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Supporting Information

Chemoproteomic Approach to Explore the Target Profile of GPCR ligands: Application to 5-HT_{1A} and 5-HT_6 Receptors

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Supporting Information

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1. Chemistry

1.1. General Experimental Details

Optical rotations were recorded on a Perkin-Elmer 241 polarimeter using a 1 dm path length and a sodium lamp (λ = 589 nm), except for rhodamine fluorescent compounds (which showed high absorbance at this wavelenght); concentrations are given as g/100 mL. Melting points (mp, uncorrected) were determined on a Stuart Scientific electrothermal apparatus. Infrared (IR) spectra were measured on a Bruker Tensor 27 instrument equipped with a Specac ATR accessory of 5200-650 cm^{-1} transmission range; frequencies (ν) are expressed in cm^{-1}. Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker Avance 700 (^{1}H, 700 MHz; ^{13}C, 175 MHz), Bruker Avance 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) or Bruker Avance 300-AM (^{1}H, 300 MHz; ^{13}C, 75 MHz) instruments at the UCM's NMR facilities. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (*J*) are in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), q (quartet), qt (quintet), m (multiplet), br (broad), app (apparent). 2D NMR experiments (HMQC and HMBC) of representative compounds were carried out to assign protons and carbons of the new structures. High resolution mass spectrometry (HRMS) was carried out on a FTMS Bruker APEX Q IV (UCM) spectrometer in electrospray ionization (ESI) mode. Elemental analyses (C, H, N, S) were obtained on a LECO CHNS-932 apparatus at the UCM's analysis services and were within 0.4% of the calculated values, confirming a purity of at least 95% for all tested compounds. Analytical thin-layer chromatography (TLC) was run on Merck silica gel plates (Kieselgel 60 F-254) with detection by UV light (254 nm), ninhydrin solution, or 10% phosphomolybdic acid solution in EtOH. Flash chromatography was performed on glass column using silica gel type 60 (Merck, particle 230-400 mesh). Unless stated otherwise, starting materials, reagents and solvents were purchased as high-grade commercial products from Sigma-Aldrich, Acros, ABCR, Bachem, Fluorochem, Scharlab, or Panreac, and were used without further

purification. Dichloromethane (DCM) was freshly distilled from calcium hydride. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were freshly distilled from sodium and benzophenone. All non-aqueous reactions were performed under an argon atmosphere in oven-dried glassware.

The following compounds were synthesized as previously described: 2-{4-[4-(1-naphthyl)piperazin-1-yl]butyl}tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione (UCM-2550, **1**),[1] 7a-(4-aminobutyl)-2-{4-[4-(1-naphthyl)piperazin-1-yl]butyl}tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione (**9**) and 7a-(7-aminoheptyl)-2-{4-[4-(1-naphthyl)piperazin-1-yl]butyl}tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione (**10**),[2] 4-benzoyl-*N*-biotinyl-L-phenylalanine (**12**),[3] 5-chloro-*N*-(4-methoxy-3-piperazin-1-ylphenyl)-3-methyl-1-benzothiophene-2-sulfonamide (SB-271046, **13**),[4] 4-methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]benzenesulfonyl chloride (**20**),[5] tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione (**29**),[6] phenyl[(3-piperazin-1-yl)phenyl]methanone (**35**),[2] *N*-[4-(4-aminobenzoyl)phenyl]biotinamide (**37**),[7] and their spectroscopic data were consistent with those reported. Collected data for compounds **2-7, 14-19**, **27**, and **28** refer to free bases, and then hydrochloride salts were prepared prior to biological assays. The free amine was characterized (yield, IR, NMR, MS), dissolved in anhydrous diethyl ether (6 mL/mmol), and a commercial 1 M HCl(g) solution in Et_2O (1 mL/mmol free amine) was

[1] López-Rodríguez. M. L., Morcillo, M. J., Fernández, E., Benhamú, B., Tejada, I., Ayala, D., Viso, A., Campillo, M., Pardo, L., Delgado, M., Manzanares, J., and Fuentes, J. A. (2005) Synthesis and structure-activity relationships of a new model of arylpiperazines. 8. Computational simulation of ligand-receptor interaction of 5-HT_{1A}R agonists with selectivity over α_1-adrenoceptors, *J. Med. Chem. 48*, 2548–2558.

[2] Alonso, D., Vázquez-Villa, H., Gamo, A. M., Martínez-Esperón, M. F., Tortosa, M., Viso, A., Fernández de la Pradilla, R., Junquera, E., Aicart, E., Martín-Fontecha, M., Benhamú, B., López-Rodríguez, M. L., and Ortega-Gutiérrez, S. (2010) Development of fluorescent ligands for the human 5-HT_{1A} receptor, *ACS Med. Chem. Lett. 1*, 249–253.

[3] Tamura, T., Tsukiji, S., and Hamachi, I. (2012) Native FKBP12 engineering by ligand-directed tosyl chemistry: labeling properties and application to photo-cross-linking of protein complexes in vitro and in living cells, *J. Am. Chem. Soc. 134*, 2216–2226.

[4] Bromidge, S. M., Brown, A. M., Clarke, S. E., Dodgson, K., Gager, T., Grassam, H. L., Jeffrey, P. M., Joiner, G. F., King, F. D., Middlemiss, D. N., Moss, S. F., Newman, H., Riley, G., Routledge, C., and Wyman, P. (1999) 5-Chloro-*N*-(4-methoxy-3-piperazin-1-ylphenyl)-3-methyl-2-benzothiophenesulfonamide (SB-271046): a potent, selective, and orally bioavailable 5-HT_6 receptor antagonist, *J. Med. Chem. 42*, 202–205.

[5] Liu, F., Majo, V. J., Prabhakaran, J., Milak, M. S., John Mann, J., Parsey, R. V., and Kumar, J. S. (2011) Synthesis and in vivo evaluation of [*O*-methyl-^{11}C] *N*-[3,5-dichloro-2-(methoxy)phenyl]-4-(methoxy)-3-(1-piperazinyl)benzenesulfonamide as an imaging probe for 5-HT_6 receptors, *Bioorg. Med. Chem. 19*, 5255–5259.

[6] Dakin, H. D. (1920) Amino acids of gelatin, *J. Biol. Chem. 44*, 499–529.

[7] Wahlstrom, J. L., Randall, M. A. Jr, Lawson, J. D., Lyons, D. E., Siems, W. F., Crouch, G. J., Barr, R., Facemyer, K. C., and Cremo, C. R. (2003) Structural model of the regulatory domain of smooth muscle heavy meromyosin, *J. Biol. Chem. 278*, 5123–5131.

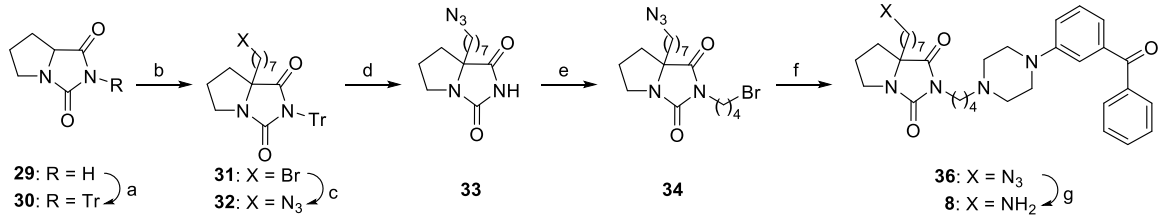
added. The hydrochloride salt was isolated by filtration or evaporation, washed with anhydrous Et_2O, dried under high vacuum, and characterized (mp, elemental analysis). Spectroscopic data of all described compounds were consistent with the proposed structures.

IUPAC rules have been followed for naming all organic compounds, except for the radical {5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoyl}, whose common name biotinyl has been employed for simplicity.

1.2. Synthesis of Probes 2-7, 27

- **Synthesis of Probes 2-6**

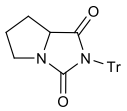
Synthesis of amine 8



Scheme S1. Reagents and conditions: (a) TrCl, NaH, DMF, 60 ºC, 96%; (b) i. *i*-Pr_2NH, *n*-BuLi, THF, 0ºC, ii. 1,7-dibromoheptane, rt, 75%; (c) NaN_3, DMF, H_2O, 50 ºC, 99%; (d) TFA, DCM, rt, 89%; (e) 1,4-dibromobutane, NaH, DMF, 110 ºC, 70%; (f) phenyl[(3-piperazin-1-yl)phenyl]methanone (**35**), Et_3N, CH_3CN, 60 ºC, 79%; (g) PPh_3, THF, H_2O, rt, 84%.

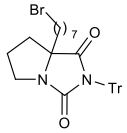
2-Trityltetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione, 30. To a solution of **29** (400 mg, 2.8 mmol) in anhydrous DMF (5 mL), NaH (126 mg, 3.1 mmol, 60% dispersion in mineral oil) was added at room temperature (rt) and the mixture was stirred at 60 ºC for 1 h. Then, a solution of trityl chloride (TrCl, 1.60 g, 5.7 mmol) in anhydrous DMF (10.7 mL) was added dropwise and the mixture was stirred at 60 ºC for 3 h. Once at rt, the solvent was evaporated under reduced pressure and the residue was resuspended in H_2O (50 mL) and extracted with DCM (3 x 50 mL). The organic extracts were washed with H_2O, dried (Na_2SO_4) and filtered. The solvent was

evaporated under reduced pressure and the residue was purified by column chromatography (hexane:EtOAc, 9:1 to 1:1 v/v) to afford the title compound **30** as a white solid (1.05 g, 96%).



R_f (hexane:EtOAc, 8:2 v/v) = 0.32; mp: 254-255 ºC; IR (ATR, cm^{-1}): 1774, 1714, 1595, 1492, 1450; ^1H NMR (300 MHz, $CDCl_3$): δ 1.30-1.43 (m, 1H, ½CH_{2cyc}), 1.67-1.94 (m, 2H, CH_{2cyc}), 2.03-2.13 (m, 1H, ½CH_{2cyc}), 3.12 (ddd, *J* = 11.5, 7.9, 5.3, 1H, ½CH_{2cyc}N), 3.58 (dt, *J* = 11.4, 7.3, 1H, ½CH_{2cyc}N), 4.15 (t, *J* = 8.0, 1H, CH_{cyc}), 7.15-7.28 (m, 9H, Ar), 7.42 (d, *J* = 7.4, 6H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.4, 27.9, 46.3 ($3CH_2$), 62.8 (CH), 73.5 (C), 126.7 (3CH), 127.6 (6CH), 128.6 (6CH), 142.4 (3C), 160.7, 174.2 (2CO); MS (ESI, *m/z*): 243.1 [Tr^+].

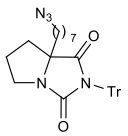
7a-(7-Bromoheptyl)-2-trityltetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione, 31. To a solution of *N,N*-diisopropyl amine (150 μL, 1 mmol) in anhydrous THF (1 mL), *n*-BuLi (440 μL, 1 mmol, 2.4 M in hexane) was added dropwise at 0 ºC and the reaction mixture was stirred at this temperature for 30 min. Then, a solution of compound **30** (200 mg, 0.52 mmol) in anhydrous THF (5 mL) was added dropwise and the mixture was stirred at 0 ºC for 1 h, before 1,7-dibromoheptane (300 μL, 2.2 mmol) was added in one portion. The reaction was allowed to warm to rt and stirred for 24 h. After this time, the mixture was quenched with a saturated aqueous solution of NH_4Cl (4 mL), basified with an aqueous solution of 20% K_2CO_3 and extracted with EtOAc (3 x 20 mL). The combined organic extracts were washed with brine and dried (Na_2SO_4). The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (hexane:DCM, 2:8 v/v) to afford the title compound **31** as a white solid (220 mg, 75%).



R_f (DCM) = 0.7; mp: 62-63 ºC; IR (ATR, cm^{-1}): 2095, 1776, 1717, 1492, 1451; ^1H NMR (300 MHz, $CDCl_3$): δ 1.44-1.79 (m, 12H, $(CH_2)_5$, CH_{2cyc}), 1.88-1.97 (m, 4H, $\underline{C}H_2CH_2Br$, CH_{2cyc}),3.09

(ddd, J = 11.9, 8.0, 5.7, 1H, ½CH_{2cyc}N), 3.46 (t, J = 6.8, 2H, CH_2Br), 3.69 (ddd, J = 11.9, 8.1, 6.1, 1H, ½CH_{2cyc}N), 7.18-7.31 (m, 9H, Ar), 7.43 (d, J = 7.2, 6H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$,): δ 24.2, 25.4, 28.1, 28.7, 29.4, 32.8, 33.8, 33.9, 35.3, 45.5 (10CH_2), 71.8, 73.6 (2C), 126.7 (3CH), 127.6 (6CH), 128.6 (6CH), 142.5 (3C), 160.5, 176.5 (2CO); MS (ESI, *m/z*): 243.1 $[Tr]^+$.

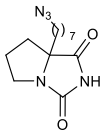
7a-(7-Azidoheptyl)-2-trityltetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione, 32. To a solution of bromo derivative **31** (280 mg, 0.50 mmol) in DMF (0.8 mL), a freshly prepared solution of sodium azide (75 mg, 2.2 mmol) in H_2O (0.3 mL) was added dropwise and the mixture was stirred at 50 ºC for 12 h. Then, a solution of sodium azide (25 mg, 0.38 mmol) in H_2O (0.1 mL) was added and the mixture was stirred at 50 ºC for additional 4 h. The mixture was quenched with H_2O (5 mL) and extracted with EtOAc (3 x 20 mL). The combined organic extracts were washed with brine and dried (Na_2SO_4). The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (hexane:EtOAc, 8:2 to 2:8 v/v) to afford the title compound **32** as an oil (258 mg, 99%).



R_f (DCM) = 0.53; IR (ATR, cm^{-1}): 2095, 1776, 1717, 1492, 1451; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41-1.77 (m, 14H, $(CH_2)_6$, CH_{2cyc}), 1.84-1.97 (m, 2H, CH_{2cyc}), 3.07 (ddd, J = 11.8, 8.0, 5.8, 1H, ½CH_{2cyc}N), 3.30 (t, J = 6.9, 2H, CH_2N_3), 3.67 (ddd, J = 11.8, 8.0, 6.1, 1H, ½CH_{2cyc}N), 7.16-7.28 (m, 9H, Ar), 7.42 (d, J = 7.3, 6H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$,): δ 24.2, 25.5, 26.7, 28.9, 29.1, 29.5, 33.8, 35.3, 45.5, 51.5 (10CH_2), 71.8, 73.6 (2C), 126.7 (3CH), 127.6 (6CH), 128.6 (6CH), 142.5 (3C), 160.5, 176.5 (2CO); MS (ESI, *m/z*): 243.1 $[Tr]^+$.

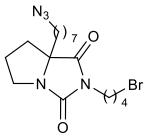
7a-(7-Azidoheptyl)tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione, 33. To a solution of compound **32** (155 mg, 0.30 mmol) in DCM (2 mL), trifluoroacetic acid (TFA, 1.6 mL, 20 mmol) was added dropwise and the reaction mixture was stirred at rt for 3 h. The solvent and

excess of trifluoroacetic acid were evaporated under reduced pressure and the residue was purified by column chromatography (DCM) to afford the title compound **33** as an oil (74 mg, 89%).



R_f (hexane:EtOAc, 1:1 v/v) = 0.4; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.17-1.44 (m, 8H, $4CH_2$), 1.51-1.60 (m, 3H, CH_2, ½CH_{2cyc}), 1.81-1.97 (m, 4H, CH_2, CH_{2cyc}), 1.99-2.12 (m, 3H, CH_2, ½CH_{2cyc}), 3.14 (dt, *J* = 11.7, 6.9, 1H, ½CH_{2cyc}N), 3.23 (t, *J* = 6.9, 2H, CH_2N_3), 3.77 (dt, *J* = 11.7, 7.4, 1H, ½CH_{2cyc}N), 8.78 (br s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$,): δ 23.8, 26.1, 26.6, 28.8, 29.0, 29.4, 32.9, 35.0, 44.5, 51.5 ($10CH_2$), 74.0 (C), 160.4, 177.2 (2CO).

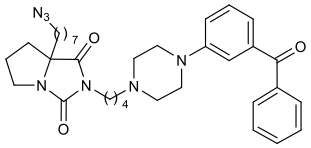
7a-(7-Azidoheptyl)-2-(4-bromobutyl)tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione, 34. To a solution of compound **33** (120 mg, 0.42 mmol), in anhydrous DMF (2 mL), NaH (20 mg, 0.50 mmol, 60% in mineral oil) was added portionwise and the reaction mixture was stirred at 60 ºC for 1 h. Then, a solution of 1,4-dibromobutane (103 µL, 0.84 mmol) in DMF (1 mL) was added dropwise an the mixture was stirred at 110 ºC for 3 h. The reaction was quenched with H_2O (5 mL) and extracted with EtOAc (3x20 mL). The organic layer was dried (Na_2SO_4), the solvents were evaporated under reduced pressure and the residue was purified by column chromatography (hexane:EtOAc, 8:2 to 7:3 v/v) to afford the title compound **34** as an oil (122 mg, 70%).



R_f (hexane:EtOAc, 7:3 v/v) = 0.42; IR (ATR, cm^{-1}): 2095, 1770, 1709; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.06-1.10 (m, 1H, ½CH_{2cyc}), 1.22-1.33 (m, 7H, $3CH_2$, ½CH_{2cyc}), 1.49-1.57 (m, 3H, CH_2, ½CH_{2cyc}), 1.68-1.91 (m, 7H, $3CH_2$, ½CH_{2cyc}), 1.98-2.07 (m, 2H, CH_2), 3.16 (ddd, *J* = 11.7, 8.0, 6.0, 1H, ½CH_{2cyc}N), 3.22 (t, *J* = 6.9, 2H, CH_2N_3), 3.39 (t, *J* = 6.4, 2H, CH_2Br), 3.44-3.55 (m, 2H, $CH_2N(CO)_2$), 3.74 (ddd, *J* = 11.7, 8.2, 6.6, 1H, ½CH_{2cyc}N); ^{13}C NMR (75 MHz, $CDCl_3$,): δ 23.9,

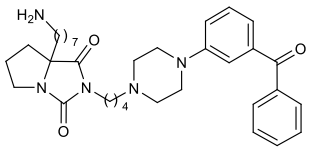
26.1, 26.6, 26.7, 28.8, 29.0, 29.3, 29.8, 32.8, 33.2, 35.1, 37.9, 44.9, 51.5 (14CH_2), 72.2 (C), 160.6, 176.5 (2CO); MS (ESI, *m/z*): 386.1 [M(^{79}Br)-N_2+H]$^+$, 388.1 [M(^{81}Br)-N_2+H]$^+$, 414.1 [M(^{79}Br)+H]$^+$, 416.1 [M(^{81}Br)+H]$^+$

7a-(7-Azidobutyl)-2-{4-[4-(3-benzoylphenyl)piperazin-1-yl]butyl}tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione, 36. To a suspension of bromo derivative **34** (54 mg, 0.13 mmol) and phenyl[(3-piperazin-1-yl)phenyl]methanone (**35**) (60 mg, 0.22 mmol) in anhydrous acetonitrile (2 mL), triethylamine (30 μL, 0.22 mmol) was added and the reaction mixture was stirred at 60 ºC for 24 h. The solvent was evaporated under reduced pressure and the residue was resuspended in H_2O and extracted with DCM (3 x 20 mL). The organic layer was dried (Na_2SO_4), the solvent was evaporated under reduced pressure and the residue was purified by column chromatography (DCM:EtOH, 99:1 to 95:5 v/v) to afford the title compound **36** as an oil (62 mg, 79%).



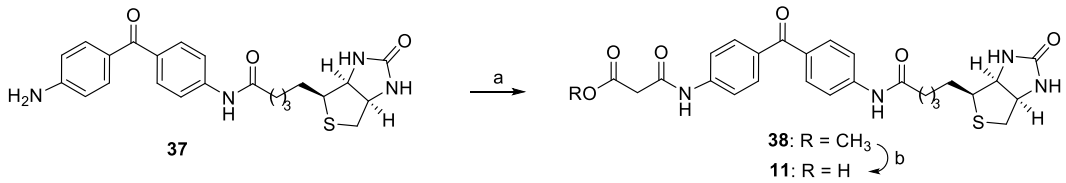
R_f (DCM:EtOH, 95:5 v/v) = 0.54; IR (ATR, cm^{-1}): 2095, 1769, 1708, 1658, 1596, 1487, 1443; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.05-1.11 (m, 1H, ½CH_{2cyc}), 1.23-1.34 (m, 7H, 3CH_2, ½CH_{2cyc}), 1.46-1.68 (m, 7H, 3CH_2, ½CH_{2cyc}), 1.80-1.90 (m, 3H, CH_2, ½CH_{2cyc}), 1.97-2.10 (m, 2H, CH_2), 2.39 (t, *J* = 7.3, 2H, CH_2N_{pip}), 2.56 (app t, *J* = 4.9, 4H, 2CH_{2pip}), 3.16 (ddd, *J* = 11.7, 8.0, 6.0, 1H, ½CH_{2cyc}N), 3.19-3.24 (m, 6H, 2CH_{2pip}, CH_2N_3), 3.40-3.55 (m, 2H, $CH_2N(CO)_2$), 3.75 (ddd, *J* = 11.7, 8.1, 6.5, 1H, ½CH_{2cyc}N), 7.11 (dd, *J* = 8.2, 1.9, 1H, Ar), 7.16 (d, *J* = 7.6, 1H, Ar), 7.30 (d, *J* = 7.9, 1H, Ar), 7.34 (m, 1H, Ar), 7.45 (t, *J* = 7.4, 2H, Ar), 7.55 (t, *J* = 7.4, 1H, Ar), 7.78 (d, *J* = 7.0, 2H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 23.8, 24.0 (2CH_2), 26.1 (2CH_2), 26.6, 28.8, 29.0, 29.3, 33.1, 35.1, 38.8, 44.9 (8CH_2), 48.9 (2CH_2), 51.4 (CH_2), 53.1 (2CH_2), 57.9 (CH_2), 72.4 (C), 116.7, 119.8, 121.5 (3CH), 128.2 (2CH), 128.9 (CH), 130.1 (2CH), 132.3 (CH), 137.9, 138.5, 151.3 (3C), 160.7, 176.5, 197.1 (3CO); MS (ESI, *m/z*): 600.4 [M+H]$^+$.

7a-(7-Aminobutyl)-2-{4-[4-(3-benzoylphenyl)piperazin-1-yl]butyl}tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione, 8. To a solution of azide **36** (62 mg, 0.10 mmol) in a mixture of THF/H_2O, 25:1 (3 mL, v/v), triphenylphosphine (54 mg, 0.21 mmol) was added portionwise and the reaction mixture was stirred at rt for 24 h. The solvent was evaporated under reduced pressure and the residue was redissolved in EtOAc and extracted with an aqueous 1 M HCl solution (2 x 30 mL). The aqueous layer was basified with and aqueous 20% K_2CO_3 solution and extracted with DCM (3 x 30 mL). The organic extracts were washed with brine and dried (Na_2SO_4). The solvents were evaporated to afford the title amine **8** as an oil (50 mg, 84%), which was used in the next step without further purification.



