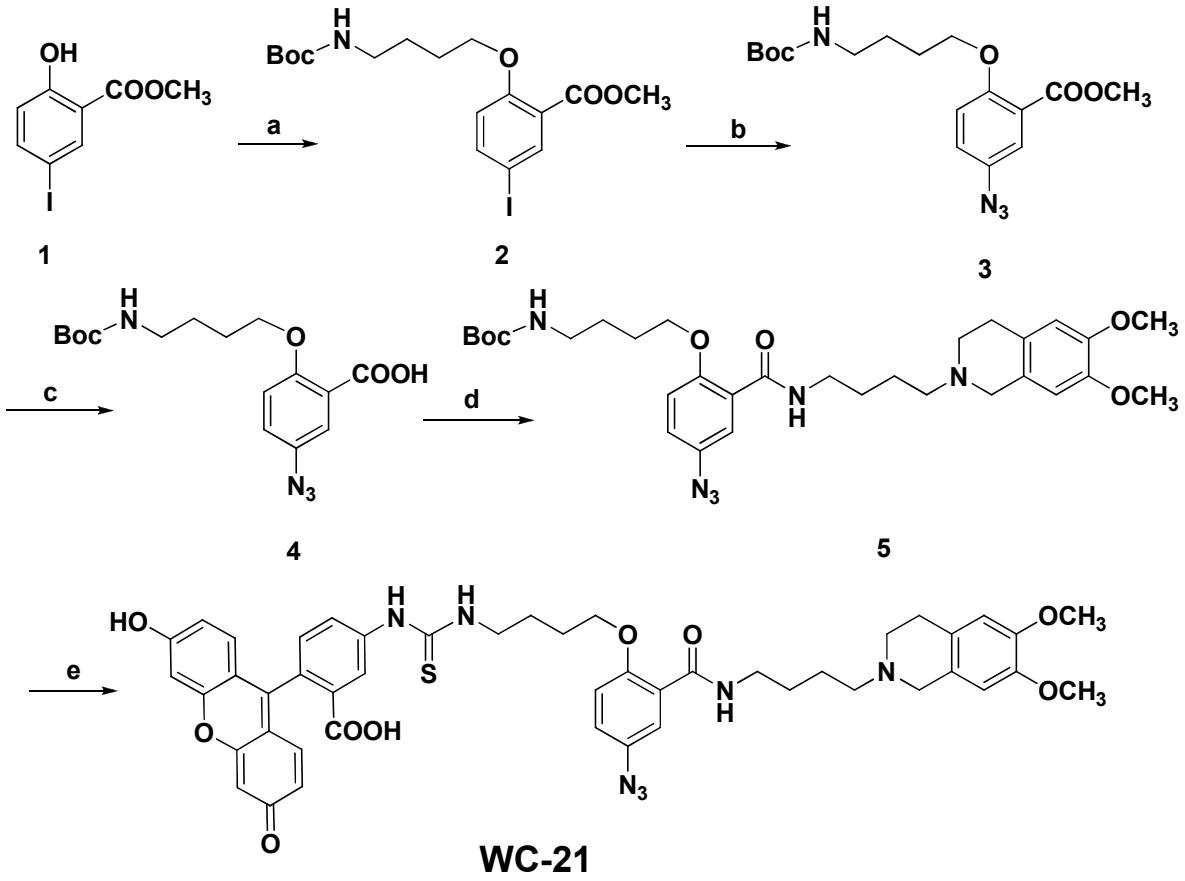
Supplementary information

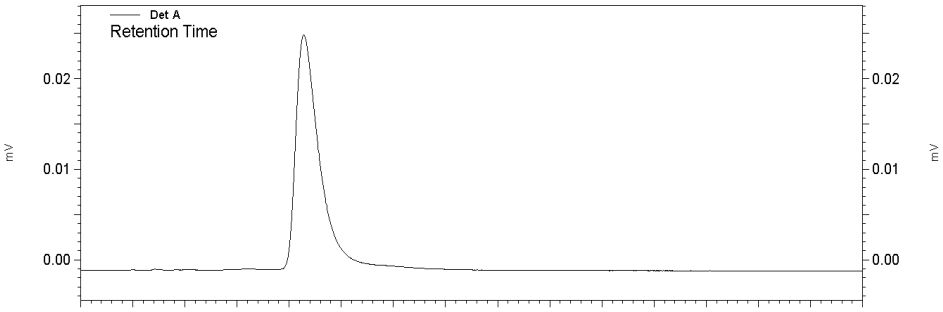
Identification of the PGRMC1 protein complex as the putative sigma-2 receptor binding site.

Xu et al.

