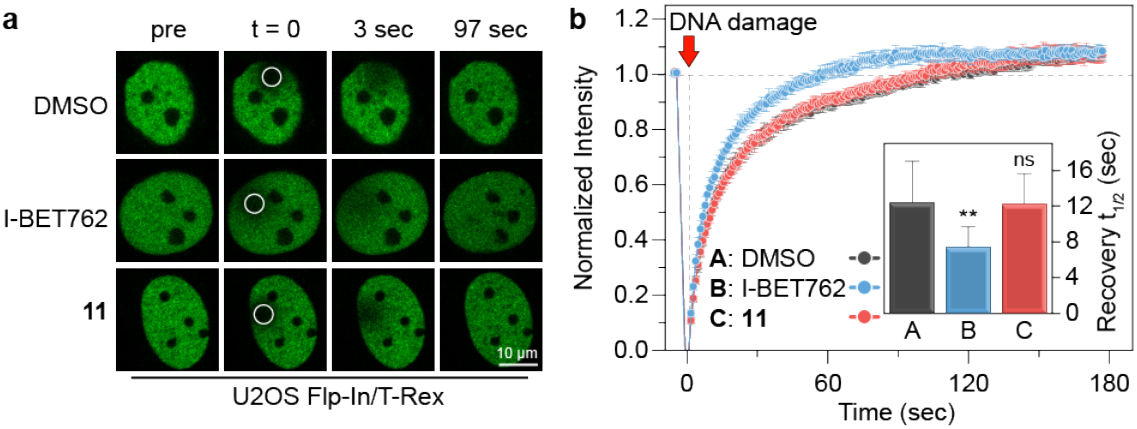


Figure S1. Cellular on-target engagement of **11**. **a**. Fluorescent recovery after photo-bleaching of GFP-FL-BRD4 in U2-OS cells following treatment with1 µM of **11** for 1 h. **b**. Quantitative comparison of time to half-maximal fluorescence recovery for BRD4 FRAP shown in a. Data represent means ± SEM (n = 20) and are annotated with p values obtained from a two-tailed t test (** $p < 0.01$).

Flow Cytometry determination of fluorescein-positive cells

LNCaP cells were seeded in 6-well plates at 3 x 10^4 cells/cm^2 and grown for 48 h before being treated with drug or vehicle. Compounds were used at concentrations of 10 µM and 50 µM and incubated for 2 h at 37 ºC. Cells were then harvested with trypsin and transferred to to FACS tubes and centrifuged for 4 min at 4,000 x *g*. Supernatant was discarded and each cell pellet was resuspended thoroughly in 300 µL of 10 % (v/v) FCS in PBS. Vials were stored at 4 ºC until analysis. The analysis of fluorescein-positive cells was performed on a FACSCalibur. A typical cell area was gated and a total of 50.000

events per sample were acquired. Results were expressed as percentage of the maximal FACS signal. Data were analyzed using FloJo software.

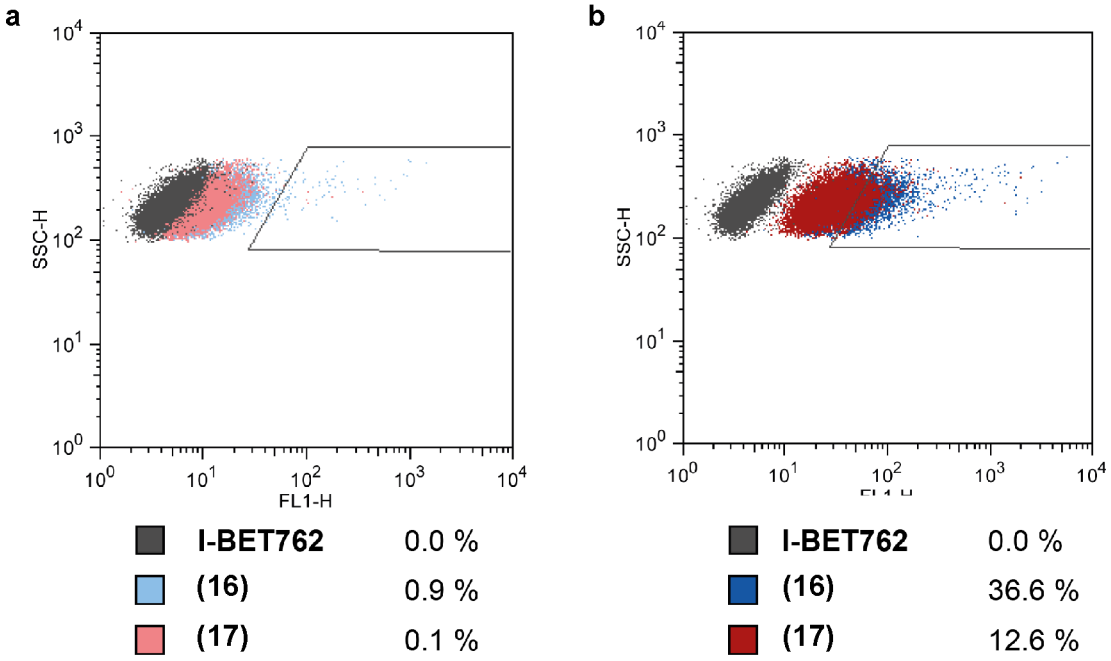


Figure S2. Flow cytometry analysis of fluorescein-positive cells after treatment with (**a**) 10 μM and (**b**) 50 μM of labelled conjugates (data represented as percentage of FITC+ cells).

In vitro assays

Cell culture conditions

LNCaP cells were maintained in RPMI 1640 medium supplemented with penicillin (50 IU/mL), streptomycin (50 µg/mL), 10 % (v/v) serum, and 10 % (v/v) Glutamax at 37 ºC and 5 % CO_2. Subculture was performed once a week and the cells reseeded in constant volumes of cell suspension and medium. The split ratio used for subculture was 1:6 to 1:10. Cells were split 20 times until discarding.

Preparation of stock compounds

All stocks of drugs used in biological assays were prepared at 50 mM in sterile DMSO. Drugs were thoroughly weighed in sterile eppendorfs, diluted in the proper amount of DMSO and stored at -80 ºC. Before each experiment drugs were left to thaw at room temperature and a working stock was prepared in cell culture medium. Unless otherwise stated DMSO from initial stocks was diluted 1000x in each assay to prevent vehicle-induced toxicity.

Cellular growth inhibition

LNCaP cells were seeded on flat bottom 96-well plates at a density of 2,500 cells/well (optimized for 6 days of growth). The outside wells of the plate were filled with 200 µL of sterile water, minimizing the edge effect. T_0 measurements were taken the following day using Cell-Titer Blue (Promega) following the manufacturer's instructions and measured on an Infinite M200 multimode plate reader (Tecan). The remaining plates were treated with DMSO or serial dilutions of compounds and incubated for 6 days. After 6 days the plates were developed as above, with LNCaP cells being incubated with Cell-Titer Blue for 2 h. Results are background correct by subtraction of values from wells containing no cells, expressed as percentage of the T_0 value, and plotted against compound concentration. The T_0 was normalised to 100 %, representing the number of viable cells at the time of compound addition. The cellular response was determined for each compound by using GraphPad Prism software. Growth IC50 (gIC50) values correspond to the concentration at the mid-point of the growth window (between DMSO and T_0 values).