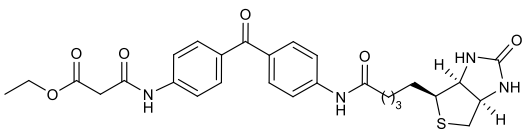
R_f (DCM:EtOH:NH_3, 5:5:0.1 v/v/v) = 0.29; IR (ATR, cm^{-1}): 3461, 1768, 1707, 1657, 1596, 1487, 1443; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.11 (m, 1H, ½CH_{2cyc}), 1.26-1.42 (m, 9H, 3CH_2, ½CH_{2cyc}, NH_2), 1.45-1.68 (m, 7H, 3CH_2, ½CH_{2cyc}), 1.79-1.90 (m, 3H, CH_2, ½CH_{2cyc}), 1.97-2.10 (m, 2H, CH_2), 2.39 (t, *J* = 7.3, 2H, CH_2N_{pip}), 2.57 (app t, *J* = 4.9, 4H, 2CH_{2pip}), 2.64 (t, *J* = 6.9, 2H, C<u>H</u>$_2NH_2$), 3.16 (ddd, *J* = 11.7, 8.0, 6.0, 1H, ½CH_{2cyc}N), 3.21-3.24 (app t, *J* = 4.8, 4H, 2CH_{2pip}), 3.40-3.55 (m, 2H, $CH_2N(CO)_2$), 3.75 (ddd, *J* = 11.7, 8.1, 6.6, 1H, ½CH_{2cyc}N), 7.11 (dd, *J* = 8.2, 2.4, 1H, Ar), 7.16 (dt, *J* = 7.6, 1.1, 1H, Ar), 7.30 (d, *J* = 7.9, 1H, Ar), 7.34 (m, 1H, Ar), 7.45 (t, *J* = 7.4, 2H, Ar), 7.56 (t, *J* = 7.4, 1H, Ar), 7.78 (d, *J* = 7.0, 2H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 23.9, 24.0 (2CH_2), 26.1 (2CH_2), 26.8, 29.4, 29.5, 33.1, 33.7, 35.1, 38.8, 42.4, 44.9 (9CH_2), 48.9 (2CH_2), 53.1 (2CH_2), 58.0 (CH_2), 72.5 (C), 116.7, 119.8, 121.6 (3CH), 128.3 (2CH), 128.9 (CH), 130.1 (2CH), 132.4 (CH), 137.9, 138.5, 151.4 (3C), 160.8, 176.6, 197.2 (3CO); MS (ESI, *m/z*): 574.4 $[M+H]^+$, 287.7 $([M+2H]/2)^+$.

Synthesis of carboxylic acid 11



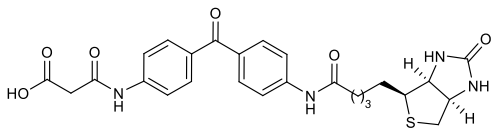
Scheme S2. Reagents and conditions: (a) 3-ethoxy-3-oxopropanoic acid, EDC, HOBt, DMF, DCM, rt, 92%; (b) 1 M methanolic NaOH, MeOH, rt, 60%.

(+)-Ethyl 3-oxo-3-({4-[4-(biotinylamino)benzoyl]phenyl}amino)propanoate, 38. To a solution of HOBt (250 mg, 1.8 mmol) and 3-ethoxy-3-oxopropanoic acid (242 mg, 1.8 mmol) in dry DCM (3 mL), EDC (355 mg, 1.8 mmol) was added portionwise and the mixture was stirred at rt for 40 min. Then, a solution of aniline **37** (270 mg, 0.62 mmol) in dry DMF (1.5 mL) was added and the reaction was stirred for additional 60 h. The mixture was diluted with EtOAc, washed with a saturated aqueous $NaHCO_3$ solution and brine and dried (Na_2SO_4). The solvents were evaporated under reduced pressure and the residue was purified by column chromatography (DCM:EtOH, 95:5 v/v) to afford the title compound **38** as a white solid (316 mg, 92%).



R_f (DCM:EtOH, 9:1 v/v) = 0.17; mp: 136-138 ºC; $[\alpha]_D^{20}$ = +47.9 (c = 0.7, DMSO); IR (ATR, cm^{-1}): 3261, 1695, 1595, 1529, 1463; ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.21 (t, J = 7.1, 3H, CH_3), 1.35-1.65 (m, 6H, $(CH_2)_3$), 2.37 (t, J = 7.3, 2H, CH_2CO), 2.58 (d, J = 12.4, 1H, ½CH_2S), 2.83 (dd, J = 12.4, 5.1, 1H, ½CH_2S), 3.10-3.16 (m, 1H, CHS), 3.51 (s, 2H, $CH_2(CO)_2$), 4.10-4.17 (m, 3H, CH_2O, C<u>H</u>NH), 4.29-4.34 (m, 1H, C<u>H</u>NH), 6.35 (br s, 1H, NH), 6.43 (br s, 1H, NH), 7.67-7.78 (m, 8H, Ar), 10.24 (br s, 1H, NH), 10.53 (br s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 14.0 (CH_3), 25.0, 28.1, 28.2, 36.3, 40.1, 43.8 ($6CH_2$), 55.4, 59.2 (2CH), 60.7 (CH_2), 61.0 (CH), 118.2 (2CH), 118.3 (2CH), 130.9 (4CH), 131.6, 132.3, 142.4, 143.2 (4C), 162.7, 164.6, 167.5, 171.8, 193.4 (5CO).

(+)-3-Oxo-3-({4-[4-(biotinylamino)benzoyl]phenyl}amino)propanoic acid, 11. A suspension of ethyl ester **38** (315 mg, 0.57 mmol) in 2 mL of NaOH (1 M in MeOH) was stirred for 2 h at rt. The solvent was then evaporated under reduced pressure and the residue was acidified with an aqueous 1 M HCl solution. The resulting white precipitate was filtered, washed with H_2O, and dried under high vacuum at 35 ºC overnight to afford title compound **11** as a white solid (177 mg, 60%).

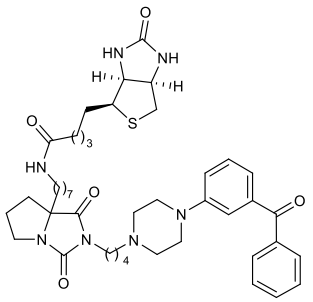


R_f (EtOAc/MeOH/AcOH, 8:2:0.1 v/v/v) = 0.25; mp: 182-186 ºC; $[\alpha]_D^{20}$ = +106.2 (c = 0.3, DCM/MeOH, 1:1); IR (ATR, cm^{-1}): 3300, 1695, 1672, 1596, 1530, 1471; ^{1}H NMR (500 MHz, DMSO-d_6,) δ: 1.36-1.65 (m, 6H, $(CH_2)_3$), 2.37 (t, *J*=7.2, 2H, CH_2CO), 2.57 (d, *J*=12.4, 1H, ½CH_2S), 2.82 (dd, *J*=12.4, 5.0, 1H, ½CH_2S), 3.09-3.16 (m, 1H, CHS), 3.40 (s, 2H, $CH_2(CO)_2$), 4.12-4.16 (m, 1H, CHNH), 4.29-4.33 (m, 1H, CHNH), 6.41 (br s, 1H, NH), 6.50 (br s, 1H, NH), 7.71-7.80 (m, 8H, Ar), 10.30 (br s, 1H, NH), 10.61 (br s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6,): δ 25.9, 29.0, 29.1, 37.2, 40.7, 45.0 ($6CH_2$), 56.2, 60.1, 61.9 (3CH), 119.1 (4CH), 131.8 (4CH), 132.5, 133.0, 143.5, 144.0 (4C), 163.6, 166.1, 170.0, 172.7, 194.2 (5CO); MS (ESI, *m/z*): 479.0 $[M\text{-}COOH]^-$, 523.1 $[M\text{-}H]^-$.

General Procedure for the Synthesis of Compounds 2-6

To a solution of biotin, carboxylic acids **11**, **12**, or Fmoc-4-benzoyl-L-phenylalanine (1.5 equiv) in anhydrous DMF (10 mL/mmol) and DCM (10 mL/mmol), 4 Å molecular sieves previously activated (35 mg/mmol carboxylic acid), HOBt (1.5 equiv) and EDC (1.5 equiv) were added and the mixture was stirred at rt for 1 h. Then, a solution of the corresponding amine **8-10** (1 equiv) in anhydrous DCM (20 mL/mmol) was added and the reaction mixture was stirred for 72 h at rt. The molecular sieves were removed by filtration and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography.

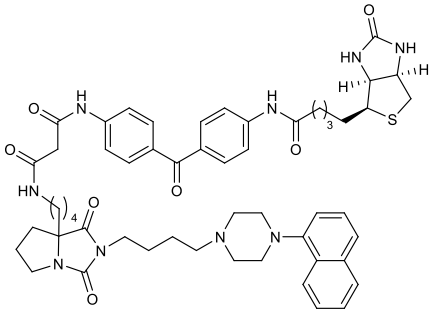
***N*-{4-[2-{4-[4-(3-Benzoylphenyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo-[1,2-*c*]imidazol-7a(5*H*)-yl]butyl}biotinamide, 2.** Obtained from biotin (51 mg, 0.21 mmol) and amine **8** (80 mg, 0.14 mmol) as an oil (43 mg, 39%). Chromatography: DCM:EtOH:NH_3, 9:1:0.1 v/v/v.



R_f (DCM:EtOH, 8:2 v/v) = 0.54; $[\alpha]_D^{20}$ = +12.56 (c = 1.0, $CHCl_3$); IR (ATR, cm^{-1}): 3286, 1767, 1703, 1655, 1596, 1573, 1445; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.82-1.71 (m, 21H, 9CH_2, 3½CH_{2cyc}), 1.81-1.90 (m, 3H, CH_2, ½CH_{2cyc}), 2.03-2.09 (m, 2H, CH_2), 2.17 (t, *J* = 7.4, 2H, CH_2CO), 2.46 (m, 2H, CH_2N_{pip}), 2.64 (m, 4H, 2CH_{2pip}), 2.72 (d, *J* = 12.8, 1H, ½CH_2S), 2.89 (dd, *J* = 12.8, 4.9, 1H, ½CH_2S), 3.09-3.27 (m, 8H, 2CH_{2pip}Ar, ½CH_{2cyc}N, C$\underline{H}_2$NHCO, CHS), 3.43-3.54 (m, 2H, $CH_2N(CO)_2$), 3.75 (dt, *J* = 11.0, 7.6, 1H, ½CH_{2cyc}N), 4.29 (dd, *J* = 7.2, 5.1, 1H, C$\underline{H}$NH), 4.49 (dd, *J* = 7.6, 4.9, 1H, C$\underline{H}$NH), 5.43 (br s, 1H, NH), 6.08 (br s, 1H, NH), 6.20 (br s, 1H, NH), 7.13 (dd, *J* = 8.2, 1.8, 1H, Ar), 7.18 (dt, *J* = 7.6, 1.1, 1H, Ar), 7.33 (t, *J* = 7.9, 1H, Ar), 7.36 (m, 1H, Ar), 7.47 (t, *J* = 7.4, 2H, Ar), 7.58 (t, *J* = 7.4, 1H, Ar), 7.79 (d, *J* = 7.0, 2H, Ar); ^{13}C NMR (125 MHz, $CDCl_3$): δ 24.0, 25.8, 26.0, 26.1, 26.9, 28.2, 28.3, 29.2, 29.5, 29.7, 29.8, 33.2, 35.1, 36.1, 38.8, 39.5, 40.7, 44.9 (18CH_2), 48.7 (2CH_2), 53.0 (2CH_2), 55.6 (CH), 57.9 (CH_2), 60.3, 61.9 (2CH), 72.6 (C), 116.8, 120.0, 121.8 (3CH), 128.4 (2CH), 129.0 (CH), 130.2 (2CH), 132.5 (CH), 137.9, 138.5, 151.3 (3C), 160.8, 163.8, 173.2, 176.6, 197.4 (5CO); MS (ESI, *m/z*): 400.5 $[(M+2H)/2]^+$, 799.8 $[M+H]^+$; analysis (calcd., found for $C_{44}H_{61}N_7O_5S\cdot HCl\cdot 4H_2O$): C (58.16, 58.52), H (7.77, 8.11), N (10.79, 10.70), S (3.53, 3.46).

(+)-*N*-{4-[2-{4-[4-(1-Naphthyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo-[1,2-*c*]imidazol-7a(5*H*)-yl]butyl}-*N*'-{4-[4-(biotinylamino)benzoyl]phenyl}malonamide, 3.

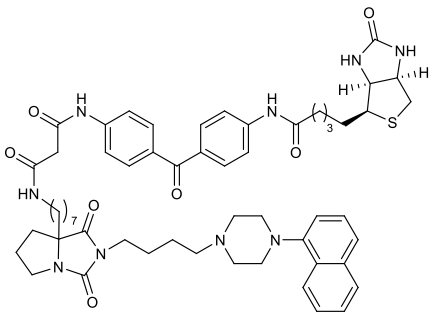
Obtained from carboxylic acid **11** (85 mg, 0.16 mmol) and amine **9** (52 mg, 0.11 mmol) as a white solid (18 mg, 17%). Chromatography: DCM:EtOH:NH_3, 9:1:0.1 v/v/v.



R_f (DCM:EtOH:NH_3, 9:1:0.1 v/v/v) = 0.5; mp: 171-172 ºC; $[\alpha]_D^{20}$ = +12.6 (c = 0.55, DCM/MeOH, 1:1); IR (ATR, cm^{-1}): 3305, 1766, 1699, 1593, 1528, 1450; ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.40-1.70 (m, 15H, 7CH_2, ½CH_{2cyc}), 1.74-1.82 (m, 3H, CH_2, ½CH_{2cyc}), 1.90-2.05 (m, 2H, CH_{2cyc}), 2.34-2.42 (m, 4H, CH_2CO, CH_2N_{pip}), 2.56-2.60 (m, 5H, 2CH_{2pip}, ½CH_2S), 2.83 (dd, *J* = 12.4, 5.1, 1H, ½CH_2S), 3.01-3.16 (m, 8H, 2CH_{2pip}Ar, C<u>H</u>$_2$NH, CHS, ½CH_{2cyc}N), 3.27 (s, 2H, ($CH_2(CO)_2$), 3.36-3.42 (m, 2H, $CH_2N(CO)_2$), 3.54-3.63 (m, 1H, ½CH_{2cyc}N), 4.12-4.16 (m, 1H, C<u>H</u>NH), 4.29-4.33 (m, 1H, C<u>H</u>NH), 6.34 (br s, 1H, NH), 6.41 (br s, 1H, NH), 7.09 (d, *J* = 7.3, 1H, Ar), 7.40 (t, *J* = 7.8, 1H, Ar), 7.46-7.50 (m, 2H, Ar), 7.56 (d, *J* = 8.1, 1H, Ar), 7.68-7.77 (m, 8H, Ar), 7.84-7.87 (m, 1H, Ar), 8.04-8.10 (m, 2H, Ar, NH), 10.22 (br s, 1H, NH), 10.43 (br s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.4, 25.0, 25.4, 25.5, 26.2, 28.1, 28.2, 28.9, 32.3, 34.0, 36.3, 38.1, 38.5, 39.9, 44.6, 44.8 (16CH_2), 52.6 (2CH_2), 53.1 (2CH_2), 55.3 (CH), 57.2 (CH_2), 59.2, 61.3 (2CH), 71.8 (C), 114.5 (CH), 118.2 (2CH), 118.3 (2CH), 123.0, 123.3, 125.3, 125.8, 126.0 (5CH), 128.1 (C), 128.2 (CH), 130.9 (4CH), 131.6, 132.0, 134.3, 142.6, 143.1, 149.3 (6C), 160.1, 162.7, 166.1, 166.3, 171.8, 175.9, 193.3 (7CO); MS (ESI, *m/z*): 492.5 $[(M+2H)/2]^+$, 984.5 $[M+H]^+$; analysis (calcd., found for $C_{54}H_{65}N_9O_7S\cdot4HCl\cdot H_2O$): C (56.49, 56.48), H (6.23, 6.14), N (10.98, 10.51), S (2.79, 2.68).

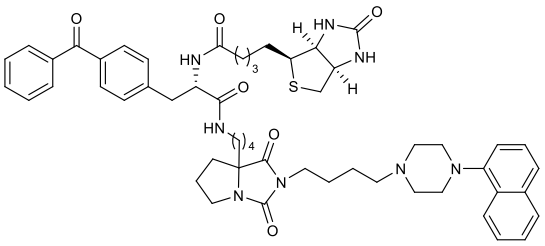
(+)-*N*-{4-[2-{4-[4-(1-Naphthyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo[1,2-*c*]imidazol-7a(5*H*)-yl]heptyl}-*N*'-{4-[4-(biotinylamino)benzoyl]phenyl}-malonamide, 4.

Obtained from carboxylic acid **11** (80 mg, 0.15 mmol) and amine **10** (51 mg, 0.10 mmol) as a white solid (36 mg, 36%). Chromatography: DCM:EtOH:NH_3, 9:1:0.1 v/v/v.



R_f (DCM:EtOH, 8:2 v/v) = 0.3; mp: 98-100 ºC; $[\alpha]_D^{20}$ = +16.3 (c = 0.55, DCM/MeOH, 1:1); IR (ATR, cm^{-1}): 3279, 1765, 1698, 1593, 1528, 1450; ^{1}H NMR (500 MHz, DMSO-d_6): δ 0.81-0.85 (m, 1H, ½CH_{2cyc}), 1.24-1.80 (m, 23H, 11CH_2, ½CH_{2cyc}), 1.91-2.08 (m, 2H, CH_{2cyc}), 2.35-2.38 (m, 4H, CH_2CO, CH_2N_{pip}), 2.57-2.63 (m, 5H, 2CH_{2pip}, ½CH_2S), 2.82 (dd, *J* = 12.4, 5.2, 1H, ½CH_2S), 3.01-3.07 (m, 6H, 2CH_{2pip}Ar, CHS, ½CH_{2cyc}N), 3.11-3.15 (m, 2H, C<u>H</u>$_2$NH), 3.29 (s, 2H, $CH_2(CO)_2$), 3.36-3.43 (m, 2H, $CH_2N(CO)_2$), 3.51-3.60 (m, 1H, ½CH_{2cyc}N), 4.13-4.15 (m, 1H, C<u>H</u>NH), 4.29-4.32 (m, 1H, C<u>H</u>NH), 6.35 (br s, 1H, NH), 6.43 (br s, 1H, NH), 7.10 (d, *J* = 7.3, 1H, Ar), 7.41 (t, *J* = 7.8, 1H, Ar), 7.46-7.51 (m, 2H, Ar), 7.57 (d, *J* = 8.1, 1H, Ar), 7.69 (dd, *J* = 8.7, 3.3, 4H, Ar), 7.75 (t ap, *J* = 8.1, 4H, Ar), 7.85-7.87 (m, 1H, Ar), 8.03-8.05 (m, 1H, NH), 8.08-8.09 (m, 1H, Ar), 10.24 (br s, 1H, NH), 10.44 (br s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.4, 23.5, 25.0, 25.4, 25.5, 26.2, 28.1, 28.2, 28.6, 28.8, 28.9, 32.4, 34.3, 36.3, 38.0, 38.7, 39.8, 44.6, 44.8 (19CH_2), 52.6 (2CH_2), 53.1 (2CH_2), 55.3 (CH), 57.2 (CH_2), 59.2, 61.0 (2CH), 71.9 (C), 114.5 (CH), 118.2 (2CH), 118.3 (2CH), 123.0, 123.2, 125.3, 125.8, 126.0 (5CH), 128.1 (C), 128.3 (CH), 130.9 (4CH), 131.6, 132.0, 134.3, 142.6, 143.1, 149.3 (6C), 160.1, 162.7, 166.0, 166.3, 171.8, 175.9, 193.3 (7CO); MS (ESI, *m/z*): 513.3 $[(M+2H)/2]^+$, 1025.4 $[M+H]^+$; analysis (calcd., found for $C_{57}H_{71}N_9O_7S{\cdot}4HCl{\cdot}7H_2O$): C (52.73, 52.83), H (6.91, 6.65), N (9.71, 9.51), S (2.47, 2.39).

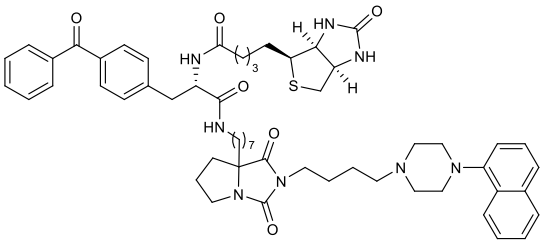
(+)-4-Benzoyl-*N*-{4-[2-{4-[4-(1-naphthyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo[1,2-*c*]imidazol-7a(5*H*)-yl]butyl}-*N*-biotinyl-L-phenylalaninamide, 5. Obtained from carboxylic acid **12** (100 mg, 0.20 mmol) and amine **9** (65 mg, 0.14 mmol) as a white solid (25 mg, 20%). Chromatography: DCM:EtOH:NH_3, 9:1:0.1 v/v/v.



R_f (DCM:EtOH:NH_3, 9:1:0.1 v/v/v) = 0.2; mp: 90-92 ºC; $[\alpha]_D^{20}$ = +14.5 (c = 0.75, DCM:MeOH, 1:1); IR (ATR, cm^{-1}): 3268, 1767, 1702, 1653, 1603, 1550, 1447; ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.08-1.80 (m, 18H, 8CH_2, CH_{2cyc}), 1.91-2.06 (m, 4H, CH_{2cyc}, CH_2CO), 2.36 (t, *J* = 6.5, 2H, CH_2N_{pip}), 2.53-2.59 (m, 5H, 2CH_{2pip}, ½CH_2S), 2.70 (dd, *J* = 12.5, 5.1, 1H, ½CH_2S), 2.78-3.03 (m, 9H, 2CH_{2pip}Ar, CH_2Ar, C<u>H</u>$_2$NH, CHS), 3.13 (ddd, *J* = 11.6, 8.4, 5.7, 1H, ½CH_{2cyc}N), 3.36-3.40 (m, 2H, $CH_2N(CO)_2$), 3.52-3.61 (m, 1H, ½CH_{2cyc}N), 4.00-4.06 (m, 1H, C<u>H</u>NH_{biotin}), 4.20-4.24 (m, 1H, C<u>H</u>NH_{biotin}), 4.49-4.55 (m, 1H, C<u>H</u>NH), 6.34 (br s, 1H, NH), 6.38 (br s, 1H, NH), 7.37-7.42 (m, 3H, Ar), 7.46-7.58 (m, 5H, Ar), 7.62-7.69 (m, 5H, Ar), 7.84-7.87 (m, 1H, Ar), 8.00-8.09 (m, 4H, Ar, 2NH); ^{13}C-RMN (75 MHz, DMSO-d_6, δ): 20.9, 23.4, 25.1, 25.4, 25.6, 27.9, 28.1, 28.9, 30.7, 32.3, 34.9, 35.0, 38.1, 38.2, 39.6, 44.6 (16CH_2), 52.6 (2CH_2), 53.1 (2CH_2), 53.5, 55.4 (2CH), 57.2 (CH_2), 59.1, 61.0 (2CH), 71.9 (C), 114.5, 123.0, 123.3, 125.4, 125.8, 126.0 (6CH), 128.1 (C), 128.3 (CH), 128.5 (2CH), 129.4 (2CH), 129.5 (2CH), 129.6 (2CH), 132.6 (CH), 134.3, 135.0, 137.2, 143.5, 149.3 (5C), 160.1, 162.7, 170.8, 171.9, 175.9, 195.5 (6CO); MS (ESI, *m/z*): 478.4 $[(M+2H)/2]^+$, 955.5 $[M+H]^+$; analysis (calcd., found for $C_{54}H_{66}N_8O_6S\cdot3HCl\cdot H_2O$): C (59.91, 59.52), H (6.61, 6.62), N (11.00, 10.71), S (3.15, 2.79).