Imaging studies after cell membrane permeabilization

LNCaP cells were seeded at 3 x 10^4 cells/cm^2 in 24 –well plates onto round glass cover slips and grown for 48 h. The medium was then removed and cells washed twice with PBS. At room temperature and outside the aseptic environment, cells were fixed with 100 µL of a solution of 4 % (v/v) paraformaldehyde in PBS. Cells were incubated with PFA for 20 min at room temperature with plate agitation. After the incubation PFA was removed and cells rinsed five times with PBS. At this point, some of the cell membranes were permeabilized with 0.2 % (v/v) Triton X-100 while others remained untreated. The incubation with Triton was performed for 10-15 min. Triton was then removed and cells incubated with PBS for 4 times during 5 min. each. All cells (permeabilized and non-permeabilized) were treated with 10 µM compounds or vehicle control and incubated for 1 h. Cells were washed 4 times to remove unspecific binding. The glass cover slips were then carefully dried for the excess PBS and mounted on glass plates with mounting medium. The glass plates were stored at 4 ºC until analysis by confocal microscopy. Imaging was done using a ZEISS LSM 710 with identical laser, filter and detector settings for all samples tested.

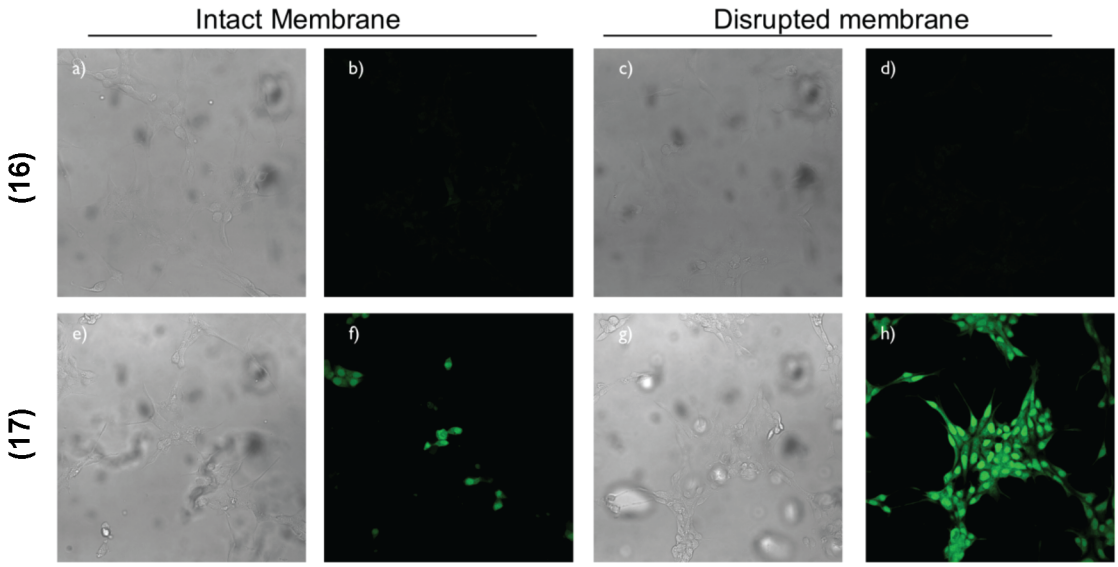


Figure S3. Confocal microscopy analysis on the internalization rate of **16** (e-h) and **17** (a-d) with and without membrane disruption. (40x magnification; Green: fluorescein; grey: transmitted light)

Imaging studies in live cells

LNCaP cells were seeded at 1 x 10^4 cells/cm^2 in 24 –well plates (2 x 10^4 cells/well) onto round glass cover slips and grown for 48 h. The medium was removed and compounds at 10 µM were added at different time points. After the drug incubation period the plates were removed from the incubator, the medium containing drugs was removed and the wells were washed with PBS (3 times). The cells were then fixed with a solution of 4 % (v/v) paraformaldehyde in PBS (100 µL/well) for 15 min. PFA was discarded and the cells were washed again with PBS (4 times) before incubation with Hoescht staining reagent (dilution of 1:5000 from the stock solution) for 15 min. Upon Hoescht removal, cells were washed with PBS, the glass cover slips were then carefully dried for the excess PBS and mounted on glass plates with mounting medium. The glass plates were stored at 4 ºC until analysis by confocal microscopy. Imaging was done using a ZEISS LSM 710 with identical laser, filter and detector settings for all samples tested.

Permeability assessment

Permeability studies were conducted at Pion, Inc. (Billerica, MA, US). The PAMPA EvolutionTM instrument was used to determine permeability. The effective permeability, Pe, of each compound was measured at pH 6.8 in the donor compartment using low-binding, low UV Prisma buffer. The drug-free acceptor compartment was filled with acceptor sink buffer containing a scavenger at the start of the test. The proprietary scavenger mimics serum proteins and blood circulation, thus creating sink conditions. In the default protocol the aqueous solutions of studied compounds were prepared by diluting and thoroughly mixing 3 µL of DMSO stock in 600 µL of Prisma HT buffer. Final concentration of organic solvent (DMSO) in aqueous buffer is 0.5% (v/v). The reference solution is identical to the donor at time zero, so that any surface adsorption effects from the plastic is compensated. The PAMPA sandwich was assembled and allowed to incubate for ~15 h. The solutions in the donor compartment were un-stirred within duration of the experiment. Thus, the thickness of the aqueous boundary layer expected to be about 1000 µm. The sandwich was then separated, and both the donor and receiver compartments were assayed for the amount of drug present by comparison with the UV spectrum obtained from reference standards. Mass balance was used to determine the amount of material remaining in the membrane filter and on the plastic (%R).

Table S5. Permeability of compounds by PAMPA assay.

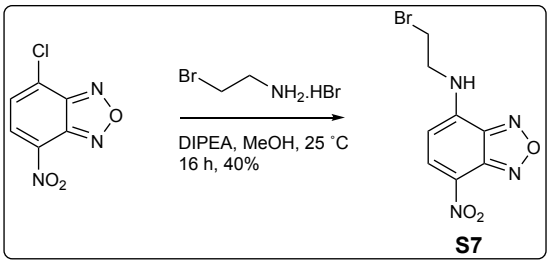
Compound	pH	Avg.Pe[a]	SD Pe	Avg. % R[b]	SD[c] % R	Avg.[d] logPe	SD logPe
I-BET762	6.8	51	5	14	10	-4.29	0.03
14	6.8	0.4	0.15	3	1	-6.42	0.17
15	6.8	0.36	0.06	7	1	-6.45	0.07
16	6.8	0.22	0.13	6	1	-6.72	0.26
17	6.8	0.20	0.07	5	1	-6.73	0.18
Propranolol	6.8	74	11	27	2	-4.14	0.06
Atenolol	6.8	<0.31	ND[e]	4	2	ND	ND

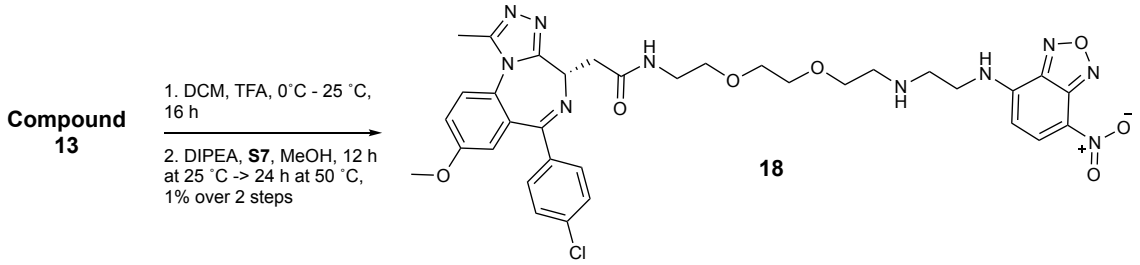
[a] Pe, effective permeability ($x10^{-6}$ cm/sec); [b] % R, membrane retention; [c] SD, Standard deviation; [d] Avg, the value is reported as average of quadruplicates; [e] ND, undetected due to weak UV absorbance of compound caused by low solubility.

Synthesis and bioimaging of nitrobenzofurazan (NBD)-labelled conjugates

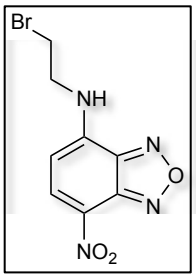
Synthesis and characterization of NBD-labelled I-BET762

Literature basis[15,16].





1. Synthesis of compound **S7**

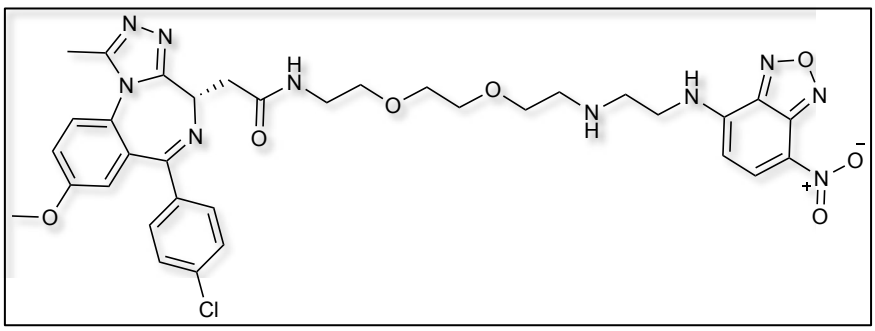


The starting material NBD-Cl (100 mg, 0.5 mmol, 1 eq) was dissolved in methanol (5 mL). 2-Bromoethylamine hydrobromide (123 mg, 0.6 mmol, 1.2 eq) and DIPEA (180 µL, 1 mmol, 2 eq) were added to the solution. The reaction mixture was stirred at room temperature for 12 h. The reaction crude was concentrated and purified over a column chromatography on silica gel with EtOAc:hexane (1:1) to afford **S7** as an orange solid (57 mg, 40%).

^{1}H-NMR (300 MHz, Methanol-*d4*) δ 8.50 (d, *J* = 8.8 Hz, 1H), 6.41 (d, *J* = 8.8 Hz, 1H), 3.96 (s, 2H), 3.68 (d, *J* = 6.6 Hz, 2H).

NMR is in accordance with the literature's report.[16]

2. Synthesis of compound **18**



Compound **13** (26 mg, 0.04 mmol) was degassed under vacuum and refilled with argon before being dissolved in DCM (1 mL). TFA (1 mL, 12 mmol, 300 eq) was added at 0 ºC. The reaction was left to warm to room temperature and stirred for

12-14 h. The reaction crude was concentrated thoroughly. To remove all TFA toluene was added several times followed by evaporation. When thoroughly dried, the reaction crude was placed under an argon flow to remove any traces of acid. The Boc-deprotected intermediate (0.04 mmol, assumed 100 % conversion, 1.1 eq) was diluted in methanol (1 mL) and stirred. DIPEA (50 µL, 0.21 mmol, 7 eq) was added and the reaction stirred for a further 5 min., time after which compound **S7** (10.4 mg, 0.036 mmol, 1 eq) was added. The reaction was stirred for 12-14 h at room temperature. According to the TLC the starting materials did not react and more DIPEA was added (10 eq). The reaction was left for a further 24 h and the formation of a new product was observed. In an attempt to consume all the starting material, the reaction was heated at 50 ºC and more 10 eq of DIPEA were added. Two preparative TLC (DCM:MeOH, 92.5:7.5) on silica gel were performed to isolate the pure product **18** (11.2 mg, 38%).
HRMS (ESI): m/z calcd $[M+H-CNO]^+$ for $C_{34}H_{37}ClN_{10}O_7$, 690.2443; found $[M+H-CNO]^+$ = 690.2443. (ionization fragmentation pattern reported for oxydized NBD)[17]

In vitro assays with NBD-labelled I-BET762

LNCaP cells were seeded at 1 x 10^4 cells/cm^2 in 24 –well plates (2 x 10^4 cells/well) onto round glass cover slips and grown for 48 h. The medium was removed and compounds at 10 µM were added at different time points. After the drug incubation period the plates were removed from the incubator, the medium containing drugs was removed and the wells were washed with PBS (3 times). The cells were then fixed with a solution of 4 % (v/v) paraformaldehyde in PBS (100 µL/well) for 15 min. PFA was discarded and the cells were washed again with PBS (4 times) before incubation with Hoescht staining reagent (dilution of 1:5000 from the stock solution) for 15 min. Upon Hoescht removal, cells were washed with PBS, the glass cover slips were then carefully dried for the excess PBS and mounted on glass plates with mounting medium. The glass plates were stored at 4 ˚C until analysis by confocal microscopy. Imaging was done using a ZEISS LSM 710 with identical laser, filter and detector settings for all samples tested