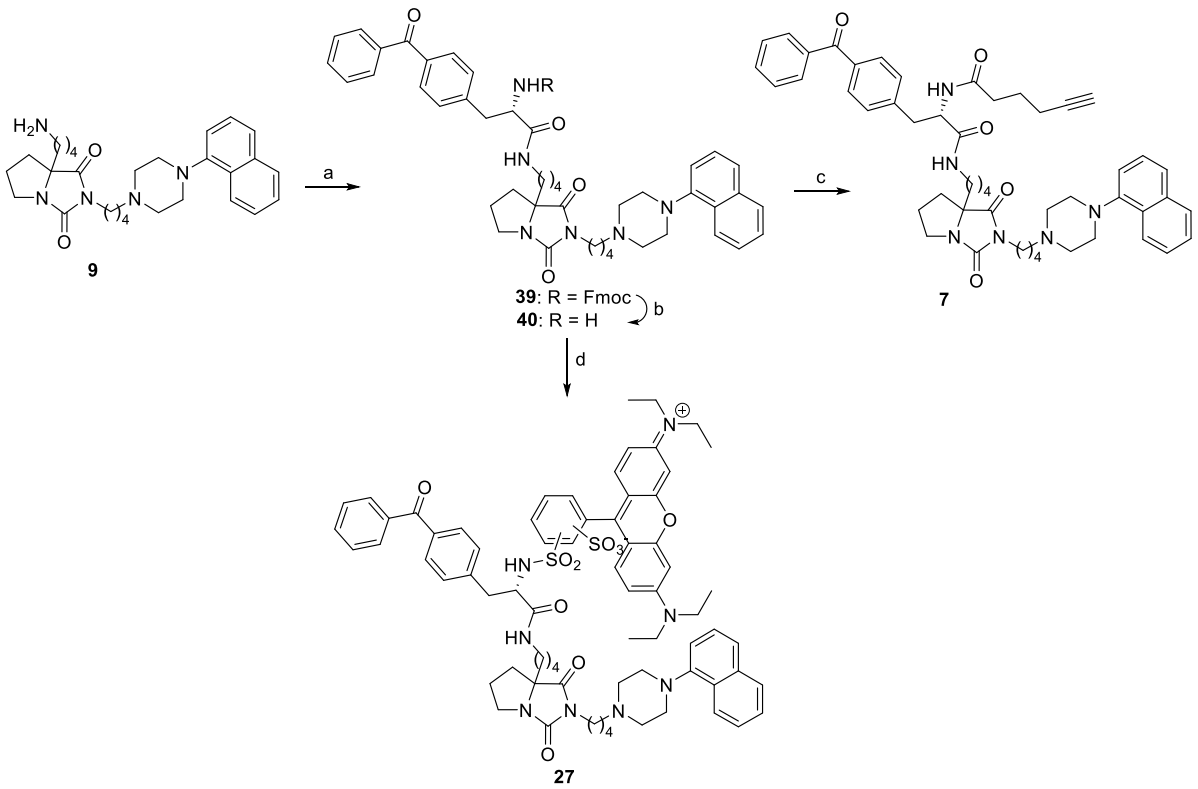
(+)-4-Benzoyl-*N*-{7-[2-{4-[4-(1-napthyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo[1,2-*c*]imidazol-7a(5*H*)-yl]heptyl}-*N*-biotinyl-L-phenylalaninamide, 6. Obtained from

carboxylic acid **12** (85 mg, 0.17 mmol) and amine **10** (57 mg, 0.11 mmol) as a white solid (52 mg, 46%). Chromatography: DCM:EtOH:NH_3, 9:1:0.1 v/v/v.



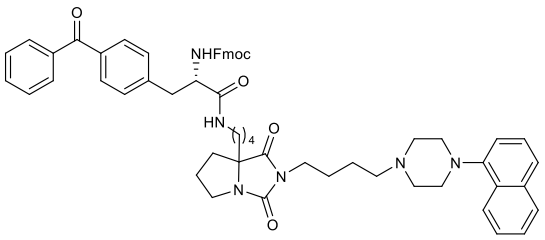
R_f (DCM:EtOH:NH_3, 9:1:0.1 v/v/v) = 0.2; mp: 102-105 ºC; $[\alpha]_D^{20}$ = +18.7 (c = 0.6, DMC/MeOH, 1:1 v/v); IR (ATR, cm^{-1}): 3270, 1766, 1702, 1653, 1604, 1547, 1446; ^1H NMR (500 MHz, DMSO-d_6): δ 1.00-1.30 (m, 12H, $6CH_2$), 1.33-1.42 (m, 5H, $2CH_2$, ½CH_{2cyc}), 1.52-1.61 (m, 4H, $2CH_2$), 1.67-1.85 (m, 3H, CH_2, ½CH_{2cyc}), 1.91-2.10 (m, 4H, CH_2CO, CH_{2cyc}), 2.38 (t, *J* = 6.9, 2H, CH_2N_{pip}), 2.55-2.60 (m, 5H, $2CH_{2pip}$, ½CH_2S), 2.73 (dd, *J* = 12.3, 4.9, 1H, ½CH_2S), 2.80-3.16 (m, 10H, $2CH_{2pip}Ar$, CH_2Ar, C<u>H</u>$_2$NH, ½$CH_{2cyc}N$, CHS), 3.39-3.48 (m, 2H, $CH_2N(CO)_2$), 3.53-3.62 (m, 1H, ½$CH_{2cyc}N$), 4.02-4.07 (m, 1H, C<u>H</u>NH_{biotin}), 4.21-4.27 (m, 1H, C<u>H</u>NH_{biotin}), 4.49-4.57 (m, 1H, C<u>H</u>NH), 6.34 (br s, 1H, NH), 6.39 (br s, 1H, NH), 7.09 (d, *J* = 6.9, 1H, Ar), 7.38 (d, *J* = 8.2, 2H, Ar), 7.41 (t, *J* = 7.9, 1H, Ar), 7.45-7.51 (m, 2H, Ar), 7.53-7.58 (m, 3H, Ar), 7.62-7.70 (m, 5H, Ar), 7.85-7.88 (m, 1H, Ar), 7.95 (m, 1H, NH), 8.07-8.10 (m, 2H, Ar, NH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.4, 23.6, 25.1, 25.2, 25.4, 25.6, 26.3, 27.9, 28.0, 28.7, 28.9, 29.0, 32.4, 34.3, 34.9, 38.1, 38.4, 39.6, 44.6 ($19CH_2$), 52.7 ($2CH_2$), 53.1 ($2CH_2$), 53.5, 55.4 (2CH), 57.3 (CH_2), 59.2, 61.0 (2CH), 71.9 (C), 114.5, 123.0, 123.3, 125.4, 125.8, 126.0 (6CH), 128.1 (C), 128.3 (CH), 128.6 (2CH), 129.4 (2CH), 129.5 (2CH), 129.6 (2CH), 132.6 (CH), 134.3, 135.0, 137.2, 143.4, 149.3 (5C), 160.1, 162.7, 170.6, 171.9, 175.9, 195.4 (6CO); MS (ESI, *m/z*): 499.5 $[(M+2H)/2]^+$, 997.5 $[M+H]^+$; analysis (calcd., found for $C_{57}H_{72}N_8O_6S \cdot 3HCl$): C (61.86, 61.49), H (6.83, 6.90), N (10.13, 9.83), S (2.90, 2.98).

- **Synthesis of Probes 7 and 27**



Scheme S3. Reagents and conditions: (a) Fmoc-4-benzoyl-L-phenylalanine, EDC, HOBt, DMF, DCM, rt, 80%; (b) piperidine, DCM, 0 ºC, 75%; (c) 5-hexynoic acid, DCC, DMAP, DCM, rt, 53%; (d) rhodamine B sulfonyl chloride (mixed isomers), Et_3N, 80 ºC, 10%.

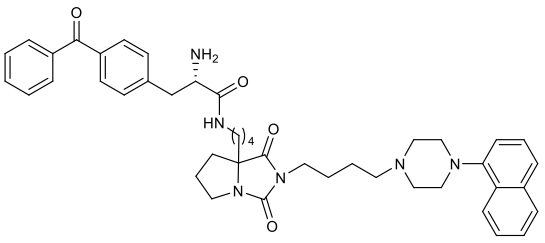
(–)-4-Benzoyl-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-*N*-{4-[2-{4-[4-(1-naphthyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo[1,2-*c*]imidazol-7a(5*H*)-yl]butyl}-L-phenylalaninamide, 39. Obtained from Fmoc-4-benzoyl-L-phenylalanine (335 mg, 0.77 mmol) and amine **9** (245 mg, 0.50 mmol) as an oil (385 mg, 80%) following the general procedures employed for the synthesis of compounds **2**-**6**. Chromatography: DCM:EtOH, 95:5 v/v.



R_f (DCM:EtOH, 95:5 v/v): 0.38; $[\alpha]_D^{20} = -5.5$ (c = 1.5, chloroform); IR (ATR, cm^{-1}): 3305, 1767, 1706, 1663, 1604, 1535, 1447; ^1H NMR (500 MHz, DMSO-d_6): δ 1.09-1.13 (m, 1H, ½CH_{2cyc}), 1.38-1.67 (m, 8H, 4CH_2), 1.80-1.85 (m, 3H, CH_2, ½CH_{2cyc}), 1.97-2.09 (m, 2H, CH_{2cyc}), 2.45 (t, *J* =

6.4, 2H, CH_2N_{pip}), 2.68 (m, 4H, $2CH_{2pip}$), 3.09-3.19 (m, 9H, $2CH_{2pip}$, CH_2Ar, $C\underline{H_2}NHCO$, ½$CH_{2cyc}N$), 3.44-3.56 (m, 2H, $CH_2N(CO)_2$), 3.75 (dt, *J* = 11.4, 7.3, 1H, ½$CH_{2cyc}N$), 4.16 (t, *J* = 6.7, 1H, CH_{Fmoc}), 4.31-4.49 (m, 3H, CH_{2Fmoc}, $C\underline{H}NH$), 5.65 (t, *J* = 10.1, 1H, NH), 6.19 (br s, 1H, NH), 7.04 (d, *J* = 7.4, 1H, Ar), 7.25-7.30 (m, 3H, Ar), 7.34-7.58 (m, 12H, Ar), 7.69-7.74 (m, 6H, Ar), 7.78-7.81 (m, 1H, Ar), 8.15-8.18 (m, 1H, Ar); ^{13}C NMR (75 MHz, DMSO-d_6): δ 21.2, 24.1, 26.1, 26.2, 29.2, 33.1, 34.4, 38.6, 38.9, 39.2, 45.0 ($11CH_2$), 47.2 (CH), 53.0 ($2CH_2$), 53.8 ($2CH_2$), 56.1 (CH), 58.2, 67.0 ($2CH_2$), 72.3 (C), 114.7 (CH), 120.1 (2CH), 123.5, 123.7 (2CH), 125.0 (2CH), 125.4, 125.8, 125.9 (3CH), 127.2 (2CH), 127.8 (2CH), 128.4 (2CH), 128.5 (CH), 128.9 (C), 129.4 (2CH), 130.0 (2CH), 130.5 (2CH), 132.5 (CH), 134.8 (C), 136.3 (2C), 137.6, 141.4, 141.8, 143.5, 143.7, 149.7 (5C), 156.0, 160.7, 170.5, 176.3, 196.3 (5CO); MS (ESI, *m/z*): 476.3 $[(M+2H)/2]^+$, 951.4 $[M+H]^+$.

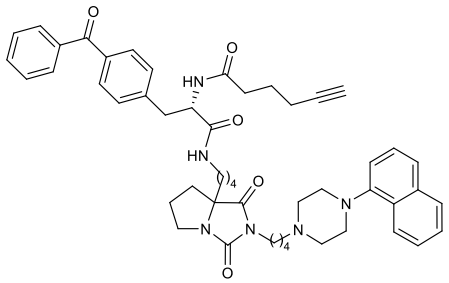
(–)-4-Benzoyl-*N*-{4-[2-{4-[4-(1-naphthyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo[1,2-*c*]imidazol-7a(5*H*)-yl]butyl}-L-phenylalaninamide, 40. To a solution of **39** (380 mg, 0.40 mmol) in anhydrous DCM (0.5 mL), a solution of piperidine (0.20 mL, 2.0 mmol) in anhydrous DCM was added dropwise at 0 ºC, and the mixture was stirred for 2 h. Then, an aqueous 5% citric acid solution (2 mL) was added and the mixture was extracted with EtOAc. The organic layer was washed with H_2O, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by column chromatography (DCM:EtOH, 95:5 to 9:1 v/v) to afford amine **40** as an oil (220 mg, 75%).



R_f (DCM:EtOH, 9:1 v/v) = 0.37; $[\alpha]_D^{20}$ = –19.6 (c = 1.0, chloroform); IR (ATR, cm^{-1}): 3372, 1767, 1706, 1659, 1603, 1535, 1447; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.12-1.24 (m, 1H,

½CH_{2cyc}), 1.31-1.71 (m, 10H, 4CH_2, NH_2), 1.81-1.95 (m, 3H, CH_2, ½CH_{2cyc}), 1.98-2.10 (m, 2H, CH_{2cyc}), 2.47 (t, *J* = 7.2, 2H, CH_2N_{pip}), 2.70-2.81 (m, 5H, 2CH_{2pip}, ½CH_{2cyc}N), 3.11-3.35 (m, 8H, 2CH_{2pip}, CH_2Ar, C$\underline{H_2}$NHCO), 3.44-3.52 (m, 2H, $CH_2N(CO)_2$), 3.60 (dd, *J* = 9.2, 3.8, 1H, C$\underline{H}NH_2$), 3.76 (dt, *J* = 10.6, 7.3, 1H, ½CH_{2cyc}N), 7.06 (d, *J* = 7.3, 1H, Ar), 7.27-7.59 (m, 9H, Ar), 7.73-7.80 (m, 5H, Ar), 8.15-8.18 (m, 1H, Ar); ^{13}C NMR (75 MHz, DMSO-d_6): δ 21.3, 24.2, 26.1, 26.3, 29.4, 33.2, 34.5, 38.7, 39.0, 41.2, 45.0 (11CH_2), 53.1 (2CH_2), 53.8 (2CH_2), 56.4 (CH), 58.2 (CH_2), 72.4 (C), 114.7, 123.5, 123.7, 125.4, 125.9, 126.0 (6CH), 128.4 (2CH), 128.5 (CH), 129.0 (C), 129.4 (2CH), 130.1 (2CH), 130.7 (2CH), 132.6 (CH), 134.8, 136.3, 137.7, 143.3, 149.8 (5C), 160.8, 173.9, 176.4, 196.4 (4CO); MS (ESI, *m/z*): 365.2 $[(M+2H)/2]^+$, 729.5 $[M+H]^+$.

(–)-4-Benzoyl-*N*-hex-3-ynoyl-*N*-{4-[2-{4-[4-(1-napthyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo[1,2-*c*]imidazol-7a(5*H*)-yl]butyl}-L-phenylalaninamide, 7. To a solution of 5-hexynoic acid (32 mg, 0.29 mmol) un anhydrous DCM (0.6 mL), a solution of DMAP (6 mg, 0.05 mmol) and DCC (60 mg, 0.29 mmol) in DCM (3 mL) was added at 0 ºC and the reaction mixture was stirred at this temperature for 30 min. Then a solution of amine **40** (115 mg, 0.19 mmol) in DCM (0.3 mL) was added and the mixture was stirred at rt for 72 h. After that, DCM (20 mL) was added and the mixture was washed with a saturated $NaHCO_3$ solution and brine. The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by column chromatography (DCM:EtOH, 95:5 v/v) to afford the title compound **7** as an oil (80 mg, 53%).

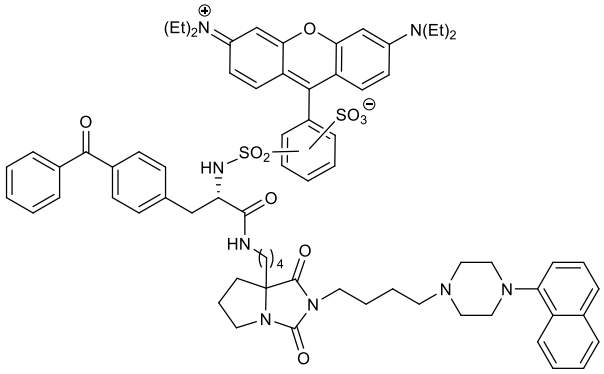


R_f (DCM:EtOH, 9:1 v/v) = 0.43; $[\alpha]_D^{20}$ = –1.2 (c = 1.0, DCM); IR (ATR, cm^{-1}): 3290, 1767, 1706, 1649, 1548, 1446, 1410; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.07-1.16 (m, 1H, ½CH_{2cyc}), 1.30-1.42

(m, 3H, CH_2, ½CH_{2cyc}), 1.52-1.70 (m, 5H, $2CH_2$, ½CH_{2cyc}), 1.73-1.90 (m, 5H, $2CH_2$, ½CH_{2cyc}), 1.96 (t, *J* = 2.6, 1H, C≡CH), 2.00-2.09 (m, 2H, CH_2), 2.13-2.19 (m, 2H, CH_2), 2.30 (t, *J* = 6.9, 2H, CH_2CO), 2.49 (t, *J* = 6.8, 2H, CH_2N_{pip}), 2.72 (m, 4H, $2CH_{2pip}$), 3.04-3.21 (m, 9H, $2CH_{2pip}$, CH_2Ar, $C\underline{H_2}NHCO$, ½$CH_{2cyc}N$), 3.41-3.49 (m, 2H, $CH_2N(CO)_2$), 3.75 (dt, *J* = 10.0, 7.3, 1H, ½$CH_{2cyc}N$), 4.65-4.72 (m, 1H, $C\underline{H}NH$), 6.33 (br s, 1H, NH), 6.40 (t, *J* = 6.3, 1H, NH), 7.06 (d, *J* = 7.4, 1H, Ar), 7.30 (d, *J* = 8.1, 2H, Ar), 7.37 (t, *J* = 7.8, 1H, Ar) 7.41-7.59 (m, 6H, Ar), 7.71-7.75 (m, 4H, Ar), 7.78-7.81 (m, 1H, Ar), 8.15-8.18 (m, 1H, Ar); ^{13}C NMR (125 MHz, $CDCl_3$): δ 17.8, 21.2 ($2CH_2$), 24.1 ($2CH_2$), 26.1, 26.2, 29.2, 33.2, 34.4, 34.8, 38.5, 38.9, 39.2, 45.0 ($10CH_2$), 52.9 ($2CH_2$), 53.8 ($2CH_2$), 54.2 (CH), 58.2 (CH_2), 69.6 (CH), 72.3, 83.3 (2C), 114.7, 123.5, 123.6, 125.4, 125.9, 126.0 (6CH), 128.4 (2CH), 128.5 (CH), 128.9 (C), 129.4 (2CH), 130.0 (2CH), 130.5 (2CH), 132.5 (CH), 134.8, 136.3, 137.6, 141.8, 149.7 (5C), 160.7, 170.6, 172.4, 176.3, 196.3 (5CO); MS (ESI, *m/z*): 412.3 $[(M+2H)/2]^+$, 823.5 $[M+H]^+$; analysis (calcd., found for $C_{50}H_{58}N_6O_5 \cdot HCl \cdot 3H_2O$): C (65.74, 66.04), H (7.17, 6.85), N (9.20, 9.14).

Mixture of 2-[6-(diethylamino)-3-(diethyliminio)-3*H*-xanten-9-yl]-5-[({4-[2-{4-[4-(3-benzoylphenyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo[1,2-*c*]imidazol-7a(5*H*)-yl]butyl}amino)sulfonyl]benzenesulfonate and 4-[6-(diethylamino)-3-(diethyliminio)-3*H*-xanten-9-yl]-3-[({4-[2-{4-[4-(3-benzoylphenyl)piperazin-1-yl]butyl}-1,3-dioxotetrahydro-1*H*-pyrrolo[1,2-*c*]imidazol-7a(5*H*)-yl]butyl}amino)sulfonyl]benzenesulfonate, 27. To a solution of amine **40** (100 mg, 0.14 mmol) and rhodamine B sulfonyl chloride, mixed isomers, (80 mg, 0.14 mmol) in anhydrous THF (2 mL), freshly distilled triethylamine (0.2 mL, 1.4 mmol) was added and the reaction mixture was stirred for 80 ºC overnight. Once at rt, the mixture was diluted with DCM (20 mL) and washed with H_2O (3 x 20 mL) and with an aqueous 1 M HCl solution (3 x 20 mL). The organic layer was dried (Na_2SO_4) and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (DCM:EtOH, 98:2 to 95:5 v/v) and

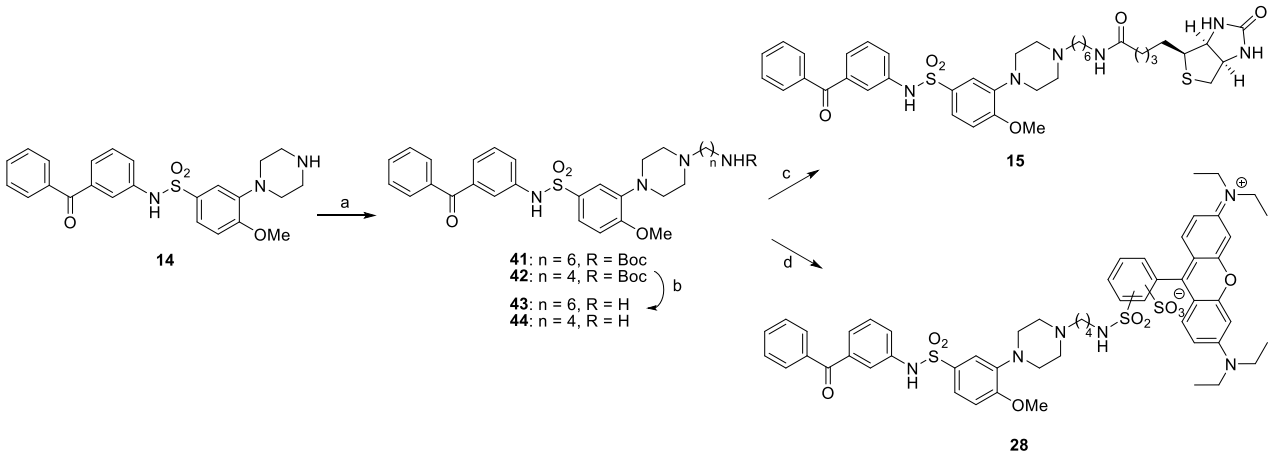
preparative TLC (DCM:EtOH, 9:1 v/v), to afford compound **27** as a purple solid (mixed isomers, 20 mg, 10%).



R_f (DCM:EtOH, 9:1 v/v) = 0.14; ^{1}H NMR (700 MHz, DMSO-d_6): δ 1.01-1.06 (m, 1H, ½CH_{2cyc}), 1.19-1.22 (m, 12H, 4CH_3), 1.25-1.31 (m, 5H, ½CH_{2cyc}, 2CH_2), 1.53-1.60 (m, 3H, ½CH_{2cyc}, CH_2), 1.64-1.68 (m, 1H, ½CH_{2cyc}), 1.72-1.78 (m, 2H, CH_2), 1.90-1.99 (m, 2H, CH_2), 2.36 (m, 2H, CH_2N_{pip}), 2.60 (m, 4H, 2CH_{2pip}), 2.82-3.10 (m, 9H, 2CH_{2pip}, CH_2Ar, C$\underline{H_2}$NHCO, ½CH_{2cyc}N), 3.33-3.38 (m, 2H, $CH_2N(CO)_2$), 3.54-3.65 (m, 9H, 4CH_2N, ½CH_{2cyc}N), 4.07-4.11 (m, 1H, C$\underline{H}$NH), 6.89-6.93 (m, 5H, Ar), 6.97 (d, *J* = 9.4, 1H, Ar), 7.09 (d, *J* = 7.5, 1H, Ar), 7.20 (t, *J* = 7.8, 1H, Ar), 7.38-7.40 (m, 3H, Ar), 7.46-7.50 (m, 4H, Ar), 7.56 (d, *J* = 8.5, 1H, Ar), 7.61-7.64 (m, 6H, Ar), 7.85 (d, *J* = 6.5, 1H, Ar), 7.98 (t, *J* = 5.0, 1H, NH), 8.08 (d, *J* = 8.7, 1H, Ar), 8.32-8.34 (m, 1H, NH), 8.39-8.40 (m, 1H, Ar); ^{13}C NMR (175 MHz, DMSO-d_6): δ 12.5 (4CH_3), 20.9, 25.3, 25.5, 28.8, 29.0, 32.3, 33.8, 38.0, 38.1, 38.2, 38.6, 44.5 (12CH_2), 45.2 (4CH_2), 52.6 (2CH_2), 55.0 (2CH_2), 57.6 (CH), 71.8 (C), 95.4 (2CH), 113.3 (2CH), 114.5 (CH), 123.1 (C), 123.2 (2CH), 125.4 (CH), 125.9 (2CH), 126.0, 126.3 (2CH), 128.0 (C), 128.3 (CH), 128.4 (2CH), 129.4 (4CH), 129.6 (2CH), 130.0 (CH), 132.6 (3CH), 134.3, 135.4, 136.9, 141.8, 142.5, 147.4, 148.4, 149.4, 151.1, 152.9, 154.9, 155.0, 157.0, 157.5 (14C_{Ar}), 160.0, 169.6, 175.8, 195.5 (4CO); HRMS (ESI, *m/z*): $[M+H]^+$ calcd. for $C_{71}H_{80}N_8O_{10}S_2$, 1269.5517, found, 1269.5512; analysis (calcd., found for $C_{71}H_{80}N_8O_{10}S_2 \cdot 3HCl$): C (61.84, 62.07), H (6.07, 6.28), N (8.13, 8.17), S (4.65, 4.38).

1.3. Synthesis of Probes 15-19, 28

- **Synthesis of Probes 15 and 28**

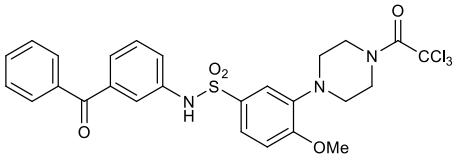


Scheme S4. Reagents and conditions: (a) *tert*-butyl 6-bromohexylcarbamate or *tert*-butyl 4-bromobutylcarbamate, NaI, DMF, 80 ºC, 60-67%; (b) TFA, DCM, rt, 95%; (c) biotin, EDC, HOBt, DMF, DCM, rt, 58%; (d) rhodamine B sulfonyl chloride (mixed isomers), Et_3N, THF, 70 ºC, 67%.

Synthesis of arylpiperazine 14

***N*-(3-Benzoylphenyl)-4-methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]benzenesulfonamide,**

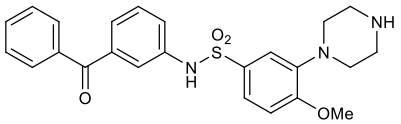
21. Pyridine (0.15 mL, 1.8 mmol) was added to a solution of 3-aminobenzophenone (178 mg, 0.90 mmol) in anhydrous DCM (2 mL) at rt. A solution of compound **20** (392 mg, 0.90 mmol) in anhydrous DCM (2 mL) was added dropwise, and the reaction mixture was stirred at rt for 12 h. The reaction was quenched with a 10% aqueous solution of $NaHCO_3$ (4 mL) and the aqueous phase was extracted with DCM (2 x 10 mL). The combined organic layers were washed with H_2O (10 mL) and brine (10 mL), dried (Na_2SO_4), and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (hexane:EtOAc, 9:1 to 1:1 v/v) to afford compound **21** (348 mg, 65%) as a yellow solid.



R_f (hexane:EtOAc, 1:1 v/v): 0.46; mp: 83-85 ºC; IR (ATR, cm^{-1}): 3241, 3019, 1670, 1501, 1258, 1157; ^1H NMR (300 MHz, $CDCl_3$): δ 3.05 (m, 4H, $2CH_2$), 3.92 (s, 3H, CH_3), 3.98 (m, 4H, $2CH_2$),

6.88 (d, J = 8.7, 1H,Ar), 7.33 (dd, J = 11.4, 9.3, 1H, Ar), 7.41 (d, J = 7.7, 1H, Ar), 7.47-7.58 (m, 5H, Ar), 7.62-7.70 (m, 1H, Ar), 7.73-7.79 (m, 3H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 45.3 ($2CH_2$), 49.8 ($2CH_2$), 55.9 (CH_3), 92.7 (C), 110.9, 117.2, 122.2, 123.6, 125.0, 126.6 (6CH), 128.4 (2CH), 129.3 (CH), 130.0 (2CH), 130.6 (C), 132.9 (CH), 136.7, 137.3, 138.4, 140.5, 155.8 (5C), 159.3, 195.8 (2CO); MS (ESI, *m/z*): 596.1 $[M+H]^+$.

***N*-(3-Benzoylphenyl)-4-methoxy-3-piperazin-1-ylbenzenesulfonamide, 14.** A 1 M aqueous solution of KOH (0.45 mL) was added over 5 min to a solution of compound **21** (100 mg, 0.17 mmol) in THF (0.45 mL) at rt. After stirring for 20 h, the reaction was cooled to 0 ºC and pH was adjusted to 7.0 by addition of concentrated HCl. The mixture was extracted with DCM (2 x 10 mL) and the combined organic layers were washed with H_2O (10 mL) and brine (10 mL), dried (Na_2SO_4), and evaporated under reduced pressure to yield compound **14** (67 mg, 87%) as a white solid.

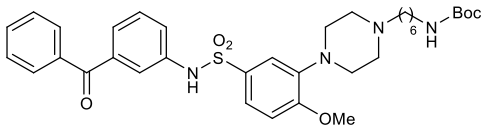


R_f (DCM:EtOH, 9:1 v/v): 0.21; mp: 193-195 ºC; IR (ATR, cm^{-1}): 3403, 1655, 1243; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.84 (m, 8H, $4CH_2$), 3.83 (s, 3H, CH_3), 7.06 (d, J = 8.5, 1H, Ar), 7.16 (d, J = 2.5, 1H, Ar), 7.34 (dd, J = 8.5, 2.5, 1H, Ar), 7.38-7.46 (m, 4H, Ar), 7.55 (t, J = 7.5, 2H, Ar), 7.59-7.61 (m, 2H, Ar), 7.69 (t, J = 7.5, 1H, Ar); ^{13}C NMR (75 MHz, DMSO-d_6): δ 46.1 ($2CH_2$), 51.4 ($2CH_2$), 56.7 (CH_3), 112.2, 116.8, 121.8, 122.7, 125.2, 125.9 (6CH), 129.4 (2CH), 130.3 (CH), 130.4 (2CH), 131.7 (C), 133.7 (CH), 137.5 (2C), 138.5, 142.4, 156.1 (3C), 196.0 (CO); MS (ESI, *m/z*): 452.3 $[M+H]^+$; analysis (calcd., found for $C_{24}H_{25}N_3O_4S \cdot 3/2HCl \cdot 1/2H_2O$): C (55.95, 56.09), H (5.38, 5.64), N (8.16, 7.79), S (6.22, 5.95).

General Procedure for the Synthesis of Compounds 41 and 42. NaI (2 equiv) and *tert*-butyl 6-bromohexylcarbamate or *tert*-butyl 4-bromobutylcarbamate (1.25 equiv) were added to a solution of piperazine **14** (1 equiv) in anhydrous DMF (14 mL/mmol) and the reaction mixture was

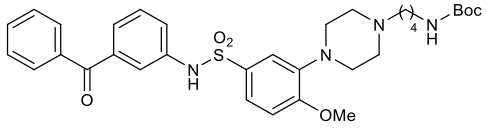
heated at 80 ºC for 24 h. Upon cooling to rt, solvent was removed under reduced pressure and the residue was purified by column chromatography, using the appropriate eluent, to afford compound **41** or **42**.

***tert*-Butyl {6-[4-(5-{[(3-benzoylphenyl)amino]sulfonyl}-2-methoxyphenyl)piperazin-1-yl]hexyl}carbamate, 41.** Obtained from **14** (503 mg, 1.1 mmol) and *tert*-butyl 6-bromohexylcarbamate (389 mg, 1.4 mmol) as a yellow solid (483 mg, 67%). Chromatography: DCM:MeOH, 80:1 to 10:1 v/v.



R_f (DCM:EtOH, 9:1 v/v): 0.37; mp: 68-70 ºC; IR (ATR, cm^{-1}): 3387, 1664, 1504, 1256, 1159; ^1H NMR (300 MHz, $CDCl_3$): δ 1.31 (m, 4H, $2CH_2$), 1.41 (s, 9H, $3CH_3$), 1.42-1.57 (m, 4H, $2CH_2$), 2.46 (t, *J* = 7.5, 2H, CH_2N_{pip}), 2.68 (m, 4H, $2CH_{2pip}$), 3.06 (m, 6H, $2CH_{2pip}$, $C\underline{H}_2NH$), 3.83 (s, 3H, CH_3), 4.74 (br s, 1H, NH), 6.79 (d, *J* = 8.7, 1H, Ar), 7.28-7.33 (m, 2H, Ar), 7.39-7.57 (m, 7H, Ar), 7.65-7.68 (m, 2H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.9, 26.4, 26.9 ($3CH_2$), 28.2 ($3CH_3$), 29.7, 40.3 ($2CH_2$), 49.4 ($2CH_2$), 52.8 ($2CH_2$), 55.6 (CH_3), 58.2 (CH_2), 78.8 (C), 110.5, 116.8, 122.3, 122.7, 124.9, 126.1 (6CH), 128.1 (2CH), 129.0 (CH), 129.8 (2CH), 130.7 (C), 132.5 (CH), 136.8, 137.5, 138.1, 141.1, 155.5 (5C), 156.0, 195.8 (2CO); MS (ESI, *m/z*): 651.3 $[M+H]^+$.

***tert*-Butyl {4-[4-(5-{[(3-benzoylphenyl)amino]sulfonyl}-2-methoxyphenyl)piperazin-1-yl]butyl}carbamate, 42.** Obtained from **14** (435 mg, 0.96 mmol) and *tert*-butyl 4-bromobutylcarbamate (301 mg, 1.2 mmol) as a yellow solid (358 mg, 60%). Chromatography: DCM:MeOH, 80:1 to 10:1 v/v.

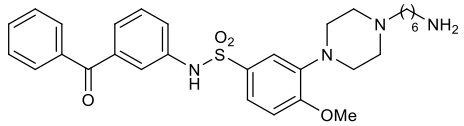


R_f (DCM:EtOH, 9:1 v/v): 0.59; mp: 73-75 ºC; IR (ATR, cm^{-1}): 3394, 1664, 1504, 1255, 1159; ^1H NMR (300 MHz, $CDCl_3$): δ 1.41 (s, 9H, $3CH_3$), 1.50-1.61 (m, 4H, $2CH_2$), 2.45-2.50 (m, 2H,

CH_2N_{pip}), 2.65 (m, 4H, $2CH_{2pip}$), 3.05 (m, 4H, $2CH_{2pip}$), 3.09-3.15 (m, 2H, $C\underline{H}_2NH$), 3.85 (s, 3H, CH_3), 5.15 (br s, 1H, NH), 6.79 (d, J = 8.7, 1H, Ar), 7.30 (m, 1H, Ar), 7.34 (d, J = 7.8, 1H, Ar), 7.41-7.50 (m, 6H, Ar), 7.57 (t, J = 7.5, 1H, Ar), 7.67-7.70 (m, 2H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 23.8, 27.9 ($2CH_2$), 28.5 ($3CH_3$), 40.4 (CH_2), 49.8 ($2CH_2$), 53.0 ($2CH_2$), 55.9 (CH_3), 57.9 (CH_2), 79.0 (C), 110.7, 117.0, 122.6, 123.0, 125.2, 126.5 (6CH), 128.4 (2CH), 129.3 (CH), 130.0 (2CH), 130.7 (C), 132.7 (CH), 137.0, 137.5, 138.5, 141.5, 155.8 (5C), 156.2, 195.8 (2CO); MS (ESI, *m/z*): 623.3 $[M+H]^+$.

General Procedure for the Synthesis of Compounds 43 and 44. TFA (20 equiv) was added to a solution of compound **41** or **42** (1 equiv) in anhydrous DCM (8 mL/mmol) and the reaction was stirred at rt for 2 h. The reaction mixture was then neutralized with a 10% aqueous solution of $NaHCO_3$, and the aqueous phase was extracted with DCM. The combined organic layers were washed with H_2O and brine, dried (Na_2SO_4), filtered, and evaporated under reduced pressure, to yield the free amine **43** or **44**, which was taken to the next step without further purification.

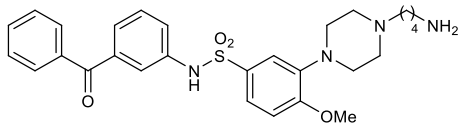
3-[4-(6-Aminohexyl)piperazin-1-yl]-*N*-(3-benzoylphenyl)-4-methoxybenzenesulfonamide, 43. Obtained from **41** (406 mg, 0.62 mmol) and TFA (0.95 mL, 12.4 mmol) as a yellow solid (324 mg, 95%).



R_f (DCM:EtOH, 9:1 v/v): 0.10; mp: 79-81 ºC; IR (ATR, cm^{-1}): 3214, 1682, 1504, 1213, 1123; ^{1}H NMR (300 MHz, acetone-d_6): δ 1.29-1.60 (m, 6H, $3CH_2$), 1.74-1.79 (m, 1H, ½CH_2), 1.88 (m, 1H, ½CH_2), 2.34 (t, J = 7.5, 2H, CH_2N_{pip}), 2.50 (m, 4H, $2CH_{2pip}$), 2.95 (m, 4H, $2CH_{2pip}$), 3.18 (t, J = 7.5, 2H, $C\underline{H}_2NH_2$), 3.89 (s, 3H, CH_3), 7.02 (d, J = 8.6, 1H, Ar), 7.23 (d, J = 2.3, 1H, Ar), 7.40 (dd, J = 8.6, 2.3, 1H, Ar), 7.46-7.58 (m, 6H, Ar), 7.65-7.69 (m, 3H, Ar); ^{13}C NMR (75 MHz, acetone-d_6): δ 27.7, 28.1, 28.3, 31.7, 51.1 ($5CH_2$), 51.8 ($2CH_2$), 54.1 ($2CH_2$), 56.2 (CH_3), 59.2 (CH_2),

111.9, 117.3, 122.7, 123.0, 125.6, 126.3 (6CH), 129.3 (2CH), 130.2 (CH), 130.6 (2CH), 132.3 (C), 133.4 (CH), 138.2, 139.3, 139.6, 142.9, 156.8 (5C), 195.9 (CO); MS (ESI, *m/z*): 551.3 $[M+H]^+$.

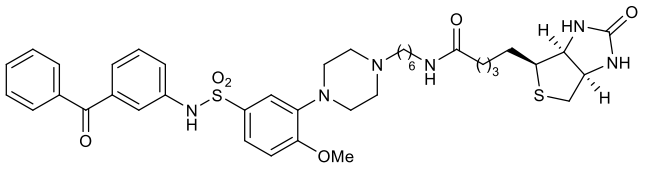
3-[4-(4-Aminobutyl)piperazin-1-yl]-*N*-(3-benzoylphenyl)-4-methoxybenzenesulfonamide, 44. Obtained from **42** (151 mg, 0.24 mmol) and TFA (0.37 mL, 4.8 mmol) as a brown syrup (119 mg, 95%).



R_f (DCM:EtOH, 9:1 v/v): 0.10; IR (ATR, cm^{-1}): 1674, 1591, 1503, 1377, 1259, 1160; ^1H NMR (500 MHz, CD_3OD): δ 1.56-1.61 (m, 4H, $2CH_2$), 2.38-2.43 (m, 2H, CH_2N_{pip}), 2.59 (m, 4H, $2CH_{2pip}$), 2.75-2.80 (m, 2H, $C\underline{H}_2NH_2$), 2.98 (m, 4H, $2CH_{2pip}$), 3.86 (s, 3H, CH_3), 6.97 (d, *J* = 8.7, 1H, Ar), 7.28 (d, *J* = 2.3, 1H, Ar), 7.33-7.34 (m, 3H, Ar), 7.40-7.49 (m, 4H, Ar), 7.58-7.63 (m, 3H, Ar); ^{13}C NMR (125 MHz, CD_3OD): δ 24.9, 30.0, 41.7 ($3CH_2$), 51.2 ($2CH_2$), 54.1 ($2CH_2$), 56.4 (CH_3), 59.3 (CH_2), 112.2, 118.1, 123.6, 123.9, 125.3, 126.9 (6CH), 129.5 (2CH), 130.2 (CH), 131.0 (2CH), 133.8 (CH), 134.2, 138.7, 139.4, 142.5, 142.7, 156.8 (6C), 198.1 (CO); MS (ESI, *m/z*): 523.2 $[M+H]^+$.

***N*-{6-[4-(5-{[(3-Benzoylphenyl)amino]sulfonyl}-2-methoxyphenyl)piperazin-1-yl]hexyl}-biotinamide, 15.** Biotin (151 mg, 0.62 mmol) was suspended in anhydrous DMF (10 mL) with 4 Å molecular sieves previously activated (35 mg/mmol carboxylic acid), and the mixture was heated until a clear solution was obtained (~45 ºC). The mixture was then cooled to rt and a solution of HOBt (84 mg, 0.62 mmol) and EDC (119 mg, 0.62 mmol) in anhydrous DMF (2 mL) was added dropwise. After stirring at rt 1 h, a solution of compound **43** (228 mg, 0.41 mmol) in anhydrous DCM (2 mL) was added and the mixture was stirred at rt for 24 h. Then, the reaction was quenched with a 10% aqueous solution of $NaHCO_3$ (3 mL) and the aqueous phase was extracted with DCM (2 x 10 mL). The combined organic layers were washed with H_2O (10 mL) and brine (10 mL), dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue

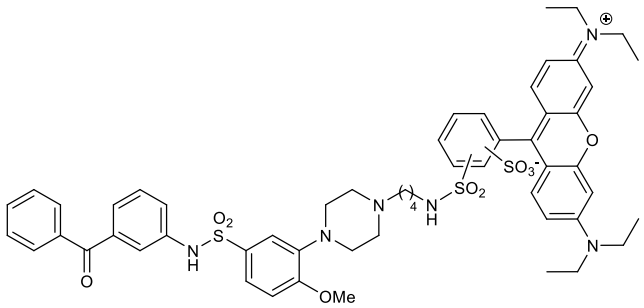
was purified by column chromatography (DCM:MeOH, 100:1 to 10:1 v/v) to afford the desired compound **15** (185 mg, 58%) as a yellow solid.



R_f (DCM:EtOH, 8:2 v/v): 0.37; mp: 115-117 ºC; $[\alpha]_D^{20}$: +21.1 (c = 1.0, MeOH); IR (ATR, cm^{-1}): 3284, 1691, 1455, 1256, 1154; ^1H NMR (300 MHz, CD_3OD): δ 1.36-1.77 (m, 14H, $7CH_2$), 2.20 (t, *J* = 7.3, 2H, CH_2CO), 2.44 (m, 2H, CH_2N_{pip}), 2.64 (m, 4H, $2CH_{2pip}$), 2.69 (d, *J* = 12.8, 1H, ½CH_2S), 2.91 (dd, *J* = 12.7, 5.0, 1H, ½CH_2S), 2.99 (m, 4H, $2CH_{2pip}$), 3.16-3.21 (m, 3H, CHS, C<u>H</u>$_2$NH), 3.87 (s, 3H, CH_3), 4.29 (dd, *J* = 7.9, 4.5, 1H, C<u>H</u>NH), 4.48 (dd, *J* = 8.0, 5.1, 1H, C<u>H</u>NH), 7.00 (d, *J* = 8.6, 1H, Ar), 7.23 (d, *J* = 2.3, 1H, Ar), 7.39-7.51 (m, 7H, Ar), 7.61-7.64 (m, 3H, Ar); ^{13}C NMR (75 MHz, CD_3OD): δ 26.9, 27.2, 27.8, 28.2, 29.5, 29.8, 30.3, 36.8, 40.3, 41.1 ($10CH_2$), 50.8 ($2CH_2$), 54.1 ($2CH_2$), 56.4 (CH_3), 57.0 (CH), 59.6 (CH_2), 61.6, 63.4, 112.2, 117.9, 123.3, 124.1, 126.5, 127.0 (8CH), 129.6 (2CH), 130.4 (CH), 130.9 (2CH), 132.3 (C), 133.9 (CH), 138.3, 139.6, 139.6, 142.5, 157.3 (5C), 166.0, 175.9, 197.5 (3CO); MS (ESI, *m/z*): 777.4 $[M+H]^+$; analysis (calcd., found for $C_{40}H_{52}N_6O_6S_2 \cdot 4HCl \cdot 3H_2O$): C (49.18, 49.51), H (6.40, 6.33), N (8.60, 8.48), S (6.56, 6.19).