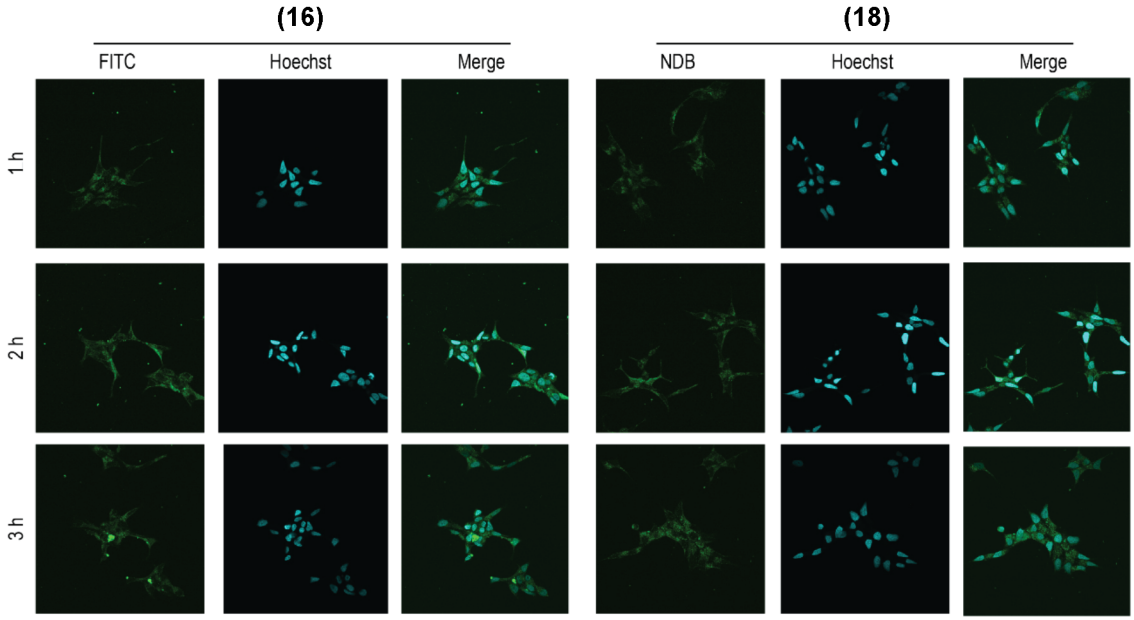
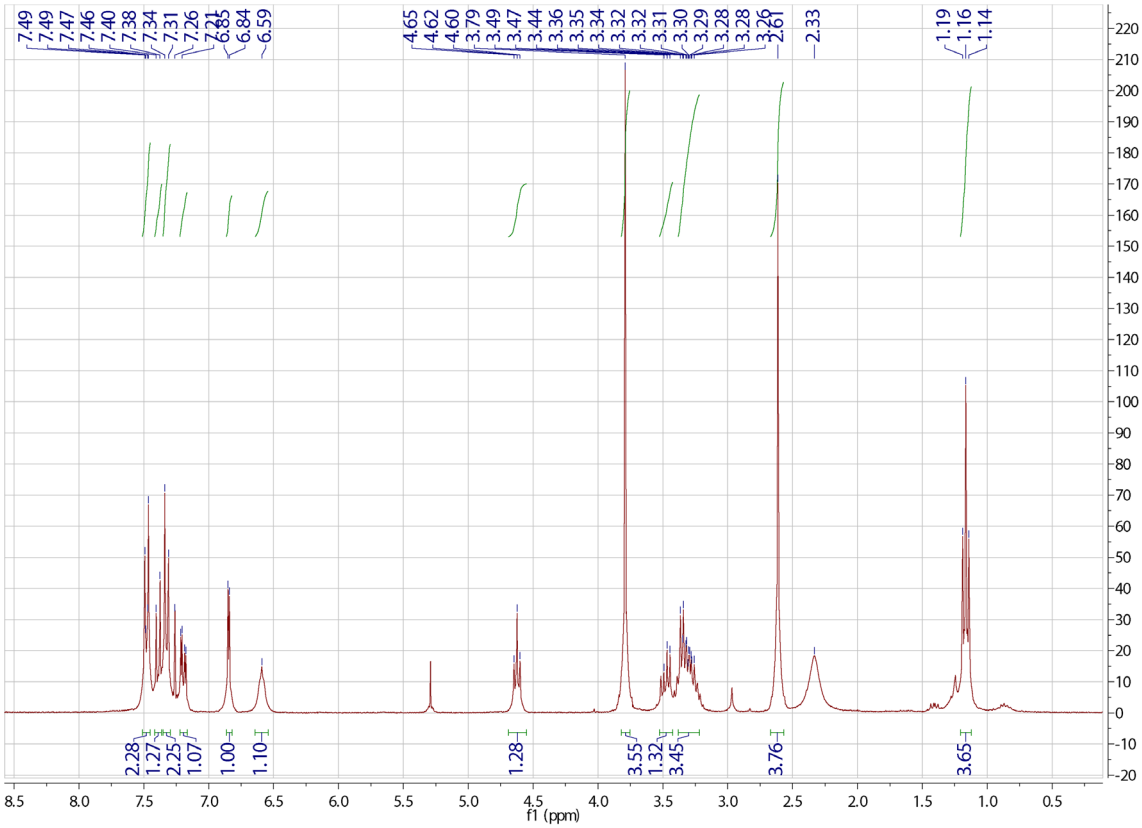
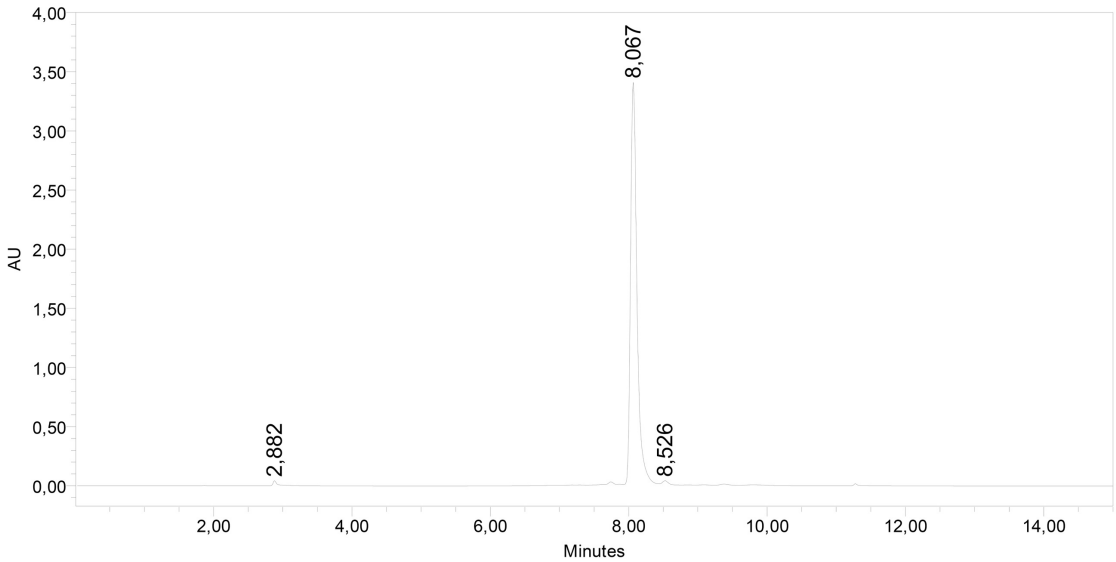


Figure S4. Confocal microscopy analysis of the rate of internalization in LNCaP cells of compound **18**. Nuclei were stained with Hoechst; Merge images represent fluorescein (FITC) and Hoechst channels over imposed.