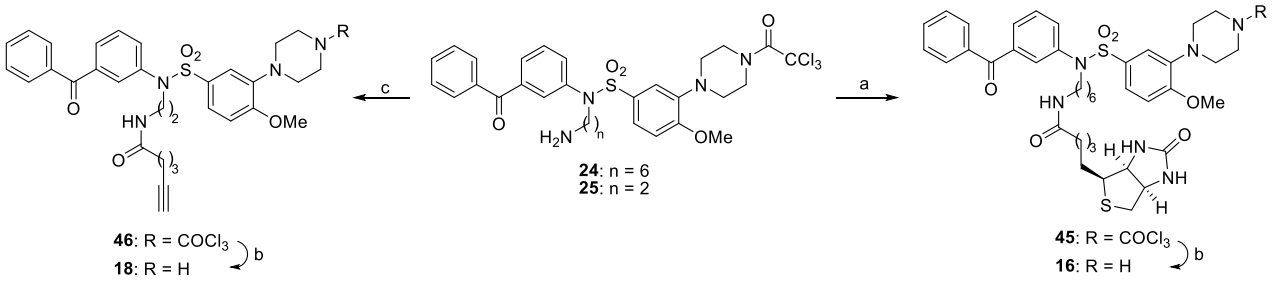
Mixture of 5-[({4-[4-(5-{[(3-benzoylphenyl)amino]sulfonyl}-2-methoxyphenyl)piperazin-1-yl]butyl}amino)sulfonyl]-2-[6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl]benzenesulfonate and 3-[({4-[4-(5-{[(3-benzoylphenyl)amino]sulfonyl}-2-methoxyphenyl)piperazin-1-yl]butyl}amino)sulfonyl]-4-[6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl]benzenesulfonate, 28. Freshly distilled triethylamine (0.51 mL, 3.7 mmol) was added to a solution of compound **44** (193 mg, 0.37 mmol) in anhydrous THF (7.5 mL). A solution of rhodamine B sulfonyl chloride (mixed isomers, 213 mg, 0.37 mmol) in anhydrous THF (1.5 mL) was added and the reaction mixture was stirred at 70 ºC for 16 h. Afterwards, the solvent was evaporated and the residue was dissolved in DCM (10 mL). The resulting solution was

successively washed with H_2O (10 mL) and brine (10 mL), dried (Na_2SO_4), filtered and evaporated under reduced pressure. Column chromatography of the residue (DCM to DCM:MeOH, 20:1 v/v) gave final compound **28** as a red solid (mixed isomers; 264 mg, 67%).



R_f (DCM:EtOH, 9:1 v/v): 0.29; IR (ATR, cm^{-1}): 3447, 1632, 1457, 1340, 1260, 1157; ^1H NMR (500 MHz, CD_3OD): δ 1.24-1.31 (m, 12H, $4CH_3$), 1.59-1.66 (m, 4H, $2CH_2$), 2.52 (t, J = 7.0, 2H, CH_2N_{pip}), 2.70 (m, 4H, $2CH_{2pip}$), 3.00 (m, 4H, $2CH_{2pip}$), 3.09 (t, J = 6.1, 2H, $C\underline{H}_2NH$), 3.59-3.73 (m, 8H, $4CH_2N$), 3.85 (s, 3H, CH_3), 6.90-7.20 (m, 8H, Ar), 7.36-7.64 (m, 11H, Ar), 8.11 (ddd, J = 8.0, 2.7, 1.6, 1H, Ar), 8.66 (s, 1H, Ar); ^{13}C NMR (125 MHz, CD_3OD): δ 12.9 ($2CH_3$), 13.9, 14.8 ($2CH_3$), 24.4, 28.9, 39.3, 44.0, 46.8, 47.1, 47.3, 47.8 ($8CH_2$), 50.7 ($2CH_2$), 54.0 ($2CH_2$), 56.4 (CH_3), 58.9 (CH_2), 97.0, 97.3 (2CH), 104.0, 108.4 (2C), 112.2, 112.4, 115.0, 115.3 (4CH), 115.7, 115.8 (2C), 116.1, 117.1, 118.0 (3CH), 118.9 (C), 123.2, 124.1, 126.4, 126.9, 127.6, 129.3, 129.6, 130.4, 130.5, 130.9 (10CH), 132.3, 132.5 (2C), 133.6, 134.0, 134.2, 134.3, 134.7, 134.8, 135.0, 135.4 (8CH), 138.3, 139.6, 139.7, 142.4, 144.2, 144.4, 144.5, 147.3, 147.4, 153.2, 153.4, 153.9, 154.2, 156.5, 157.1, 157.3, 157.8, 158.0, 158.2, 158.6, 159.3, 159.5, 159.9 (23C), 197.4 (CO); HRMS (ESI, *m/z*): $[M+H]^+$ calcd. for $C_{55}H_{62}N_6O_{10}S_3$, 1063.3762, found, 1063.3771; analysis (calcd., found for $C_{55}H_{62}N_6O_{10}S_3 \cdot 2HCl \cdot 4H_2O$): C (54.67, 54.69), H (6.01, 5.71), N (6.96, 6.98), S (7.96, 7.71).

- **Synthesis of Probes 16 and 18**

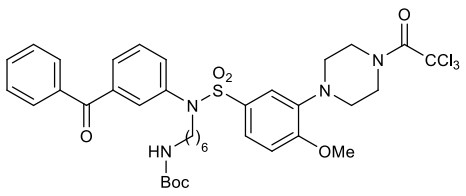


Scheme S5. Reagents and conditions: (a) biotin, EDC, HOBt, DMF, DCM, rt, 82%; (b) 1 M aq. KOH, THF, rt, 76-82%; (c) 5-hexynoic acid, DCC, DMAP, DCM, rt, 61%.

Synthesis of amines 24 and 25

General Procedure for the Synthesis of 22 and 23. To a suspension of sulfonamide **21** (1 equiv) and Cs_2CO_3 (2 equiv) in anhydrous DMF (10 mL/mmol), *tert*-butyl 6-bromohexylcarbamate or *tert*-butyl 2-bromoethylcarbamate (2 equiv) was added, and the mixture was stirred at rt overnight. Then, H_2O was added, and the solution was extracted with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was purified by column chromatography, using the appropriate eluent, to afford compound **22** or **23**.

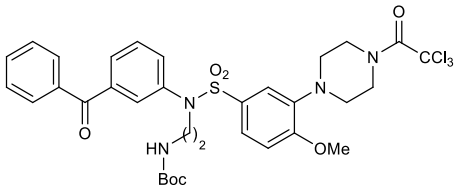
***tert*-Butyl {6-[(3-benzoylphenyl)({4-methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]phenyl}-sulfonyl)amino]hexyl}carbamate, 22.** Obtained from **21** (346 mg, 0.58 mmol) and *tert*-butyl 6-bromohexylcarbamate (325 mg, 1.2 mmol) as a yellow solid (341 mg, 74%). Chromatography: hexane:EtOAc, 15:1 to 2:1 v/v.



R_f (hexane:EtOAc, 1:1 v/v): 0.51; mp: 69-71 ºC; IR (ATR, cm^{-1}): 3395, 1680, 1504, 1254, 1160; ^1H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 9H, $3CH_3$), 1.20-1.38 (m, 8H, $4CH_2$), 2.93-3.00 (m, 6H, $2CH_{2pip}$, $C\underline{H}_2NH$), 3.43 (t, J = 6.7, 2H, CH_2NSO_2), 3.83 (m, 4H, $2CH_{2pip}$), 3.87 (s, 3H, CH_3), 4.56

(bs r, 1H, NH), 6.83-6.87 (m, 2H, Ar), 7.28-7.43 (m, 6H, Ar), 7.50-7.55 (m, 1H, Ar), 7.62-7.66 (m, 3H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.0, 26.2, 28.0 ($3CH_2$), 28.4 ($3CH_3$), 29.9 (CH_2), 40.4 (CH_2), 49.8 ($2CH_2$), 50.0 ($2CH_2$), 56.0 (CH_3), 60.3 (CH_2), 78.9, 92.9 (2C), 110.9, 117.7, 123.7 (3CH), 128.5 (2CH), 129.0, 129.1, 129.5 (3CH), 129.9 (C), 130.0 (2CH), 132.9, 133.4 (2CH), 136.9, 138.3, 139.7, 140.4, 155.7 (5C), 156.0, 159.3, 195.2 (3CO); MS (ESI, *m/z*): 795.0 [M(^{35}Cl)+H]$^+$, 797.1 [M(^{37}Cl)+H]$^+$.

***tert*-Butyl {2-[(3-benzoylphenyl)({4-methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]phenyl}-sulfonyl)amino]ethyl}carbamate, 23.** Obtained from **21** (215 mg, 0.36 mmol) and *tert*-butyl 2-bromoethylcarbamate (163 mg, 0.72 mmol) as a yellow solid (216 mg, 81%). Chromatography: hexane:EtOAc, 15:1 to 2:1 v/v.

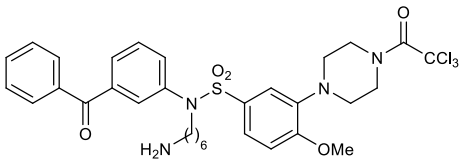


R_f (hexane:EtOAc, 1:1 v/v): 0.48; mp 63-65 ºC; IR (ATR, cm^{-1}): 3396, 2925, 2851, 1679, 1503, 1253, 1160; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.31 (s, 9H, $3CH_3$), 3.01 (m, 4H, $2CH_{2pip}$), 3.15 (q, *J* = 6.0, 2H, $C\underline{H}_2NH$), 3.58 (t, *J* = 6.0, 2H, CH_2NSO_2), 3.85 (m, 4H, $2CH_{2pip}$), 3.87 (s, 3H, CH_3), 4.93 (br s, 1H, NH), 6.84-6.87 (m, 2H, Ar), 7.28-7.44 (m, 6H, Ar), 7.52 (m, 1H, Ar), 7.65-7.67 (m, 3H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.3 ($3CH_3$), 39.5 (CH_2), 45.9 ($2CH_2$), 49.8 ($2CH_2$), 50.3 (CH_2), 56.0 (CH_3), 79.5, 92.9 (2C), 111.0, 117.7, 123.7 (3CH), 128.5 (2CH), 129.2, 129.3 (2CH), 129.7 (C), 129.8 (CH), 130.0 (2CH), 132.9, 133.4 (2CH), 136.8, 138.5, 140.0, 140.6 (4C), 155.8 (CO), 155.9 (C), 159.3, 195.1 (2CO); MS (ESI, *m/z*): 739.1 [M(^{35}Cl)+H]$^+$, 741.1 [M(^{37}Cl)+H]$^+$.

General Procedure for the Synthesis of 24 and 25. TFA (20 equiv) was added to a solution of compound **22** or **23** (1 equiv) in anhydrous DCM (8 mL/mmol) and the reaction was stirred at rt for 2 h. The reaction mixture was then neutralized with a 10% aqueous solution of $NaHCO_3$, and the aqueous phase was extracted with DCM. The combined organic layers were washed with H_2O

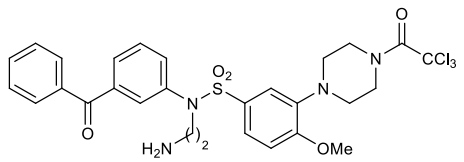
and brine, dried (Na_2SO_4), filtered, and evaporated under reduced pressure, to yield the free amine **24** or **25**, which was taken to the next step without further purification.

***N*-(6-Aminohexyl)-*N*-(3-benzoylphenyl)-4-methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]-benzenesulfonamide, 24.** Obtained from **22** (446 mg, 0.56 mmol) and TFA (0.86 mL, 11.2 mmol) as a yellow solid (363 mg, 93%).



R_f (DCM:EtOH, 9:1 v/v): 0.11; mp: 65-67 ºC; IR (ATR, cm^{-1}): 1677, 1592, 1256, 1156; ^1H NMR (300 MHz, $CDCl_3$): δ 1.16-1.38 (m, 8H, $4CH_2$), 2.57 (t, J = 6.8, 2H, CH_2NH_2), 2.95 (m, 4H, $2CH_{2pip}$), 3.44 (t, J = 6.8, 2H, CH_2N), 3.84 (m, 4H, $2CH_{2pip}$), 3.87 (s, 3H, CH_3), 6.84-6.87 (m, 2H, Ar), 7.29-7.44 (m, 6H, Ar), 7.51-7.56 (m, 1H, Ar), 7.63-7.67 (m, 3H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.2, 26.3, 28.1, 33.3, 41.9 ($5CH_2$), 45.7 ($2CH_2$), 49.9 ($2CH_2$), 50.0 (CH_2), 56.0 (CH_3), 92.9 (C), 110.9, 117.7, 123.7 (3CH), 128.5 (2CH), 129.0, 129.1, 129.5 (3CH), 129.6 (C), 130.0 (2CH), 132.9, 133.5 (2CH), 136.9, 138.3, 139.8, 140.5, 155.7 (5C), 159.3, 195.3 (2CO); MS (ESI, *m/z*): 695.4 $[M(^{35}Cl)+H]^+$, 697.2 $[M(^{37}Cl)+H]^+$.

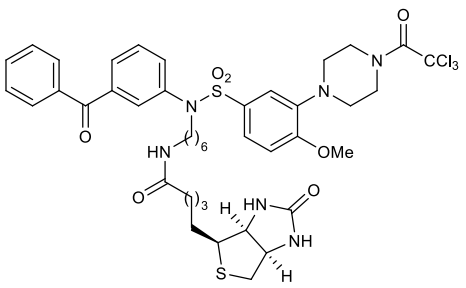
***N*-(2-Aminoethyl)-*N*-(3-benzoylphenyl)-4-methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]-benzenesulfonamide, 25.** Obtained from **23** (637 mg, 0.86 mmol) and TFA (1.32 mL, 17.2 mmol) as a yellow solid (500 mg, 91%).



R_f (DCM:EtOH, 9:1 v/v): 0.10; mp: 160-162 ºC; IR (ATR, cm^{-1}): 3389, 1680, 1500, 1230, 1157; ^1H NMR (300 MHz, $CDCl_3$): δ 2.70 (t, J = 6.0, 2H, $C\underline{H}_2NH_2$), 2.95 (m, 4H, $2CH_{2pip}$), 3.52 (t, J = 6.0 Hz, 2H, CH_2NSO_2), 3.82 (m, 4H, $2CH_{2pip}$), 3.84 (s, 3H, CH_3), 6.84-6.87 (m, 2H, Ar), 7.30-7.43 (m, 6H, Ar), 7.51 (m, 1H, Ar), 7.65-7.67 (m, 3H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 40.3 (CH_2),

45.8 ($2CH_2$), 49.9 ($2CH_2$), 53.5 (CH_2), 56.0 (CH_3), 92.9 (C), 111.0, 117.7, 123.8 (3CH), 128.5 (2CH), 129.1, 129.2, 129.8 (3CH), 129.9 (C), 130.0 (2CH), 132.9, 133.5 (2CH), 136.8, 138.5, 139.9, 140.6, 155.8 (5C), 159.3, 195.2 (2CO); MS (ESI, *m/z*): 639.1 $[M(^{35}Cl)+H]^+$, 641.1 $[M(^{37}Cl)+H]^+$.

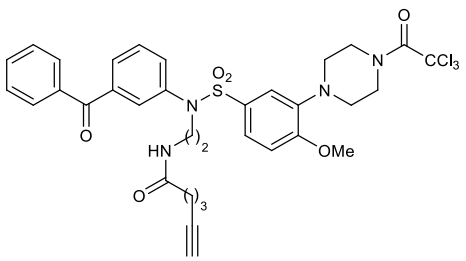
***N*-{6-[(3-Benzoylphenyl)({4-methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]phenyl}-sulfonyl)amino]hexyl}biotinamide, 45.** Biotin (187 mg, 0.765 mmol) was suspended in anhydrous DMF (10 mL) with 4 Å molecular sieves previously activated (35 mg/mmol carboxylic acid), and the mixture was heated until a clear solution was obtained (~45 ºC). The mixture was then cooled to rt and a solution of HOBt (103 mg, 0.77 mmol) and EDC hydrochloride (147 mg, 0.77 mmol) in anhydrous DMF (2 mL) was added dropwise. After stirring at rt for 1 h, a solution of compound **24** (356 mg, 0.51 mmol) in anhydrous DCM (2 mL) was added and the mixture was stirred at rt for 24 h. Then, the reaction was quenched with a 10% aqueous solution of $NaHCO_3$ (3 mL) and the aqueous phase was extracted with DCM (2 x 10 mL). The combined organic layers were washed with H_2O (10 mL) and brine (10 mL), dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was purified by column chromatography (DCM/MeOH, 70:1 to 10:1 v/v) to afford the desired compound **45** (386 mg, 82%) as a yellow solid.



R_f (DCM:EtOH, 9:1 v/v): 0.49; mp: 104-106 ºC; $[\alpha]_D^{20}$: +19.2 (*c* = 1.2, MeOH); IR (ATR, cm^{-1}): 3289, 3068, 1679, 1434, 1256, 1156; ^1H NMR (300 MHz, CD_3OD):δ 1.28-1.78 (m, 14H, $7CH_2$), 2.18 (t, *J* = 7.3, 2H, CH_2CO), 2.68 (d, 1H, *J* = 12.8, ½CH_2S), 2.90 (dd, *J* = 12.8, 4.9, 1H, ½CH_2S), 2.99 (m, 4H, $2CH_{2pip}$), 3.12 (t, *J* = 6.8, 2H, C<u>H</u>$_2$NH), 3.17 (m, 1H, CHS), 3.57 (t, *J* = 6.3, 2H, CH_2NSO_2), 3.91 (m, 4H, $2CH_{2pip}$), 3.94 (s, 3H, CH_3), 4.28 (dd, *J* = 7.9, 4.4, 1H, C<u>H</u>NH), 4.47

(dd, J = 7.8, 4.9, 1H, CHNH), 6.86 (d, J = 2.2, 1H, Ar), 7.11 (d, J = 8.6, 1H, Ar), 7.35-7.59 (m, 6H, Ar), 7.61-7.77 (m, 4H, Ar); ^{13}C NMR (75 MHz, CD_3OD): δ 27.0, 27.1, 27.4, 29.1, 29.6, 29.8, 30.3, 36.9, 40.2, 41.1 ($10CH_2$), 49.4 ($2CH_2$), 51.0 ($3CH_2$), 56.7 (CH_3), 57.0, 61.7, 63.4 (3CH), 94.1 (C), 112.6, 118.8, 125.0 (3CH), 129.8 (2CH), 130.4 (CH), 130.5 (2CH), 130.7 (C), 131.0 (2CH), 134.2, 134.8 (2CH), 138.3, 139.5, 141.1, 142.0, 157.6 (5C), 160.8, 166.1, 176.0, 197.0 (4CO); MS (ESI, *m/z*): 921.0 [M(^{35}Cl)+H]$^+$, 923.2 [M(^{37}Cl)+H]$^+$.

***N*-{2-[(3-Benzoylphenyl)({4-methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]phenyl}-sulfonyl)amino]ethyl}hex-5-ynamide, 46.** To a solution of 5-hexynoic acid (43 mg, 0.38 mmol) in anhydrous DCM (2 mL), a solution of DCC (79 mg, 0.38 mmol) and DMAP (8.5 mg, 0.07 mmol) in anhydrous DCM (2 mL) was added at 0 ºC. The mixture was stirred at this temperature for 30 min before a solution of compound **25** (160 mg, 0.25 mmol) in anhydrous DCM (0.3 mL) was added. The mixture was stirred at rt for 16 h, and the solvent was evaporated under reduced pressure. The residue was dissolved in DCM (10 mL) and washed with a 5% aqueous solution of $NaHCO_3$ (10 mL). The aqueous layer was extracted with DCM (2 x 10 mL), and the combined organic layers were dried (Na_2SO_4), filtered, and evaporated under reduced pressure. Column chromatography of the residue (DCM:MeOH, 70:1 to 10:1 v/v) afforded compound **46** (112 mg, 61%) as a white solid.

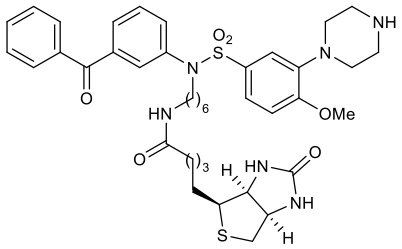


R_f (DCM:EtOH, 9:1 v/v): 0.37; mp: 80-82 ºC; IR (ATR, cm^{-1}): 3291, 1674, 1502, 1255, 1159; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.82 (qt, J = 7.0, 2H, CH_2), 1.98 (t, J = 2.6, 1H, CH), 2.23 (td, J = 6.9, 2.7, 2H, CH_2), 2.31 (t, J = 7.4, 2H, CH_2), 3.03 (m, 4H, $2CH_{2pip}$), 3.34 (q, J = 5.3, 2H, CH_2), 3.67 (t, J = 3.6, 2H, CH_2), 3.90 (m, 4H, $2CH_{2pip}$), 3.95 (s, 3H, CH_3), 6.05 (bs r, 1H, NH), 6.92-6.95

(m, 2H, Ar), 7.38 (dd, *J* = 8.5, 2.2, 1H, Ar), 7.42-7.52 (m, 5H, Ar), 7.61 (m, 1H, Ar), 7.71-7.75 (m, 3H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.9, 24.1, 34.9, 38.3 ($4CH_2$), 45.6 ($2CH_2$), 49.9 ($2CH_2$), 50.1 (CH_2), 56.1 (CH_3), 69.3 (CH), 83.5, 92.9 (2C), 111.9, 117.8, 123.8 (3CH), 128.6 (2CH), 129.2, 129.5, 129.6 (3CH), 129.7 (C), 130.0 (2CH), 133.0, 133.5 (2CH), 136.8, 138.6, 139.9, 140.6, 156.0 (5C), 159.4, 172.7, 195.1 (3CO); MS (ESI, *m/z*): 733.2 [M(^{35}Cl)+H]$^+$, 735.2 [M(^{37}Cl)+H]$^+$.

General Procedure for the Synthesis of 16 and 18. A 1 M aqueous solution of KOH (2.2 mL/mmol) was added over 5 min to a stirred solution of compound **45** or **46** (1 equiv) in THF (2.2 mL/mmol) at rt. After stirring for 20 h, the reaction was cooled to 0 ºC and pH was adjusted to 7.0 by addition of concentrated HCl. The mixture was extracted with DCM and the combined organic layers were washed with H_2O and brine, dried (Na_2SO_4), and evaporated under reduced pressure to yield compound **16** or **18**.

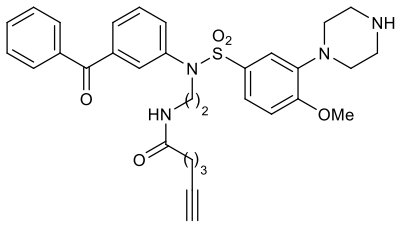
***N*-(6-{(3-Benzoylphenyl)[(4-methoxy-3-piperazin-1-ylphenyl)sulfonyl]amino}hexyl)-biotinamide, 16.** Obtained from **45** (286 mg, 0.31 mmol) as a white solid (183 mg, 76%).



R_f (DCM:EtOH, 9:1 v/v): 0.12; mp: 85-87 ºC; $[\alpha]_D^{20}$: +24.2 (c = 1.2, MeOH); IR (ATR, cm^{-1}): 3288, 3079, 1698, 1447, 1254, 1157; ^{1}H NMR (300 MHz, CD_3OD) δ 1.28-1.78 (m, 14H, $7CH_2$), 2.18 (t, *J* = 7.3, 2H, CH_2CO), 2.68 (d, *J* = 12.7, 1H, ½CH_2S), 2.87-2.93 (m, 9H, ½CH_2S, $4CH_{2pip}$), 3.13 (t, *J* = 7.0, 2H, CH_2N), 3.16-3.21 (m, 1H, CHS), 3.58 (t, *J* = 6.3, 2H, C<u>H</u>$_2$NH), 3.92 (s, 3H, CH_3), 4.28 (dd, *J* = 7.9, 4.4, 1H, C<u>H</u>NH), 4.47 (dd, 1H, *J* = 8.0, 5.0, C<u>H</u>NH), 6.89 (d, *J* = 2.3, 1H, Ar), 7.08 (d, *J* = 8.6, 1H, Ar), 7.32-7.37 (m, 2H, Ar), 7.40-7.43 (m, 1H, Ar), 7.50-7.57 (m, 3H, Ar), 7.63-7.76 (m, 4H, Ar); ^{13}C NMR (75 MHz, CD_3OD) δ 26.9, 27.0, 27.3, 29.0, 29.5, 29.7, 30.2,

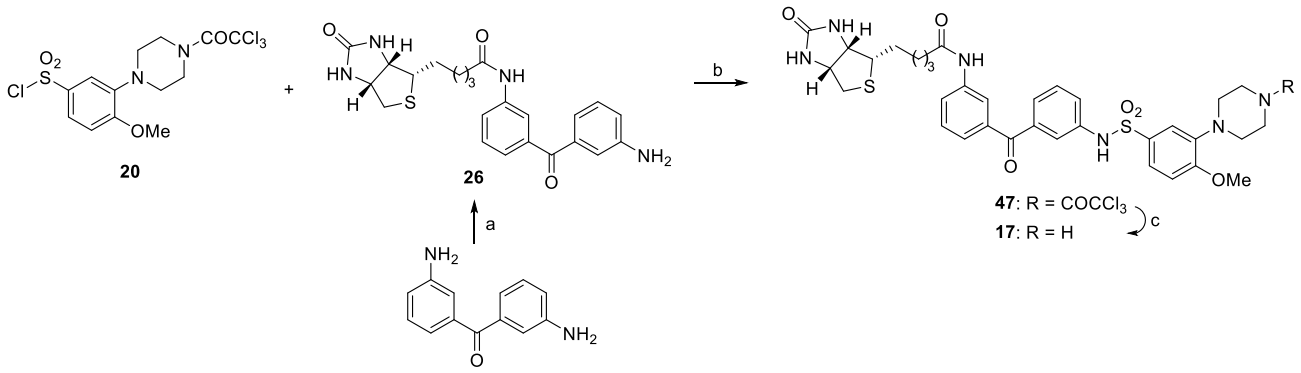
36.8, 40.1, 41.1 (10CH_2), 46.3 (2CH_2), 51.0 (CH_2), 51.9 (2CH_2), 56.5 (CH_3), 57.0, 61.6, 63.3, 112.4, 118.5, 124.5 (6CH), 129.7 (2CH), 130.2, 130.4 (2CH), 130.6 (C), 130.9 (CH), 131.0 (2CH), 134.1, 134.3 (2CH), 138.2, 139.5, 140.9, 143.0, 157.6 (5C), 166.0, 175.9, 197.0 (3CO); MS (ESI, *m/z*): 777.5 $[M+H]^+$; analysis (calcd., found for $C_{40}H_{52}N_6O_6S_2 \cdot 7HCl \cdot 3H_2O$): C (44.23, 43.99), H (6.03, 5.65), N (7.74, 7.38), S (5.90, 5.42).

***N*-(2-{(3-Benzoylphenyl)[(4-methoxy-3-piperazin-1-ylphenyl)sulfonyl]amino}ethyl)hex-5-ynamide, 18.** Obtained from **46** (73 mg, 0.10 mmol) as a brown syrup (48 mg, 82%).



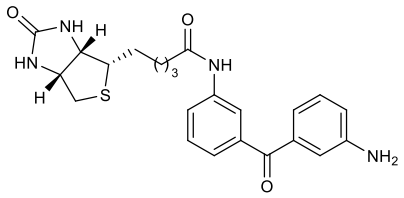
R_f (DCM:EtOH, 9:1 v/v): 0.19; IR (ATR, cm^{-1}): 3292, 1655, 1443, 1346, 1159; ^1H NMR (300 MHz, CD_3OD): δ 1.60 (qt, *J* = 7.2, 2H, CH_2), 2.05 (td, *J* = 7.0, 2.7, 2H, CH_2), 2.12 (t, *J* = 7.3, 2H, CH_2), 2.13 (t, *J* = 2.7, 1H, CH), 2.81 (m, 8H, 4CH_{2pip}), 3.17 (t, *J* = 5.9, 2H, CH_2), 3.62 (t, *J* = 5.6, 2H, CH_2), 3.82 (s, 3H, CH_3), 6.80 (d, *J* = 2.2, 1H, Ar), 6.97 (d, *J* = 8.6, 1H, Ar), 7.23 (dd, *J* = 8.6, 2.2, 1H, Ar), 7.33-7.37 (m, 2H, Ar), 7.40-7.47 (m, 3H, Ar), 7.55 (m, 1H, Ar), 7.60-7.67 (m, 3H, Ar); ^{13}C NMR (75 MHz, CD_3OD): δ 18.7, 25.8, 35.8, 39.7 (4CH_2), 46.4 (2CH_2), 50.9 (CH_2), 52.0 (2CH_2), 56.5 (CH_3), 70.3 (CH), 84.3 (C), 112.5, 118.5, 124.5 (3CH), 129.7 (2CH), 130.5, 130.7 (2CH), 131.1 (CH, C), 131.3 (2CH), 134.1, 134.6 (2CH), 138.2, 139.6, 141.4, 143.1, 157.7 (5C), 175.7, 197.0 (2CO); MS (ESI, *m/z*): 589.2 $[M+H]^+$; analysis (calcd., found for $C_{32}H_{36}N_4O_5S \cdot 2HCl \cdot 2H_2O$): C (55.09, 54.83), H (6.07, 5.90), N (8.03, 7.89), S (4.60, 4.46).

- **Synthesis of Probe 17**



Scheme S6. Reagents and conditions: (a) biotin, DCC, HOBt, DMAP, DMF, DCM, 60 ºC to rt, 53%; (b) pyridine, DMF, DCM, rt, 51%; (c) aq. 1 M KOH, THF, rt, 98%.

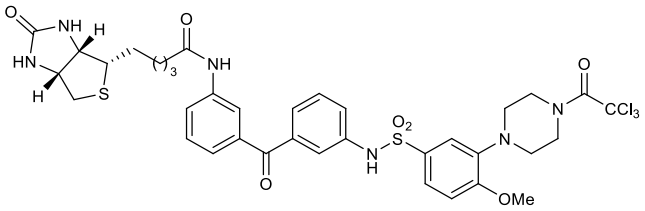
***N*-[3-(3-Aminobenzoyl)phenyl]biotinamide, 26.** Biotin (575 mg, 2.4 mmol) and HOBt (318 mg, 2.4 mmol) were suspended in anhydrous DMF (4 mL) with 4 Å molecular sieves previously activated (35 mg/mmol carboxylic acid), and the mixture was heated until a clear solution was obtained (~45 ºC). The mixture was then cooled to rt and a solution of DCC (535 mg, 2.6 mmol) in anhydrous DCM (2 mL) was added dropwise, and the reaction was stirred at rt for 3 h. Bis(3-aminophenyl)methanone (1.00 g, 4.7 mmol) and DMAP (29 mg, 0.24 mmol) were added, and the mixture was stirred at 60º C for 4 h and then at rt for 24 h. The mixture was filtered and washed with DCM/MeOH (1/1 v/v, 5 mL), and the filtrate was concentrated and purified by column chromatography (DCM:MeOH, 100:1 to 10:1 v/v) to afford compound **26** (545 mg, 53%) as an off-white solid.



R_f (DCM:EtOH, 9:1 v/v): 0.34; mp: 136-138 ºC; IR (ATR, cm^{-1}): 3396, 3355, 1690, 1545; ^1H NMR (300 MHz, DMSO-d_6): δ 1.22-1.70 (m, 6H, $3CH_2$), 2.32 (t, J = 7.4, 2H, CH_2CO), 2.57 (d, J = 12.4, 1H, ½CH_2S), 2.82 (dd, J = 12.4, 5.0, 1H, ½CH_2S), 3.12 (ddd, J = 8.4, 6.1, 4.4, 1H, CHS),

4.13 (ddd, *J* = 7.7, 4.5, 1.8, 1H, C<u>H</u>NH), 4.30 (dd, *J* = 7.8, 5.0, 1H, C<u>H</u>NH), 5.42 (bs r, 2H, NH), 6.38 (s, 1H, NH), 6.46 (s, 1H, NH), 6.82 (d, *J* = 7.8, 2H, Ar), 6.93 (s, 1H, Ar), 7.18 (t, *J* = 7.8, 1H, Ar), 7.36 (m, 1H, Ar), 7.45 (t, *J* = 7.8, 1H, Ar), 7.90 (dt, *J* = 8.0, 1.1, 1H, Ar), 7.94 (s, 1H, Ar), 10.11 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.0, 28.1, 28.2, 36.2, 39.8 (5CH_2), 55.3, 59.2, 61.0, 114.4, 117.1, 117.9, 119.8, 122.5, 123.9, 128., 128.9 (11CH), 137.8, 138.1, 139.3, 148.8 (4C), 162.7, 171.4, 196.2 (3CO); MS (ESI, *m/z*): 439.2 $[M+H]^+$.

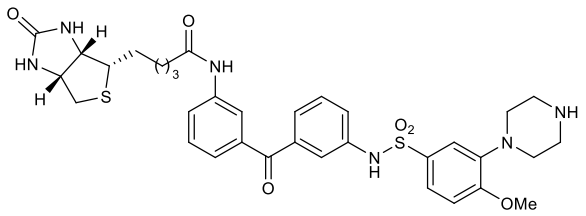
***N*-(3-{3-[({4-Methoxy-3-[4-(trichloroacetyl)piperazin-1-yl]phenyl}sulfonyl)amino]-benzoyl}phenyl)biotinamide, 47.** Pyridine (42 μL, 0.51 mmol) was added to a solution of aniline **26** (110 mg, 0.25 mmol) in anhydrous DMF (2 mL) at rt. A solution of sulfonyl chloride **20** (110 mg, 0.25 mmol) in anhydrous DCM (2 mL) was added dropwise, and the reaction mixture was stirred at rt for 16 h. The reaction was quenched with a 10% aqueous solution of $NaHCO_3$ (3 mL) and the aqueous phase was extracted with DCM (2 x 10 mL). The combined organic layers were washed with H_2O (10 mL) and brine (10 mL), dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was purified by column chromatography (DCM:EtOH, 100:1 to 10:1 v/v) to yield compound **47** (106 mg, 51% yield) as a beige solid.



R_f (DCM:EtOH, 9:1 v/v): 0.43; mp: 180-183 ºC; IR (ATR, cm^{-1}): 1741, 1678, 1462; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32-1.68 (m, 6H, 3CH_2), 2.32 (t, *J* = 7.4, 2H, CH_2CO), 2.58 (d, *J* = 12.5, 1H, ½CH_2S), 2.82 (dd, *J* = 12.5, 5.1, 1H, ½CH_2S), 2.99 (m, 4H, 2CH_{2pip}), 3.11 (dt, *J* = 8.5, 5.7, 1H, CHS), 3.84 (s, 3H, CH_3), 3.76-3.94 (m, 4H, 2CH_{2pip}), 4.13 (dd, *J* = 7.8, 4.5, 1H, C<u>H</u>NH), 4.30 (m, 1H, C<u>H</u>NH), 6.35 (s, 1H, NH), 6.42 (s, 1H, NH), 7.09 (d, *J* = 8.6, 1H, Ar), 7.20-7.22 (m, 2H, Ar), 7.36-7.47 (m, 6H, Ar), 7.88 (d, *J* = 7.8, 1H, Ar), 7.97 (s, 1H, Ar), 10.11 (s, 1H, NH), 10.30 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.9, 29.0, 29.1, 37.1, 39.9 (5CH_2), 50.3 (2CH_2), 56.2

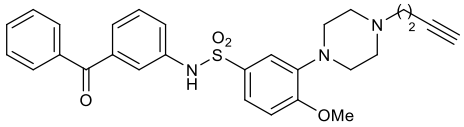
(2CH_2), 56.8 (CH), 56.9 (CH_3), 60.1, 61.9 (2CH), 93.5 (C), 112.4, 117.3, 120.6, 121.6, 123.4 (5CH), 123.9 (2CH), 125.0 (C), 129.8 (2CH), 130.4, 131.6 (2CH), 138.0, 138.6, 139.2, 140.4, 141.1, 156.1 (6C), 159.3, 163.6, 172.4, 195.8 (4CO); MS (ESI, *m/z*): 837.1 $[M(^{35}Cl)+H]^+$, 839.1 $[M(^{37}Cl)+H]^+$.

***N*-[3-(3-{[(4-Methoxy-3-piperazin-1-ylphenyl)sulfonyl]amino}benzoyl)phenyl]biotinamide, 17.** A 1 M aqueous solution of KOH (2 mL) was added over 5 min to a stirred solution of compound **47** (100 mg, 0.12 mmol) in THF (2 mL) at rt. After stirring for 20 h, the reaction was cooled to 0 ºC and pH was adjusted to 7.0 by addition of concentrated HCl. The mixture was extracted with DCM (2 x 10 mL) and the combined organic layers were washed with H_2O (10 mL) and brine (10 mL), dried (Na_2SO_4), and evaporated under reduced pressure to yield compound **17** (81 mg, 98%) as a yellow solid.



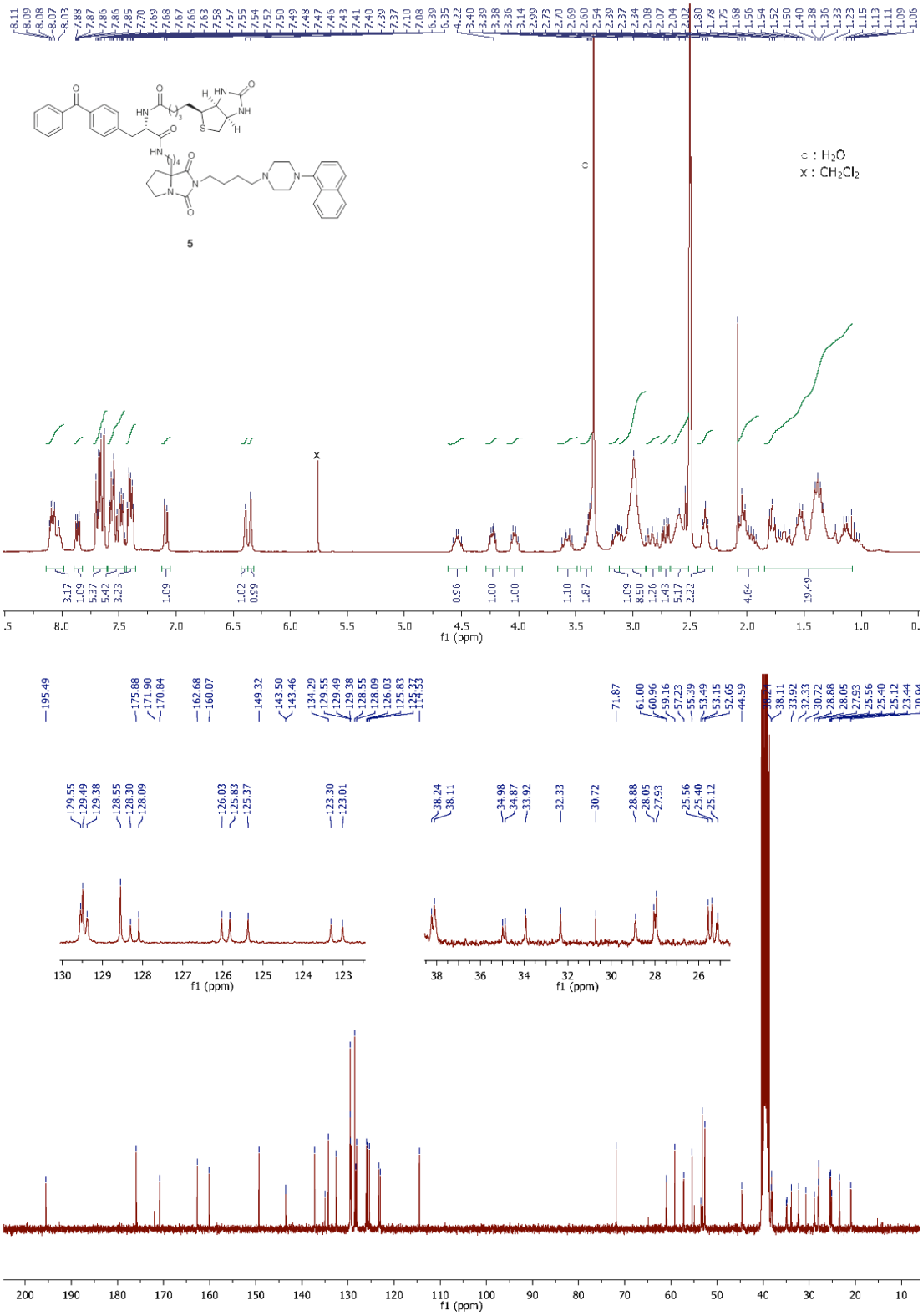
R_f (DCM:EtOH, 9:1 v/v): 0.17; mp: 213-215 ºC; $[\alpha]_D^{20}$: +25.3 (c = 0.6, MeOH); IR (ATR, cm^{-1}): 3402, 1682, 1590, 1552, 1324, 1126; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32-1.66 (m, 6H, 3CH_2), 2.33 (t, *J* = 7.3, 2H, CH_2CO), 2.58 (d, *J* = 12.5, 1H, ½CH_2S), 2.79 (m, 8H, 4CH_{2pip}), 2.85 (m, 1H, ½CH_2S), 3.12 (m, 1H, CHS), 3.79 (s, 3H, CH_3), 4.13 (ddd, *J* = 7.5, 4.5, 1.9, 1H, CHNH), 4.30 (dd, *J* = 7.8, 4.9, 1H, CHNH), 6.37 (s, 1H, NH), 6.44 (s, 1H, NH), 6.99 (d, *J* = 8.6, 1H, Ar), 7.16-7.46 (m, 8H, Ar), 7.92 (d, *J* = 7.8, 1H, Ar), 7.98 (s, 1H, Ar), 10.27 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.9, 29.0, 29.5, 30.0, 37.2 (5CH_2), 41.2 (2CH_2), 46.3 (2CH_2), 51.9 (CH), 56.6 (CH_3), 60.1, 61.9, 112.0, 116.9, 120.5, 121.7, 122.3 (7CH), 123.8 (2CH), 124.1 (C), 125.0 (2CH), 129.6, 130.0 (2CH), 138.2, 138.4 (2C), 140.4 (2C), 142.4, 155.2 (2C), 163.6, 172.4, 195.6 (3CO); MS (ESI, *m/z*): 693.2 $[M+H]^+$; analysis (calcd., found for $C_{34}H_{40}N_6O_6S_2 \cdot HCl \cdot 3H_2O$): C (52.13, 51.80), H (6.05, 5.89), N (10.73, 10.57), S (8.19, 8.31).

- **Synthesis of *N*-(3-benzoylphenyl)-3-(4-but-3-yn-1-ylpiperazin-1-yl)-4-methoxybenzene-sulfonamide, 19.** Sodium iodide (147 mg, 0.97 mmol) and 4-bromobutyne (60 μL, 0.61 mmol) were added to a solution of compound **14** (221 mg, 0.49 mmol) in anhydrous DMF (5 mL) and the reaction mixture was heated at 80 ºC for 24 h. Upon cooling to rt, solvent was removed under reduced pressure and the residue was purified by column chromatography (DCM:MeOH, 100:1 to 20:1 v/v) to afford pure compound **19** (116 mg, 47%) as a white solid.