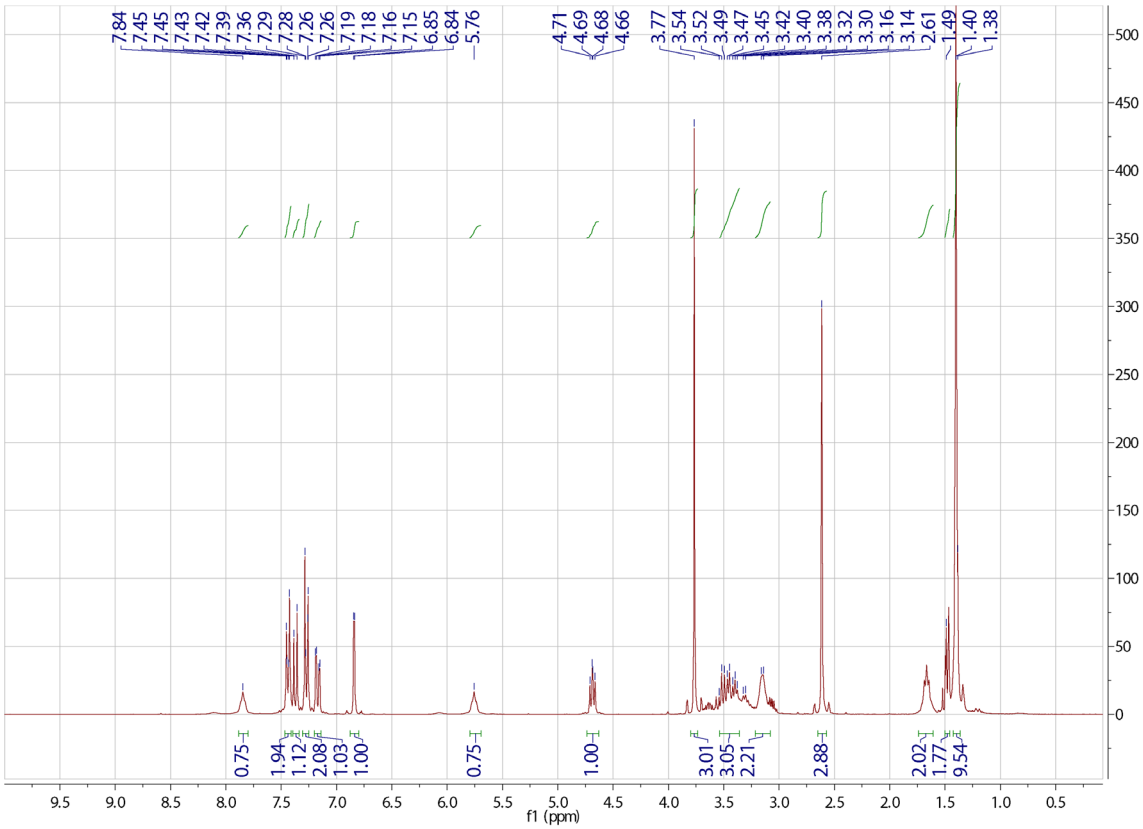
SMALL MOLECULE SPECTRA



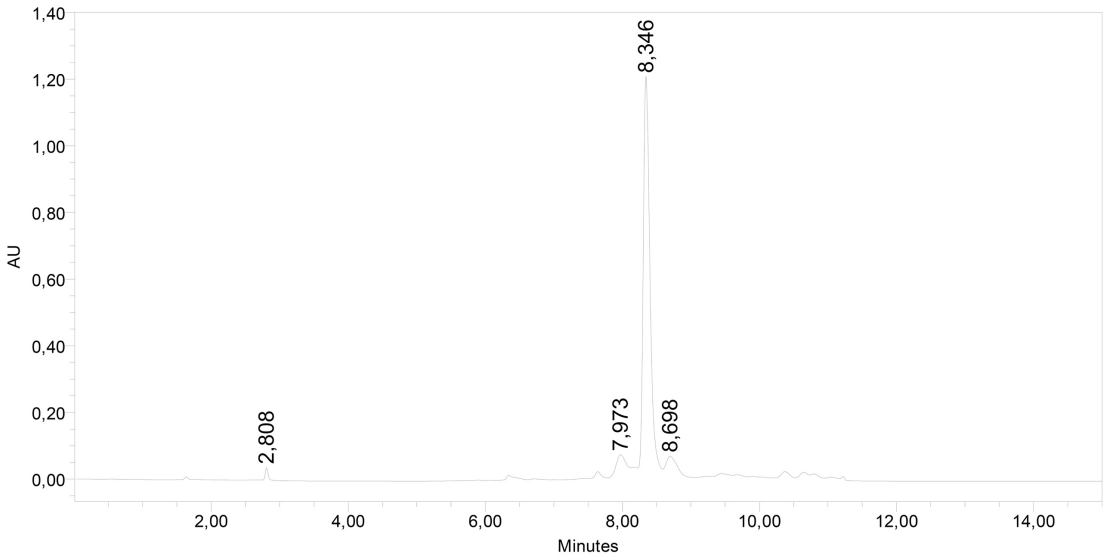
^{1}H-NMR of **6**.



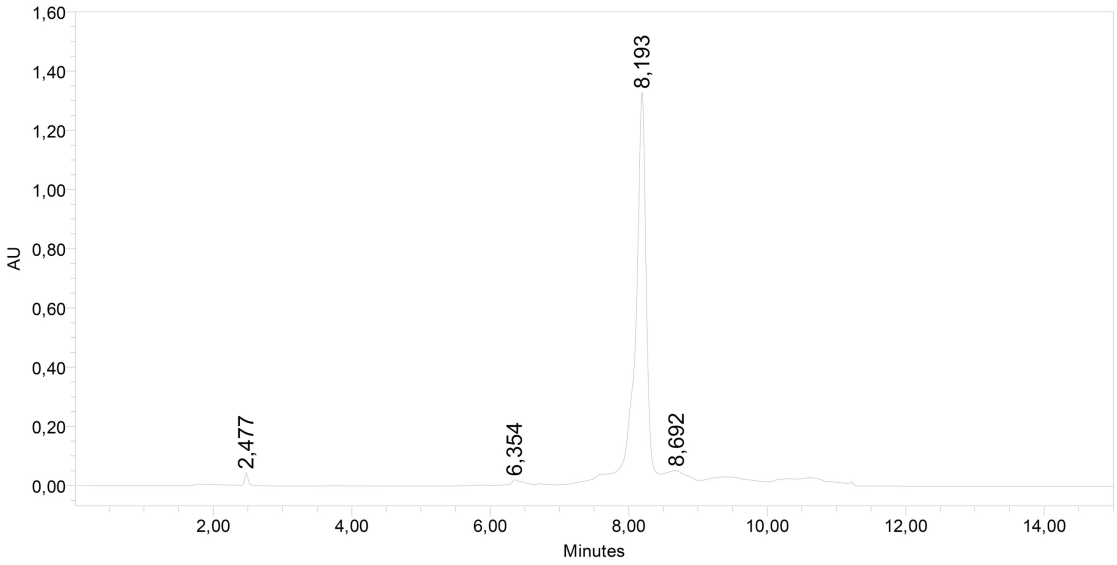
HPLC of **6**.



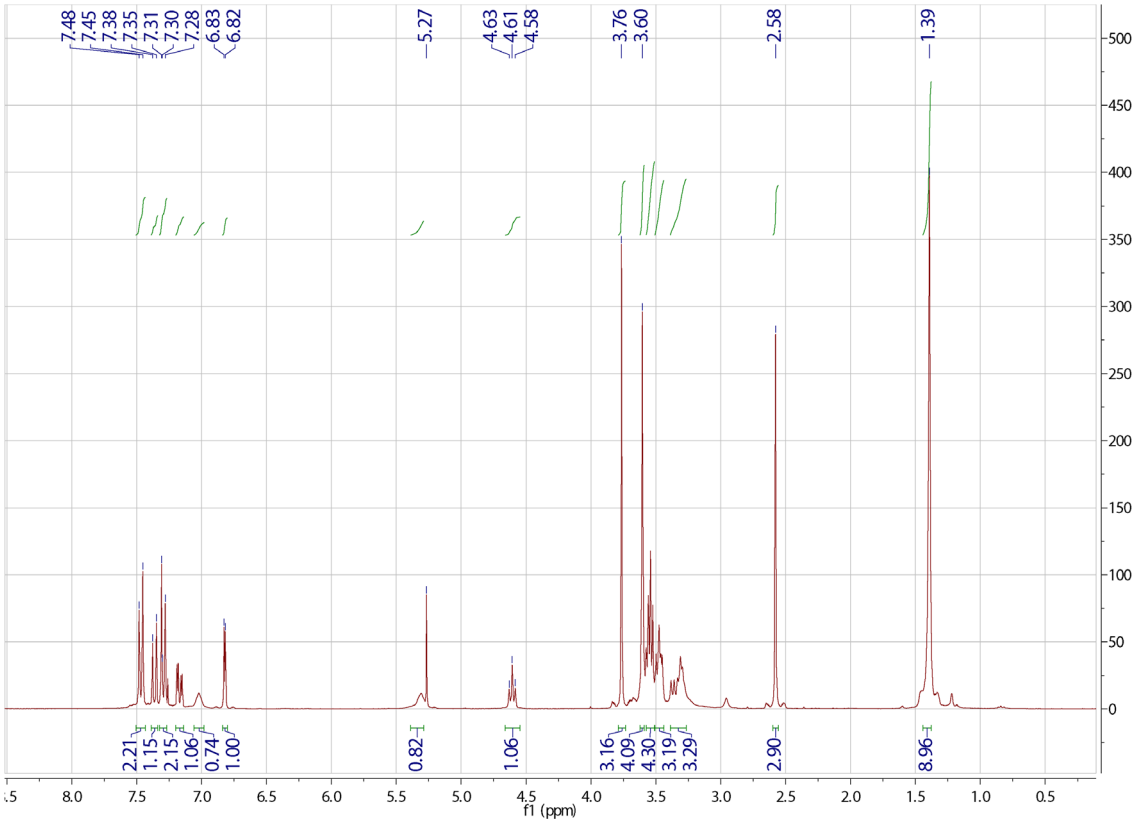
^{1}H-NMR of **11**.



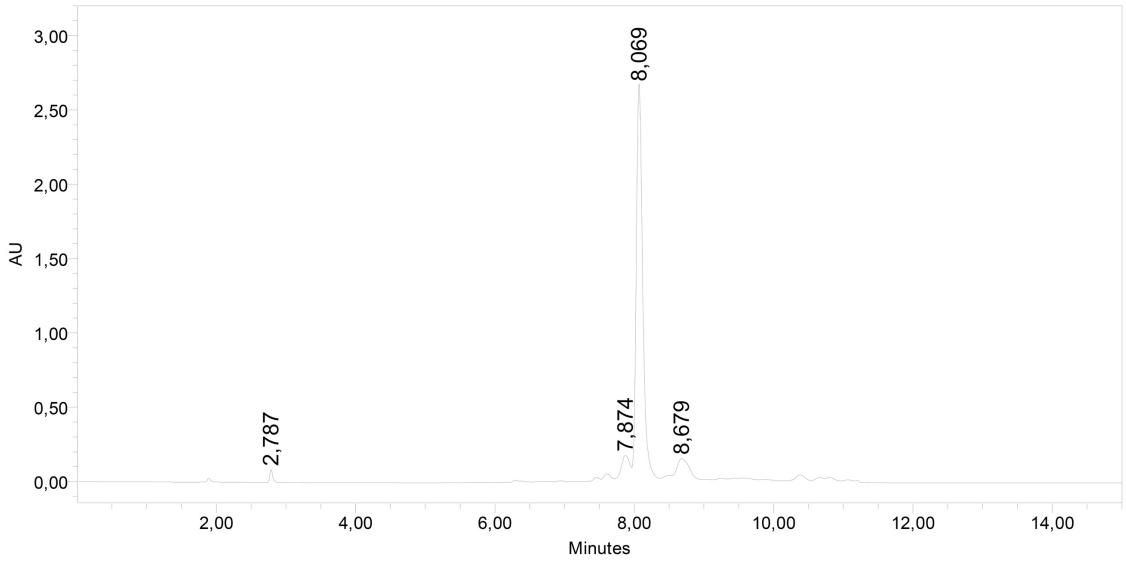
HPLC of **11**.



HPLC of **12**.



^{1}H-NMR of **13**.