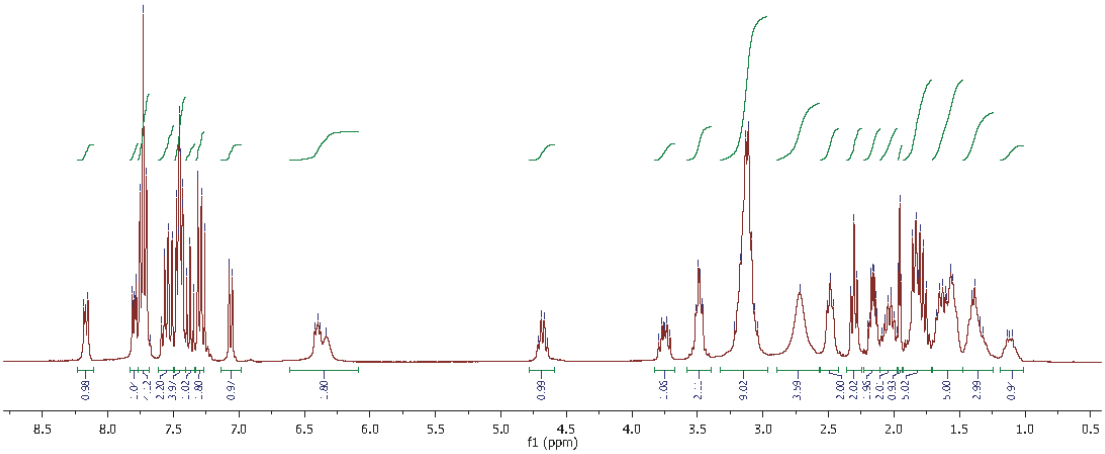


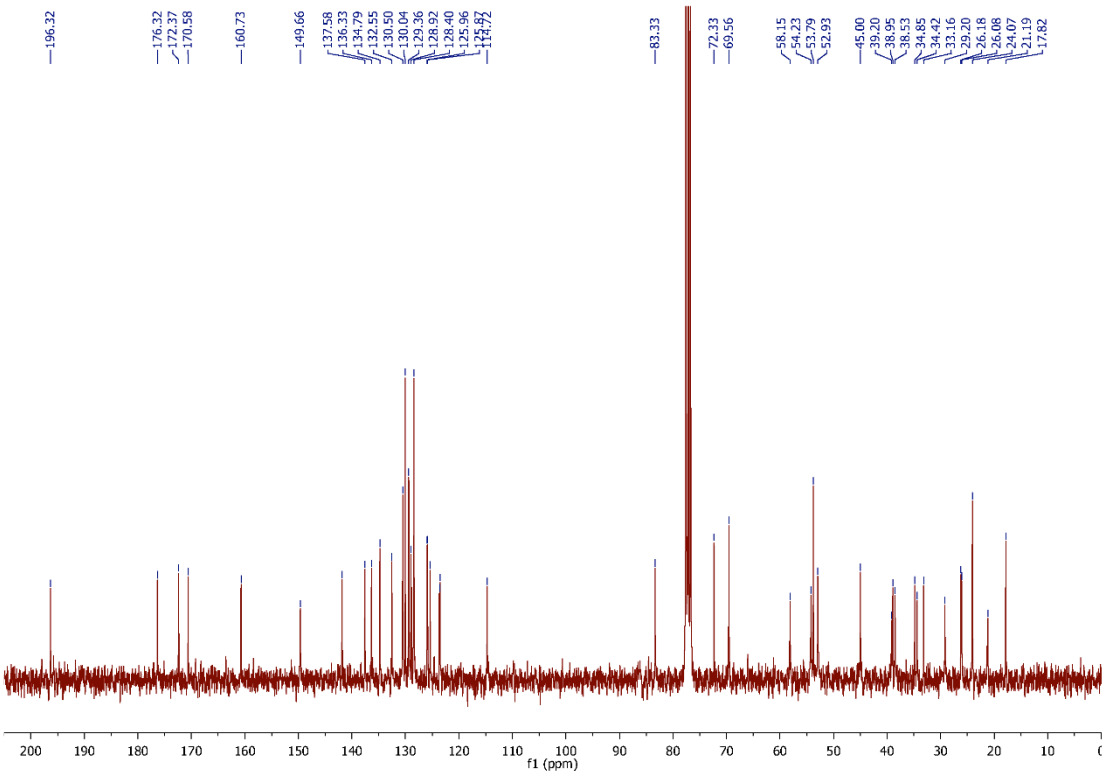
R_f (hexane:EtOAc, 3:1 v/v): 0.46; mp: 65-67 ºC; IR (ATR, cm^{-1}): 3274, 1658, 1586, 1502, 1228, 1100; ^1H NMR (300 MHz, $CDCl_3$): δ 1.99 (t, *J* = 2.7, 1H, CH), 2.40 (dt, *J* = 7.8, 2.7, 2H, $C\underline{H}_2CCH$), 2.62-2.67 (m, 6H, CH_2N_{pip}, $2CH_{2pip}$), 2.99 (m, 4H, $2CH_{2pip}$), 3.87 (s, 3H, CH_3), 6.81 (d, *J* = 8.7, 1H, Ar), 7.24 (d, *J* = 2.3, 1H, Ar), 7.33-7.52 (m, 7H, Ar), 7.58 (m, 1H, Ar), 7.69-7.72 (m, 2H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.7 (CH_2), 50.1 ($2CH_2$), 52.8 ($2CH_2$), 55.9 (CH_3), 56.9 (CH_2), 69.2 (CH), 82.6 (C), 110.7, 117.0, 122.5, 122.8, 125.1, 126.7 (6CH), 128.4 (2CH), 129.3 (CH), 130.1 (2CH), 130.6 (C), 132.8 (CH), 137.0, 137.3, 138.6, 141.7, 156.0 (5C), 195.7 (CO); MS (ESI, *m/z*): 504.2 $[M+H]^+$; analysis (calcd., found for $C_{28}H_{29}N_3O_4S \cdot 2HCl \cdot 1H_2O$): C (57.23, 57.48), H (5.80, 5.47), N (6.90, 7.08), S (5.27, 5.18).

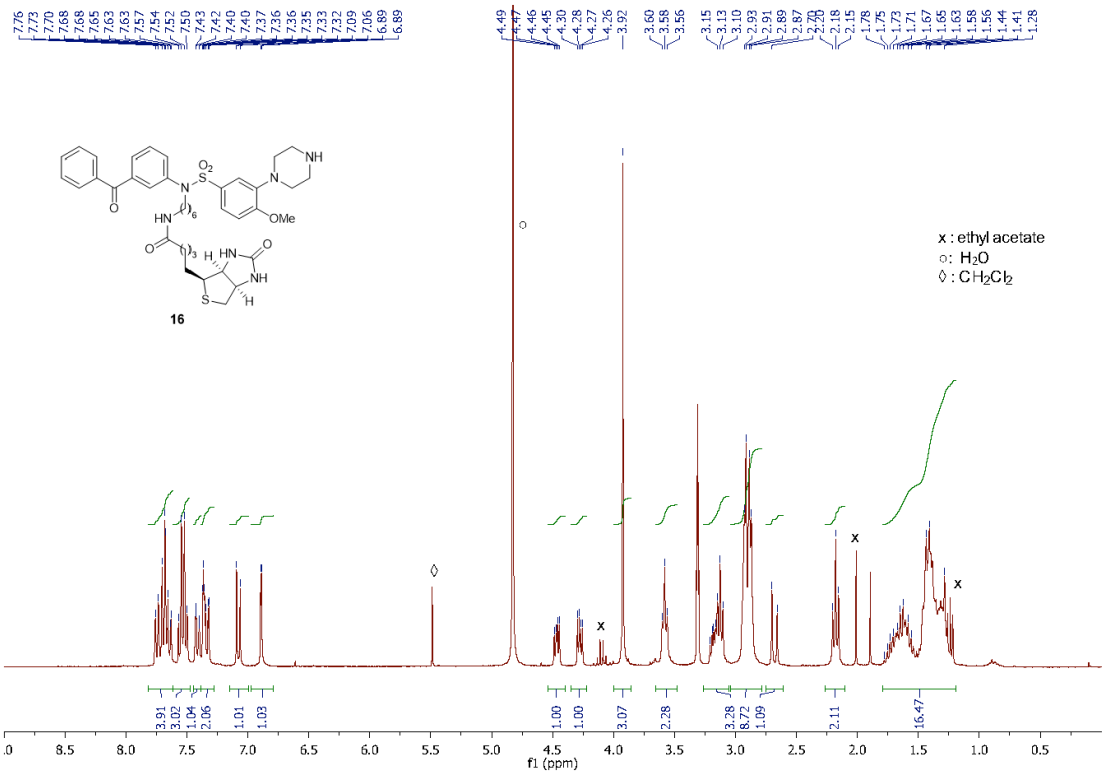
1.4. NMR Spectra of Probes 5, 7, 16, 18, 27, 28

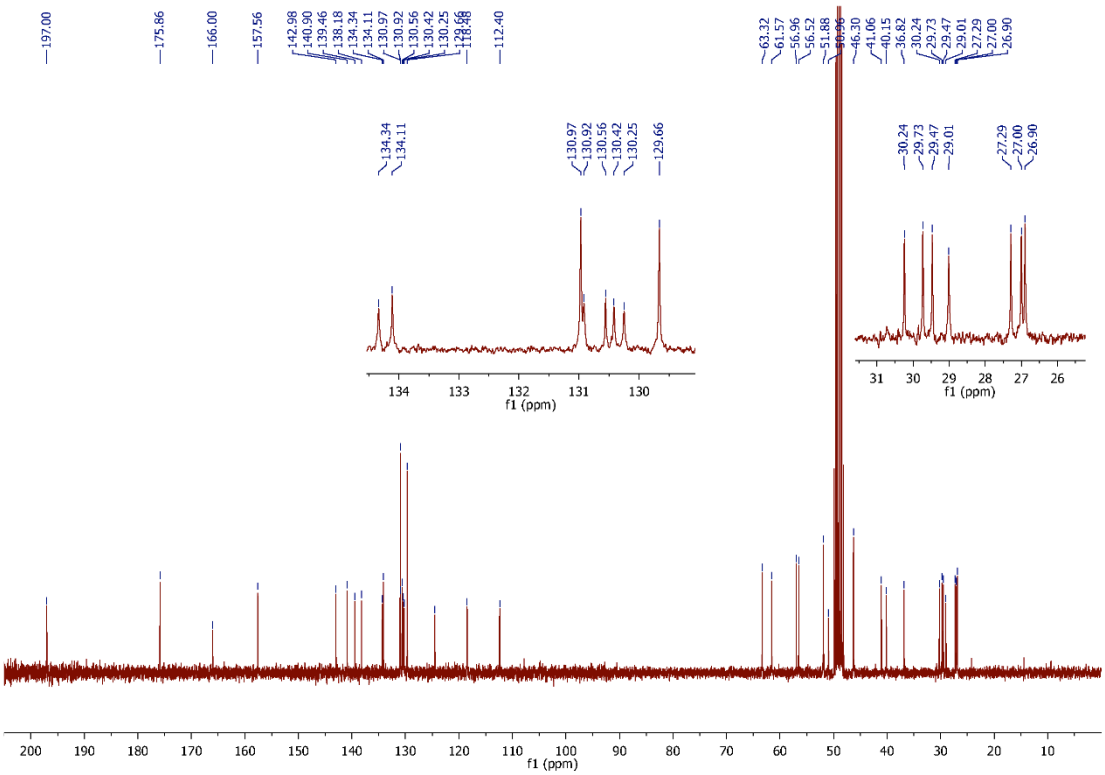


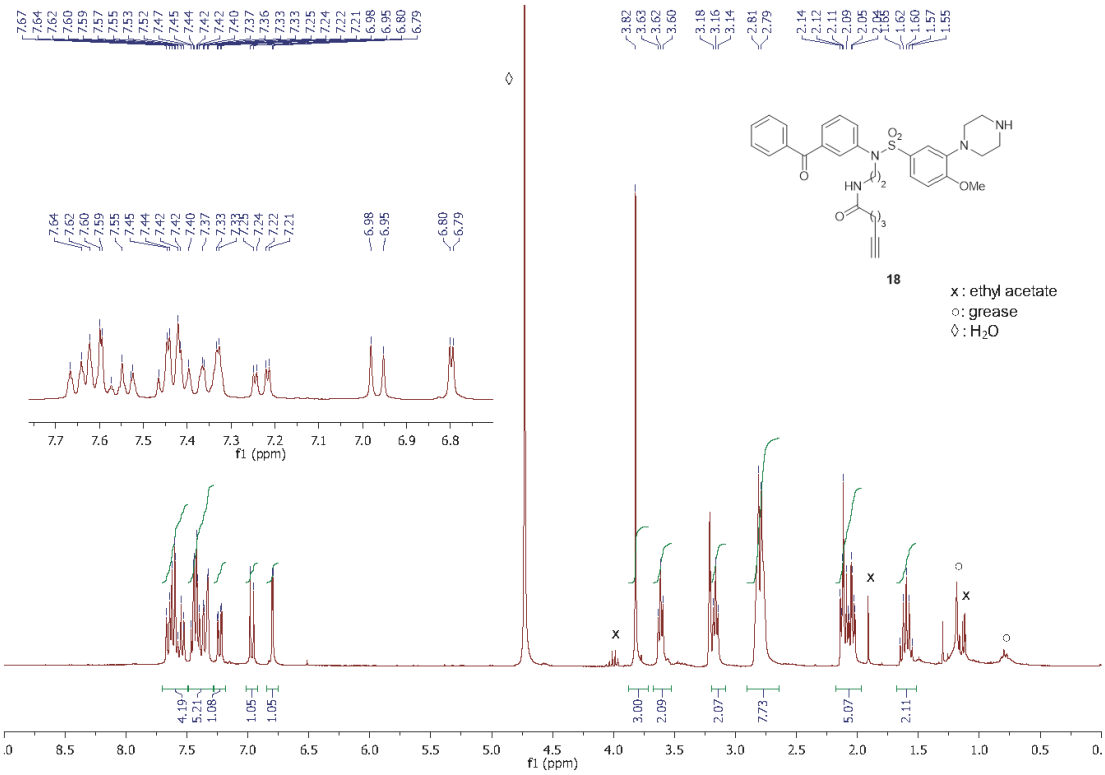
7

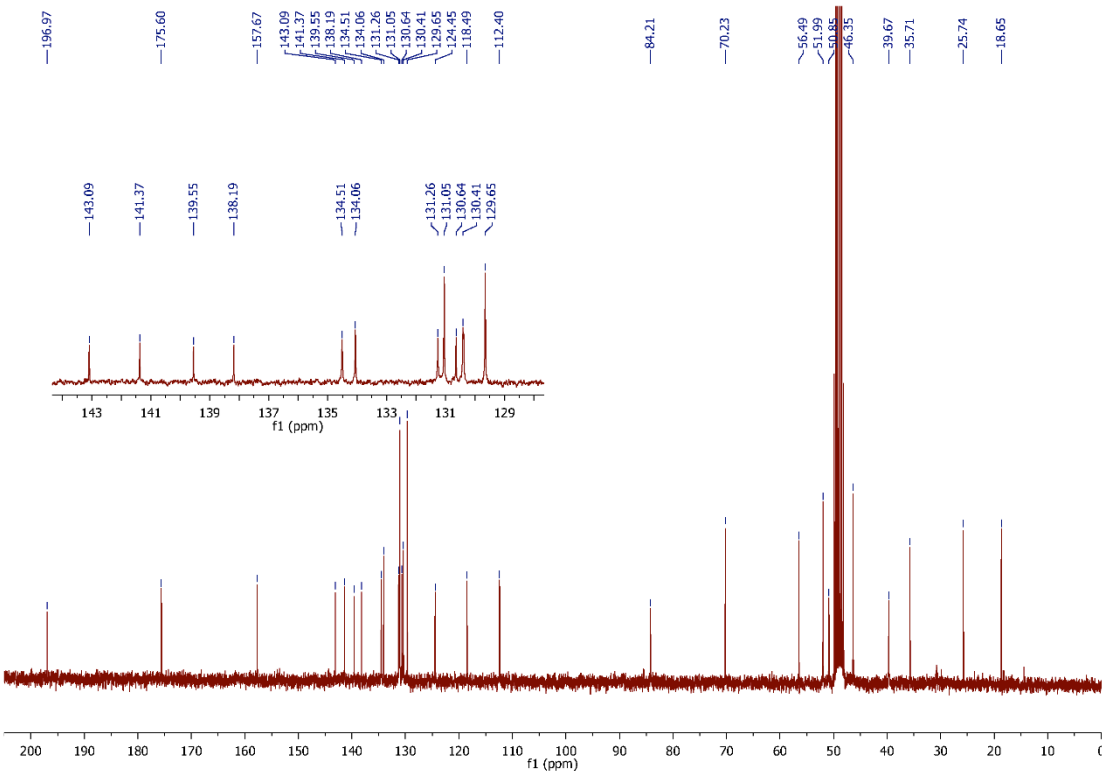

0.98
1.00
2.20
3.97
1.02
1.80
0.97
1.80
0.99
1.08
2.11
9.02
3.99
2.00
2.02
1.96
2.01
0.93
5.03
5.00
2.99
0.97
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

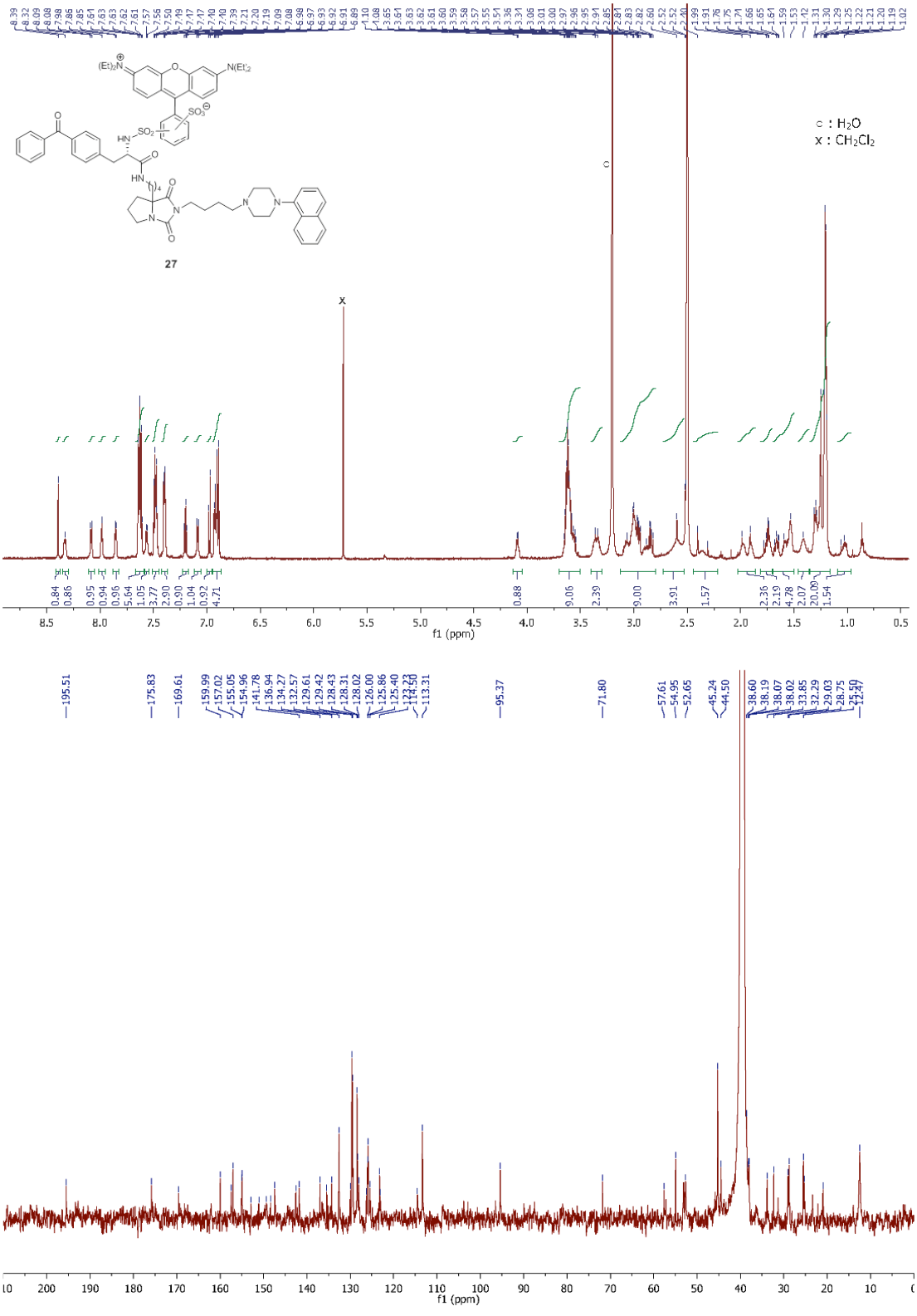

196.32
176.32
172.37
170.58
160.73
149.66
137.58
136.33
134.79
132.55
130.50
130.04
129.36
128.92
128.40
125.96
125.82
124.92
83.33
72.33
69.56
58.15
54.23
53.79
52.93
45.00
39.20
38.95
38.53
34.85
34.42
33.16
29.20
26.18
26.08
24.07
21.19
17.82
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

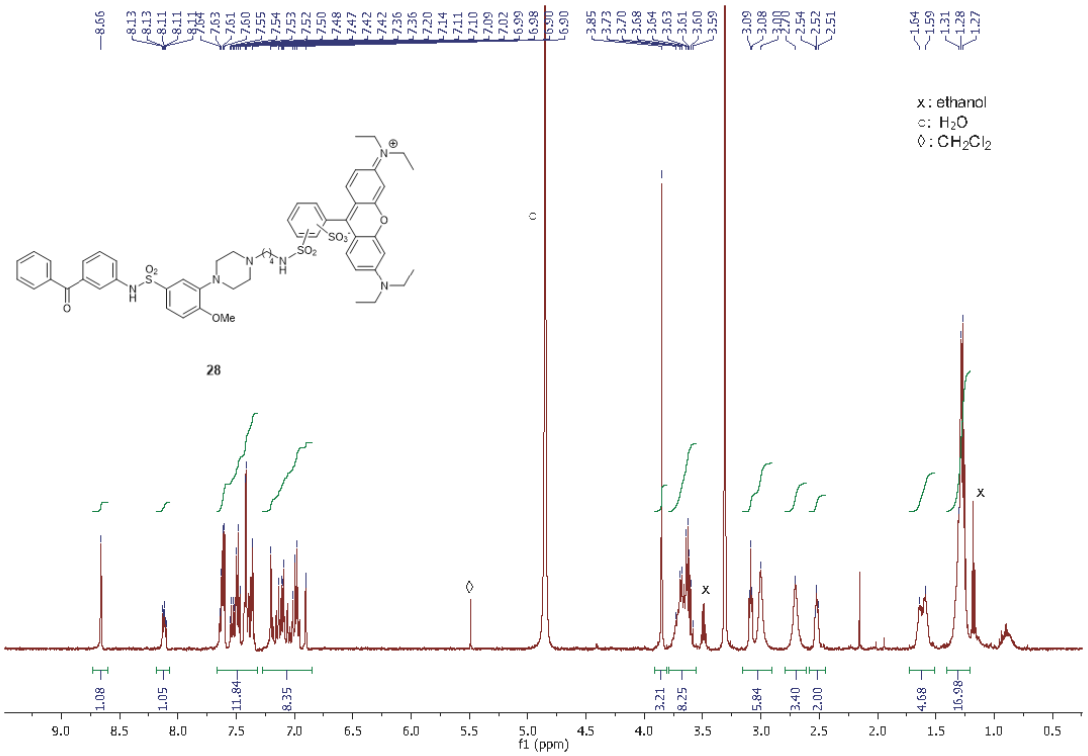

16
x : ethyl acetate
○: H2O
◊ : CH2Cl2
f1 (ppm)

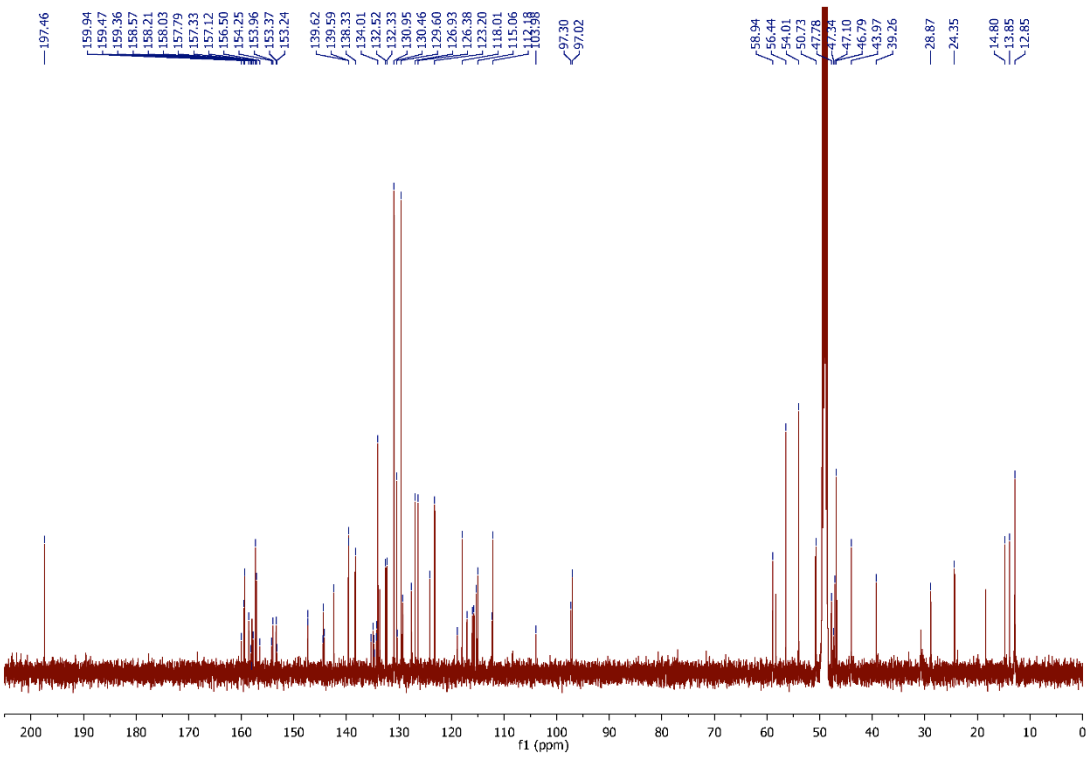

f1 (ppm)


18
x : ethyl acetate
○ : grease
◊ : H2O
f1 (ppm)


f1 (ppm)


27
○ : H2O
x : CH2Cl2
f1 (ppm)
f1 (ppm)


x : ethanol
○ : H2O
◊ : CH2Cl2
28
f1 (ppm)


f1 (ppm)

2. Binding Assays

Membranes from HEK-293-EBNA (5-HT_{1A}) and HEK-293 (5-HT_6 receptor) cells expressing the indicated human receptors were purchased from Perkin-Elmer and conserved at -80 ºC in packaging buffer for subsequent use. Competitive inhibition assays were performed according to standard procedures, briefly detailed below.

5-HT_{1A} Receptor. Cell membranes (6.4 mg/mL) were homogenized using a glass dounce homogenizer in 7 volumes of assay buffer (50 mM Tris-HCl, 0.5 mM $MgSO_4$, pH 7.4 at 25 ºC). Fractions of 20 µL of the membrane suspension were incubated at 37 ºC for 120 min with 2 nM [^{3}H]-8-hydroxy-DPAT (170.2 Ci/mmol, Perkin-Elmer) in the presence or absence of the compounds under study (ranging from 10^{-5} to 10^{-10} M) in a final volume of 200 µL of assay buffer. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 10 µM serotonin and represented less than 10% of total binding.

5-HT_6 Receptor. Cell membranes (6.0 mg/mL) were homogenized using a glass dounce homogenizer in 7 volumes of assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4 at 25 ºC). Fractions of 20 µL of the membrane suspension were incubated at 37 ºC for 60 min with 2.5 nM [^{3}H]LSD (79.2 Ci/mmol, Perkin-Elmer) in the presence or absence of the compounds under study (ranging from 10^{-5} to 10^{-10} M) in a final volume of 200 µL of assay buffer. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 100 µM serotonin and represented less than 10% of total binding.

For all binding assays, competing drug and nonspecific, total, and radioligand bindings were determined in triplicate. Incubation was terminated by rapid vacuum filtration through Wallac Filtermat A filters (PerkinElmer), presoaked in 0.5% polyethylenimine, using a FilterMate Unifilter 96-Harvester (PerkinElmer). The filters were then washed 9 times with 500 µL of the corresponding ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 25 ºC) and air-dried. Then, a MeltiLex solid scintillator sheet (PerkinElmer) was immediately melted onto the filter and the radioactivity

bound to the filters was quantified by scintillation spectrometry, using a Microbeta TopCount instrument (PerkinElmer). The data were analyzed by an iterative curve-fitting procedure using GraphPad Prism program and K_i values were calculated from the IC_{50} values using the Cheng-Prusoff equation[8] and are expressed as the average and standard error obtained from two to four independent experiments carried out in triplicate.

3. Gel Profiling of Cell Membranes

Membranes of HEK-293-EBNA (6.4 mg/mL, 5-HT_{1A} receptor) and HEK-293 (6 mg/mL, 5-HT_6 receptor) cells expressing the indicated human receptors (PerkinElmer) were homogenized in incubation buffer (50 mM Tris-HCl, 0.5 mM $MgSO_4$, pH 7.4 at 25 ºC for 5-HT_{1A} receptor; 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4 at 25 ºC for 5-HT_6 receptor) and protein concentration was adjusted to 3.2 mg/mL (5-HT_{1A} receptor) or 3 mg/mL (5-HT_6 receptor). Each sample was incubated in the absence or presence of the probe (5 μM) in a final volume of 50 μL of incubation buffer in a 96-well polyestyrene plate. After incubation at 37 ºC for 30 min, samples were irradiated at 360 nm using an UV lamp on ice for 1 h. Then, 50 μL of a 5% β-mercaptoethanol solution in Laemmli loading buffer (BioRad) was added and the samples were completely solubilized by heating at 80 ºC for 10 min. Finally, aliquots of 50 μL of each sample were loaded and resolved with 10% SDS-PAGE gels. Fluorescence images were obtained with a Typhoon 9400 scanner.

[8] Cheng, Y. C., and Prusoff, W. H. (1973) Relationship between the inhibition constant (*K*i) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction, *Biochem. Pharmacol. 22*, 3099–3108.

4. Mass Spectrometry Profiling of Ligand-Binding Proteins

Incubation and Photo-cross-linking of Samples. Membrane homogenates of HEK-293-EBNA (500 μL of 3.2 mg/mL for 5-HT_{1A}) and HEK-293 (500 μL of 3 mg/mL for 5-HT_6 receptor) were incubated in the absence or presence of the probe (5 μM) in a final volume of 600 μL of incubation buffer in a 24-well polyestyrene plate. After incubation at 37 ºC for 30 min, samples were irradiated at 360 nm using an UV lamp on ice for 1 h. Then, samples were transferred to microtubes (eppendorf) and 100 μL of a solution of 7% Triton X-100 in PBS were added to solubilize proteins and shaken at rt for 1 h, before 50 μL of a solution of 4% SDS in H_2O and 50 μL of PBS were added. The samples were incubated with 100 μL of streptavidin-agarose beads (Pierce) for 12 h at 4 ºC, transferred to falcon tubes and the beads were washed consecutively with 0.2% SDS in PBS (1×5 mL), PBS (3×5 mL), and H_2O (3×5mL). The beads were settled down by centrifugation (2500 rpm, 2 min) after each wash. The washed beads were suspended in 500 μL of 6 M urea in PBS and 25 μL of a 250 mM solution of tris(2-carboxyethyl)phosphine in H_2O were added and shaken at rt for 30 min. Then, 25 μL of a 400 mM iodoacetamide solution in H_2O were added and allowed to react at rt for 30 min in the darkness. Following reduction and alkylation, the beads were diluted in 800 μL of PBS, pelleted by centrifugation (4000 rpm, 3 min) and washed with PBS (3×1 mL).

Mass Spectrometry Analysis. Proteins from pull-down samples were loaded onto SDS-PAGE and run in a unique band that concentrated all the proteins in the sample, stained with Coomassie Brilliant Blue G-250 and excised in 1 mm^3 small pieces, which were washed in ultrapure H_2O. Samples were subjected to reduction and alkylation, and in-gel digested overnight at 37 ºC by adding modified porcine trypsin (Promega) at a final ratio of 1:20 (trypsin:protein). After digestion, peptides were vacuum-dried and finally dissolved in 0.1% formic acid for LC-MS/MS analysis. The tryptic peptide mixtures were subjected to nano-liquid chromatography coupled to mass spectrometry (MS) for protein identification. Peptides were injected onto a C-18 reversed

phase (RP) nano-column (100 mm I.D. and 12 cm, Mediterranea sea, Teknokroma) and analyzed in a continuous acetonitrile gradient consisting of 0-50% B in 90 min, 50-90% B in 1 min (B=95% acetonitrile, 0.5% acetic acid). A flow rate of 300 nL/min was used to elute peptides from the RP nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution = 30000) followed by the MS/MS spectra from most intense three parent ions (dissociated using CID activation) was analyzed along the chromatographic run (130 min). Dynamic exclusion was set at 1 min.

A second set of samples were processed as before, but the tryptic peptide mixtures were dissolved in 0.1% formic acid (Gradient Buffer A), injected onto a C-18 reversed phase (RP) precolumn (Acclaim PepMap100 nanoViper Column, C18, 3 μm, 75 μm i.d. x 2 cm) and analyzed in a continuous acetonitrile gradient consisting of 8-31% B in 120 min, 31-90% B in 1 min (B=90% acetonitrile, 0.1% formic acid) using an Acclaim PepMap100, C18, 3 μm, 100Å, 75 μm i.d. x 25 cm nanocolumn. A flow rate of 200 nL/min was used to elute peptides from the RP nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on an Orbitrap Elite mass spectrometer (Thermo Fisher).

Database Searching. Tandem mass spectra were extracted by Proteome Discoverer v1.3 (for Orbi XL data) or Proteome Discoverer v1.4 (for Elite data) (Thermo Fisher). Charge state deconvolution and deisotoping were not performed. For protein identification, fragmentation spectra were searched against a curated subset of a human database (human_ref.fasta; 2003, April; 39414 entries) using Sequest (Thermo Fisher Scientific version 1.0.43.2) and X-Tandem (The GPM, thegpm.org; version 2007.01.01.1) engines. Sequest and X-Tandem were searched allowing two missed trypsin cleavages, and a tolerance of 15 ppm or 0.8 Da was set for full MS or MS/MS spectra searches, respectively. Iodoacetamide alkylation of cysteine residues was selected as fixed modification and oxidation of methionine was allowed as variable modification. Finally, Scaffold

v.3.00.02 software (Proteome Software Inc) was used to validate MS/MS based peptide and protein identifications. Protein and peptides probabilities were assigned by the Protein and Peptide Prophet algorithm, respectively. Both protein and peptide identification were accepted if they could be established at greater than 95.0% probability. Proteins must contain at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The Prophet FDR was between 0.08% and 0.1% in all cases for peptide and protein identification, respectively.

5. In-Gel Hit Validation

Pure HSP60 (2 μg) or prohibitin (1 μg) were incubated in the absence or presence of different concentrations of the dual probes **27** (0.5-5 μM) or **28** (5-20 μM) in a final volume of 50 μL of PBS in a 96-well polystyrene plate. After incubation at 37 ºC for 30 min, samples were irradiated at 360 nm using an UV lamp on ice for 1 h. Then, 50 μL of a 5% β-mercaptoethanol solution in Laemmli loading buffer (BioRad) were added and the samples were completely solubilized by heating at 80 ºC for 10 min. Finally, aliquots of 50 μL of each sample were loaded and resolved with 10% SDS-PAGE gels. Fluorescence images were obtained with a Typhoon 9400 scanner.

For click chemistry reactions followed by SDS/PAGE experiments, pure protein (2 μL of a 1 mg/mL in PBS) was incubated in the absence or presence of the dual probe **7** (10 μM) or **18** (10 μM) in a final volume of 45 μL of PBS in a 96-well polystyrene plate (45 μL reactions were set up so that, once the cycloaddition reagents were added, the total reaction volume would be 50 μL). After incubation at 37 ºC for 30 min, samples were irradiated at 360 nm using an UV lamp on ice for 1 h. Then, samples were transferred to microtubes (eppendorf) and 1 μL of a 5 mM DMSO solution of the rhodamine-biotin-azide derivative $TriN_3$[9] (final concentration 100 μM) was added,

[9] Speers, A. E., and Cravatt, B. F. (2004) Profiling enzyme activities in vivo using click chemistry methods, *Chem. Biol. 11*, 535–546.

followed by the addition of 1 μL of a 2.5 mM aqueous solution of sodium ascorbate (final concentration 50 μM) and 2 μL of a 2.5 mM solution of *tris*[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) ligand in DMSO:^{t}BuOH 1:4 (final concentration 100 μM). Samples were gently vortexed and 1 μL of a 50 mM aqueous solution of $CuSO_4$ (final concentration 1 mM) was added to each proteome sample, making the total reaction volume 50 μL. Samples were vortexed again and allowed to react at rt for 1 h. Then, 50 µL of a 5% β-mercaptoethanol solution in Laemmli loading buffer (BioRad) were added and the samples were completely solubilized by heating at 80 ºC for 10 min. Finally, aliquots of 50 µL of each sample (1 μg of protein/gel lane) were loaded and resolved with 10% SDS-PAGE gels. Fluorescence images were obtained with a Typhoon 9400 scanner.

6. In situ (whole cell) vs in vitro (homogenates) labelling

For in situ labelling, MDA-MB-231 cells were plated in 6 cm dishes and grown to 100% confluency, washed with PBS, and treated with 10 µM of probe **7**, **18**, or **27** or 50 µM of probe **28** in 1 mL of serum-free media. After 30 min at 37 ºC, media was aspirated off and cells were irradiated (360 nm, 4 ºC, 1 h) and scrapped in 1 mL of cold PBS. Cell pellets were isolated by centrifugation (3000 rpm, 3 min), rinsed with PBS and centrifuged twice (3000 rpm, 3 min), and lysed by probe sonication. Protein concentration was determined and adjusted to 1 mg/mL in 50 µL of final volume. For probes **27** and **28**, 13 µL of Laemli loading buffer was added. For probes **7** and **18**, 50 µg of lysate were conjugated to rhodamine azide (Rh-N_3) by treating with 6 µL of a pre-mixed solution containing 3 µL of 1.7 mM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) in 4:1 DMSO:t-BuOH, 1 µL of 50 mM $CuSO_4$ in water, 1 µL of 50 mM tris(2-carboxyethyl)phosphine (TCEP) in water (freshly prepared), and 1 µL of 1.25 mM Rh-N_3 in DMSO. After 1 h at rt in the dark, samples were mixed with 17 µL of Laemmli loading buffer. The

samples were solubilized by heating at 80 ºC for 10 min and 20 μg of protein were loaded into each gel lane and resolved using SDS-PAGE.

For in vitro labelling, MDA-MB-231 cells were lysed in PBS and 50 μL of the lysate adjusted at 1 mg/mL was treated with 0.5 μL of probe in DMSO to reach a final concentration of 10 μM for probes **7**, **18** or **27**, or 50 μM of probe **28**. After 30 min at 37 ºC, the samples were irradiated (360 nm, 4 ºC, 1 h) and then either treated with 13 μL of Laemli loading buffer (samples treated with probes **27** and **28**), or conjugated to rhodamine azide (samples treated with probes **7** and **18**) as described above, and mixed with 17 μL of Laemmli loading buffer. The proteins were solubilized by heating at 80 ºC for 10 min and 20 μg were loaded into each gel lane and resolved using SDS-PAGE. Fluorescence images were obtained with a Typhoon 9400 scanner.

7. Heat denaturation experiments

Recombinant or denatured (heated at 100 ºC for 10 min) HSP60 or prohibitin (2 μg) were incubated in the presence of probes **27** (2 μM) or **28** (10 μM) in a final volume of 50 μL of PBS in a 96-well polystyrene plate. After incubation at 37 ºC for 30 min, samples were irradiated at 365 nm using a Stratagene Stratalinker 1800 for 1 min at 4 ºC. Then, 50 μL of a 5% β-mercaptoethanol solution in Laemmli loading buffer (BioRad) were added and the samples were completely solubilized by heating at 80 ºC for 10 min. Finally, aliquots of 50 μL of each sample were loaded and resolved with 10% SDS-PAGE gels. Fluorescence images were obtained with a Typhoon 9400 scanner.

Table S1. Proteins identified with probe **5**

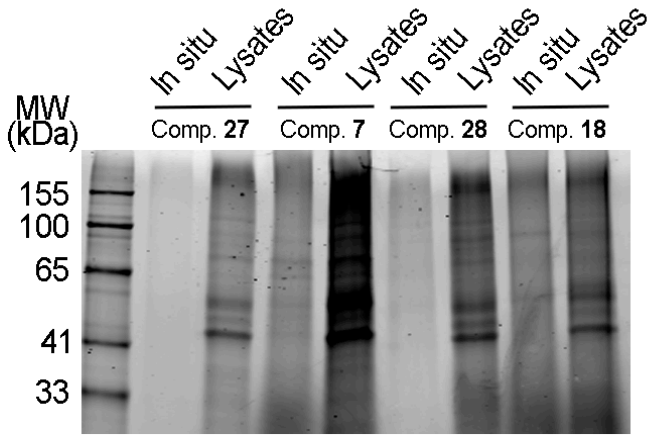
Accession	Symbol	Description
P08908	HTR1A	5-hydroxytryptamine receptor 1A
P10809	HSPD1	60 kDa heat shock protein, mitochondrial
A2TKE6	TTN	Titin isoform N2-A
P60709	ACTB	Actin, cytoplasmic 1
P40939	HADHA	Trifunctional enzyme subunit alpha, mitochondrial
P08107	HSPA1A	Heat shock 70 kDa protein 1A/1B
P09874	PARP1	Poly [ADP-ribose] polymerase 1
B4DGP8	CANX	Calnexin
P55084	HADHB	Trifunctional enzyme subunit beta, mitochondrial
P45880	VDAC2	Voltage-dependent anion-selective channel protein 2
P68363	TUBA1B	Tubulin alpha-1B chain
B4DPF9	PCCA	Propionyl-CoA carboxylase alpha chain, mitochondrial
P04843	RPN1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase
P05141	SLC25A5	ADP/ATP translocase 2
P05023	ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1
Q9P035	PTPLAD1	3-hydroxyacyl-CoA dehydratase 3
Q02224	CENPE	Isoform 1 of Centromere-associated protein E
P06576	ATP5B	ATP synthase subunit beta, mitochondrial
P28288	ABCD3	ATP-binding cassette sub-family D member 3
P14625	HSP90B1	Endoplasmin
P20700	LMNB1	Lamin-B1
P49411	TUFM	Elongation factor Tu, mitochondrial
P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K
Q06830	PRDX1	Peroxiredoxin-1
P04181	OAT	Ornithine aminotransferase, mitochondrial
Q12931	TRAP1	Heat shock protein 75 kDa, mitochondrial
P25705	ATP5A1	ATP synthase subunit alpha, mitochondrial
P52272	HNRNPM	Heterogeneous nuclear ribonucleoprotein M
P51659	HSD17B4	Peroxisomal multifunctional enzyme type 2
P38646	HSPA9	Stress-70 protein, mitochondrial

Identified proteins have been found in at least 4 independent experiments and are absent in DMSO controls (n = 4).

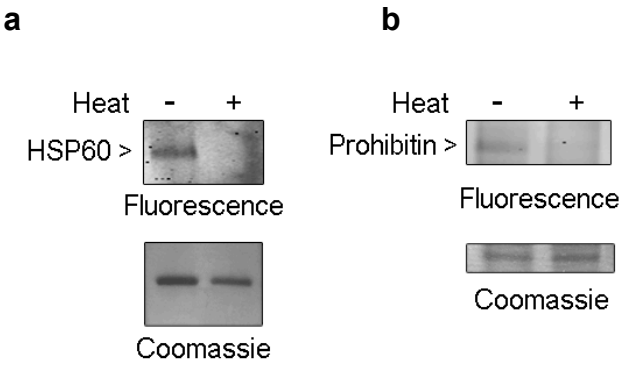
Table S2. Proteins identified with probe **16**

Accession	Symbol	Description
P50406	HTR6	5-hydroxytryptamine receptor 6
P35232	PHB	Prohibitin
P63261	ACTG1	Actin, cytoplasmic 2
P08107	HSPA1A	Heat shock 70 kDa protein 1
B4DGP8	CANX	Calnexin
P05141	SLC25A5 (ANT2)	ADP/ATP translocase 2
P40939	HADHA	Trifunctional enzyme subunit alpha, mitochondrial
P14625	HSP90B1	Endoplasmin
P68104	EEF1A1	Elongation factor 1-alpha 1
Q06830	PRDX1	Peroxiredoxin-1
P45880-2	VDAC2	Isoform 2 of Voltage-dependent anion-selective channel protein 2
P55084	HADHB	Trifunctional enzyme subunit beta, mitochondrial

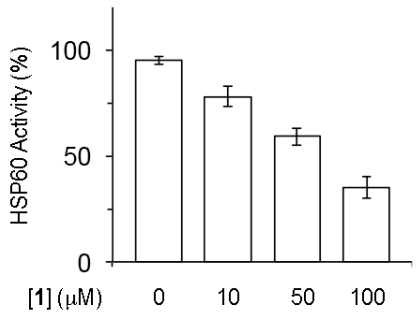
Identified proteins have been found in 4 independent experiments and are absent in DMSO controls (n = 4).



Supporting Figure S1. Gel analysis of labelling of whole cells (in situ) and lysates of MDA-MB-231 cells. The image shows the SDS-PAGE analysis of MDA-MB-231 cells treated in situ with the indicated compounds and the labelling of MDA-MB-231 lysates with the same compounds (see methods in this section for detailed experimental information). The four probes perform better in lysates than in whole cells. Compounds **7**, **18**, and **27** were used at 10 μM and compound **28** was used at 50 μM. In all cases, samples were irradiated at 360 nm for 1 h on ice.



Supporting Figure S2. (a) Probe **27** (2 μM) does not label HSP60 if the protein has been previously denatured by heat. (b) Probe **28** (10 μM) does not label prohibitin if the protein has been previously denatured by heat. In all cases, samples were irradiated at 365 nm for 1 min using a Stratagene Stratalinker 1800.



Supporting Figure S3. Inhibition of the ATPase activity of recombinant HSP60 by different concentrations of compound **1**. All activity values obtained in the presence of compound are statistically significant ($p<0.05$) vs control without compound.