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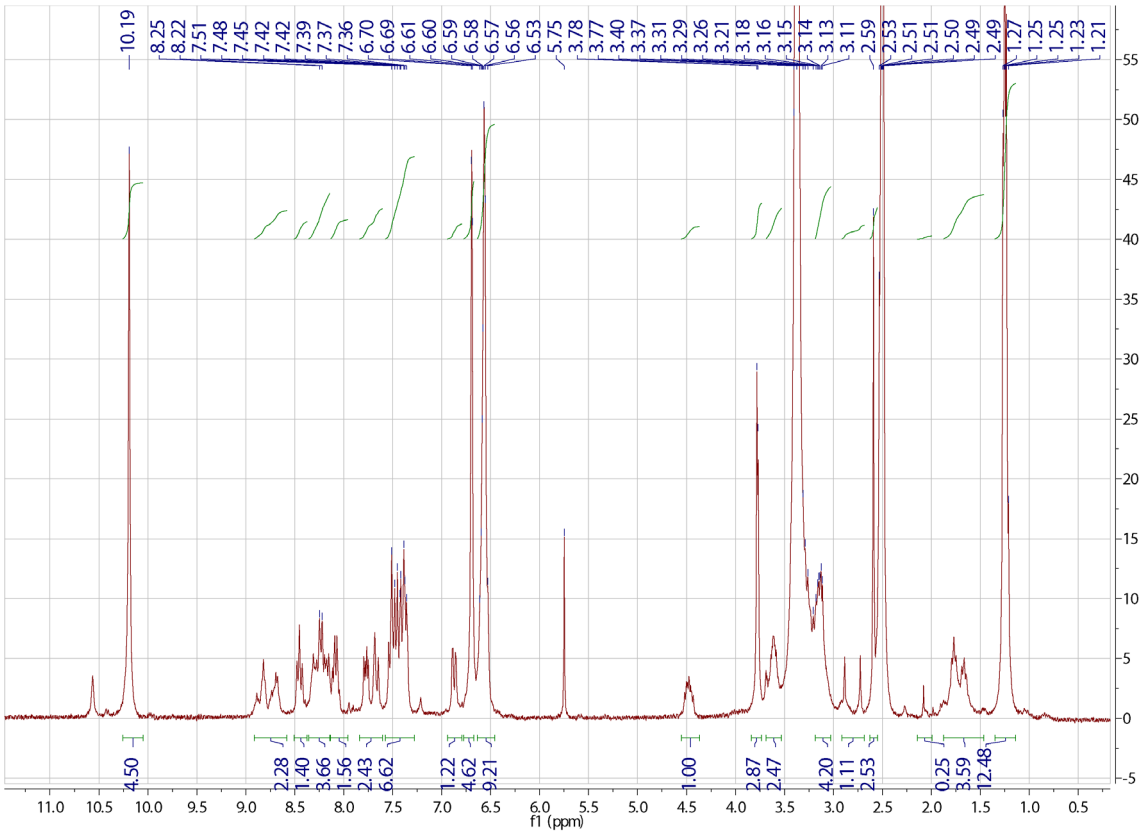
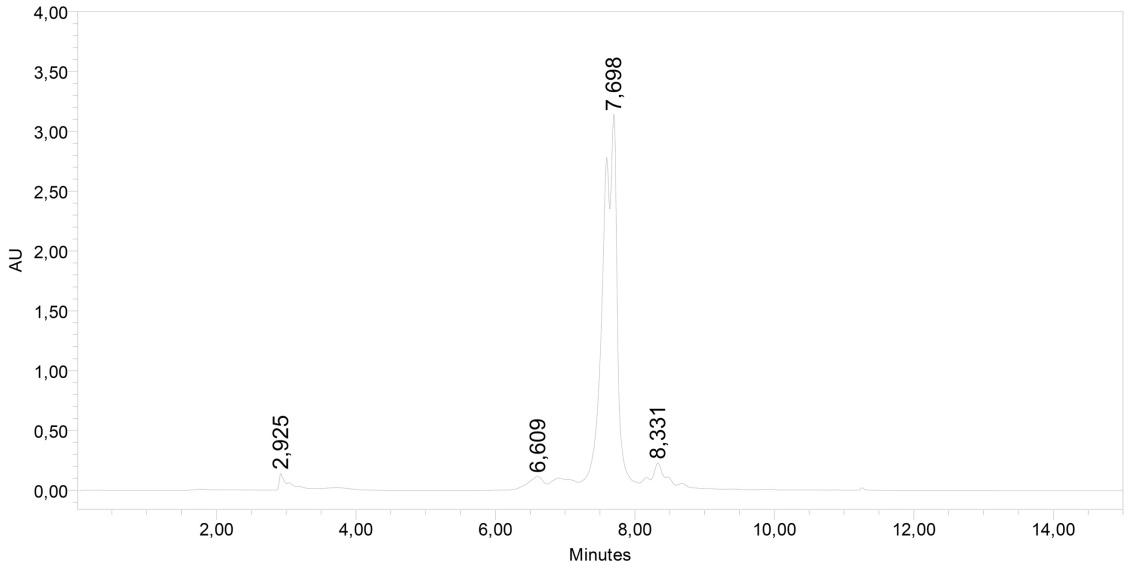
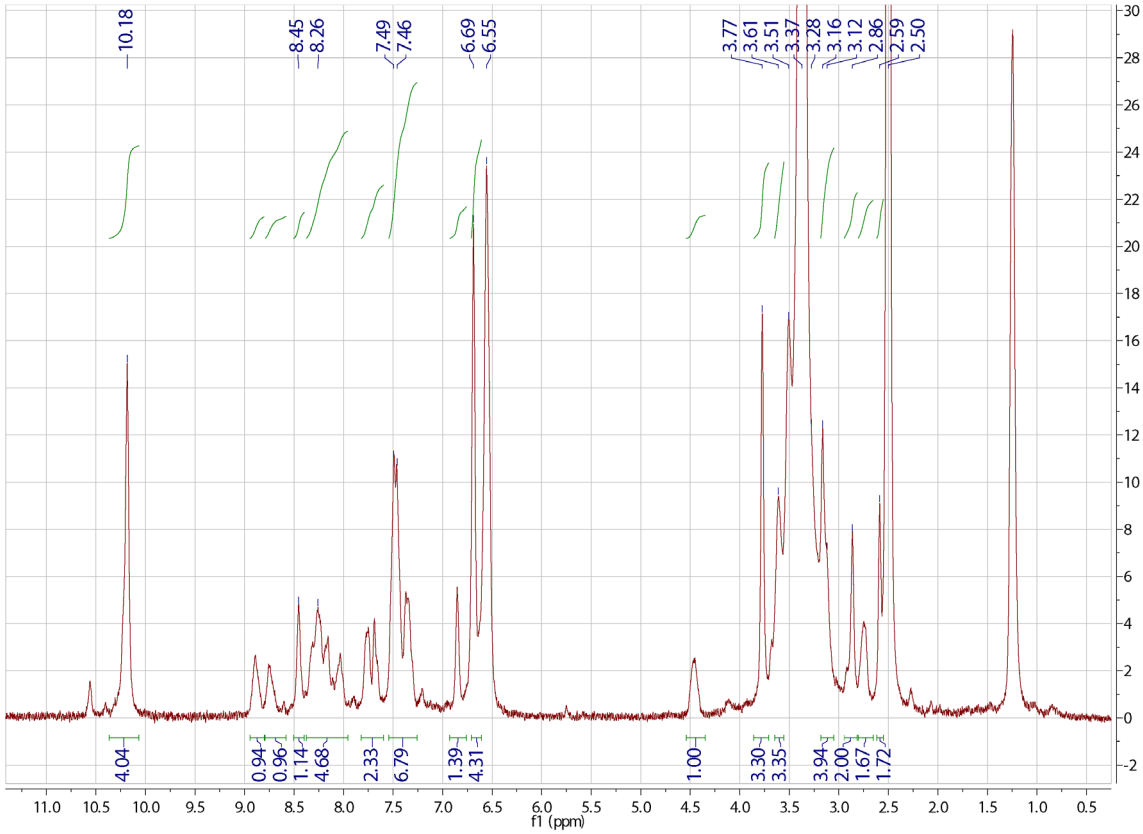


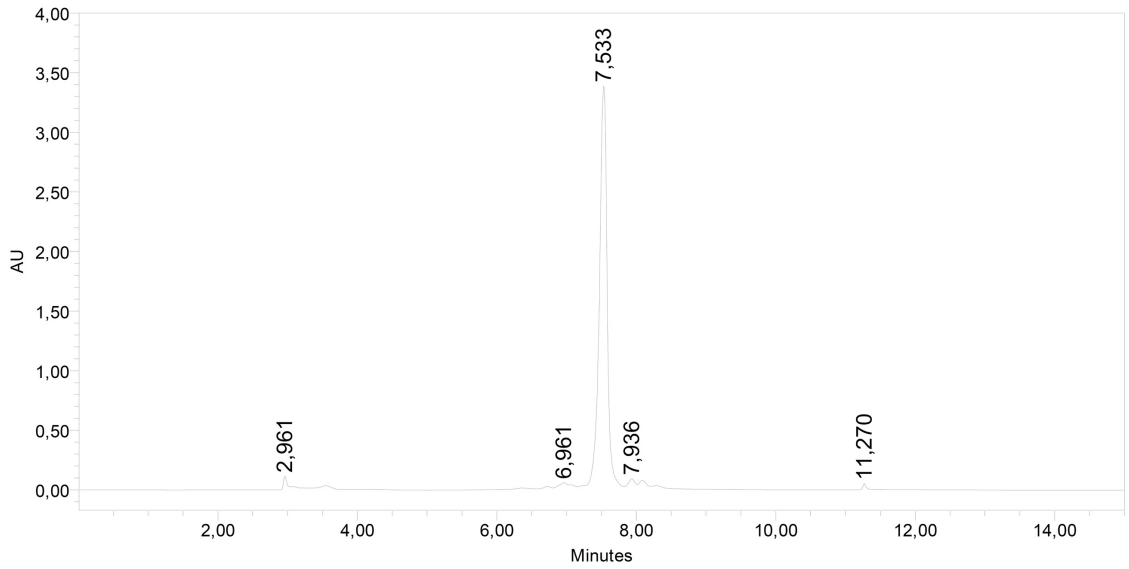
^{1}H-NMR of **14**.



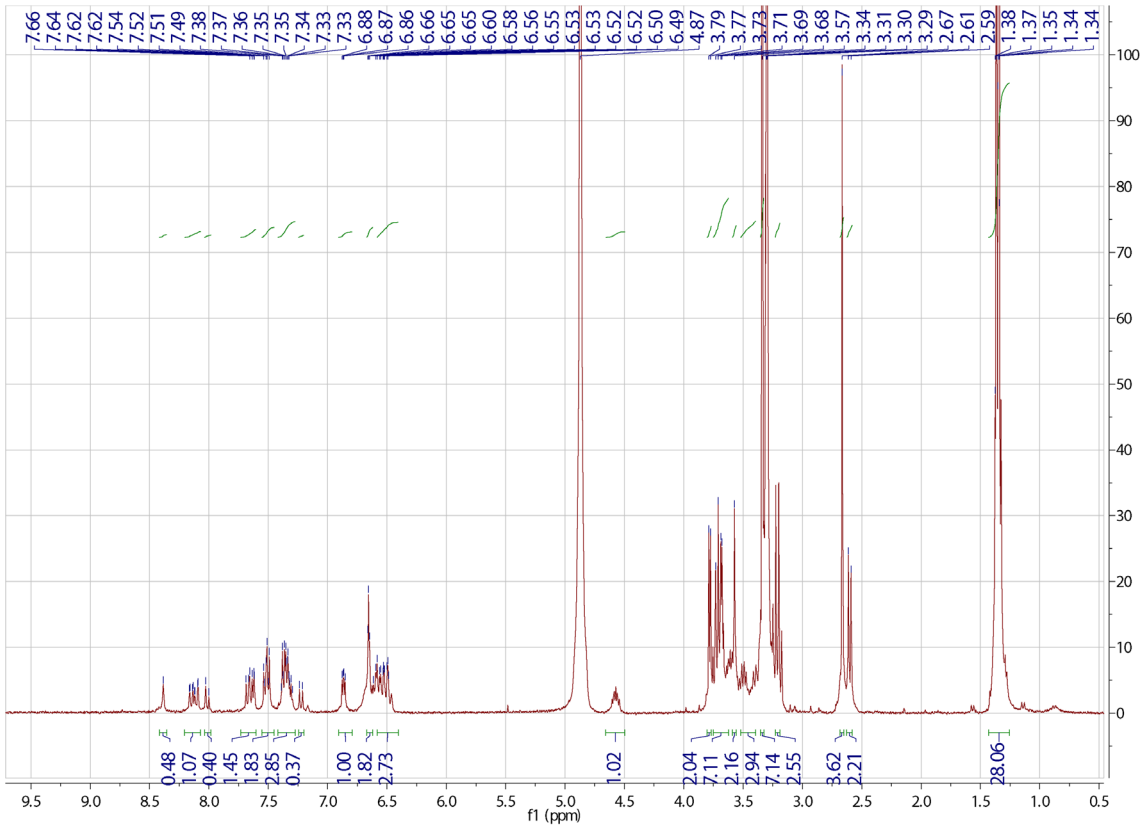
HPLC of **14**.



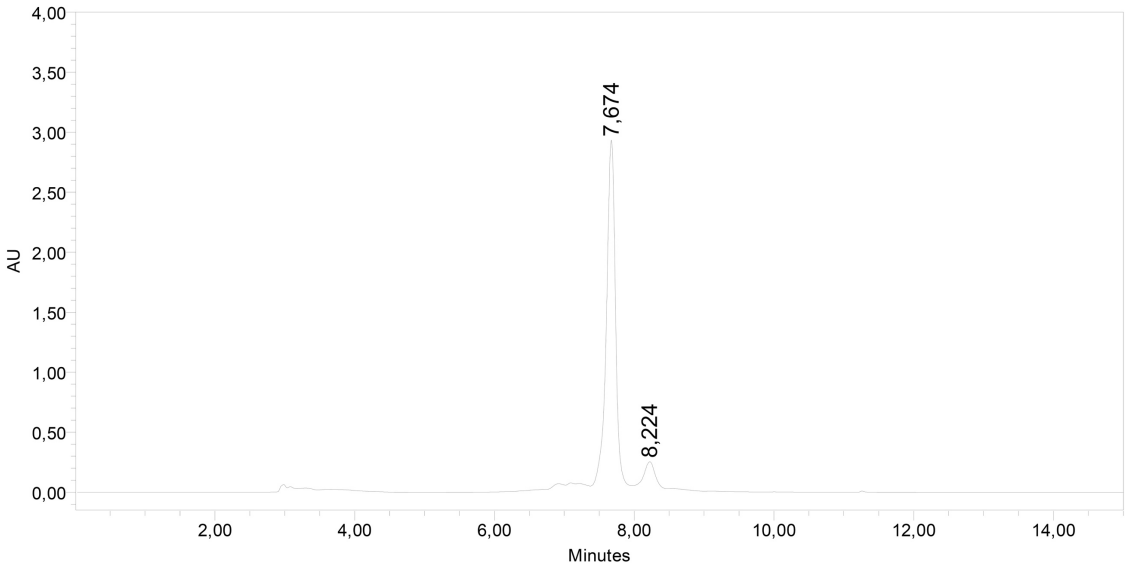
^{1}H-NMR of **15**.



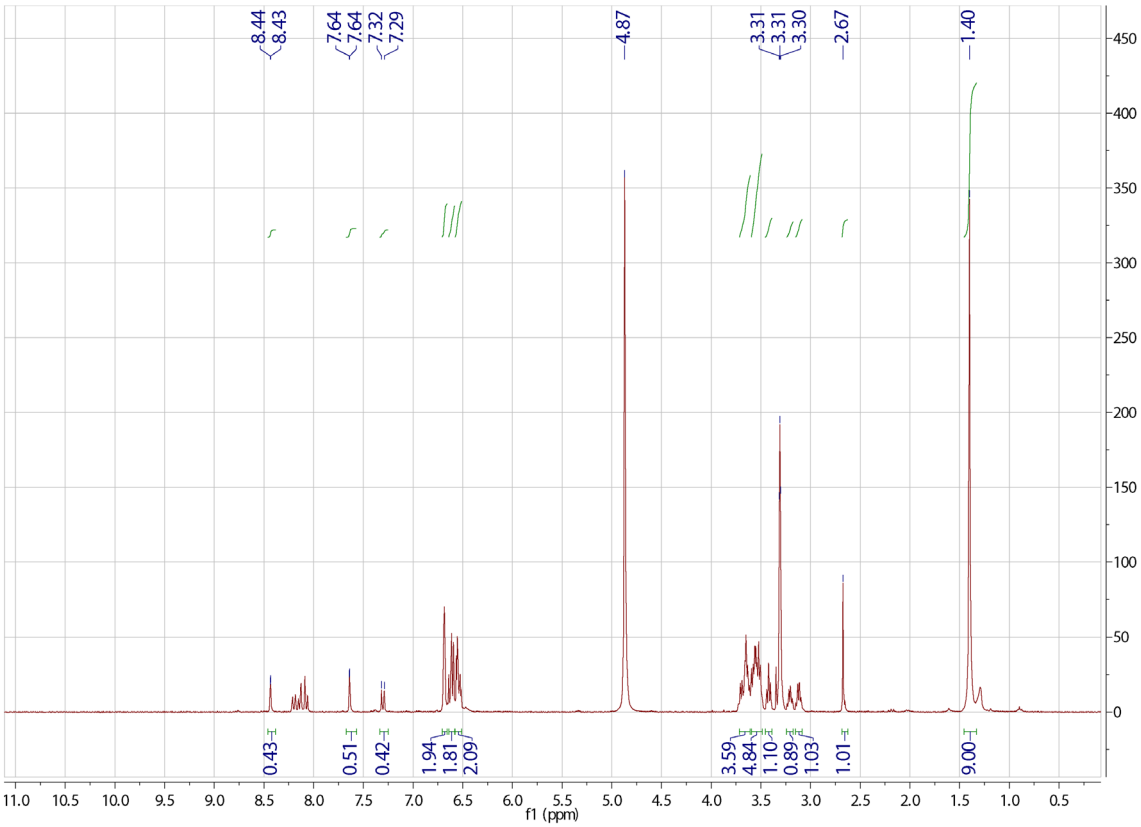
HPLC of **15**.



^{1}H-NMR of **16**.



HPLC of **16**.



^{1}H-NMR of **17**.

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