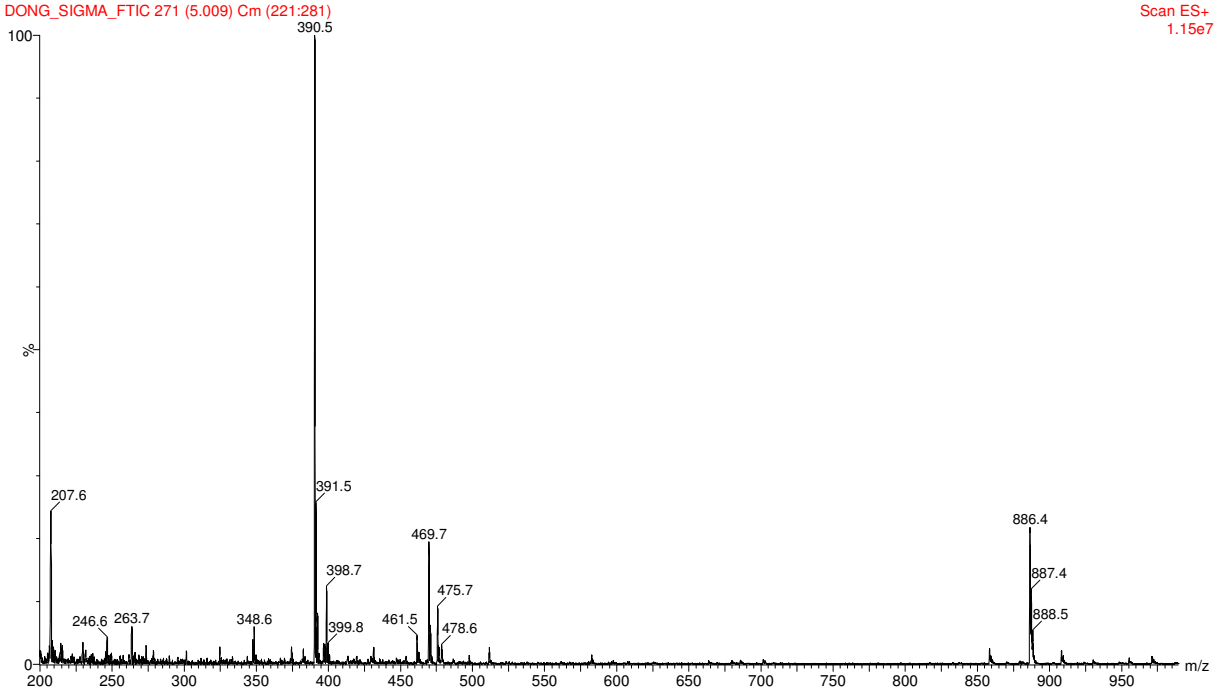


Reagents: (a) tert-butyl 4-bromobutylcarbamate, K_2CO_3, acetone; (b) CuI, sodium azide, L-proline, NaOH, DMSO; (c) NaOH, water, methanol; (d) 4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butan-1-amine, DCC, HOBt; (e) i) TFA; ii) FITC, TEA.

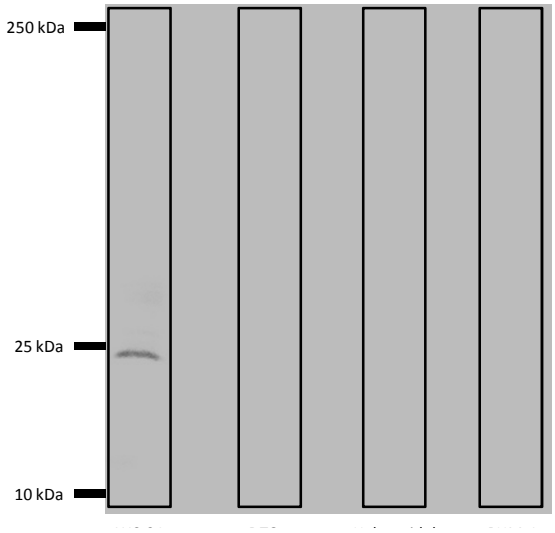
Supplementary Figure S1. Synthesis of the novel photoaffinity probe WC-21. WC-21 was synthesized from compound **1**, the methyl 2-hydroxy-5-iodobenzoate (**1**) and *tert*-butyl 4-bromobutylcarbamate in acetone was heated to reflux with K_2CO_3 as a base to give compound **2**, then the iodo of **2** was substituted with azido to afford **3**. The methyl ester of **3** was hydrolyzed with NaOH in methanol and water, acidified with HCl (1:1) to give the acid **4**, which was coupled with 4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butan-1-amine by using DCC and HOBt as coupling agents to afford **5**. The *N*-Boc group of compound **5** was removed with TFA, and the free base reacted with fluorescein thioisocyanate (FITC) to give the final compound **WC-21**. ^{1}H and ^{13}C NMR spectrums of compounds **2**, **3**, **4**, **5** and **WC-21**were shown in the supplementary Figures S10-S19.



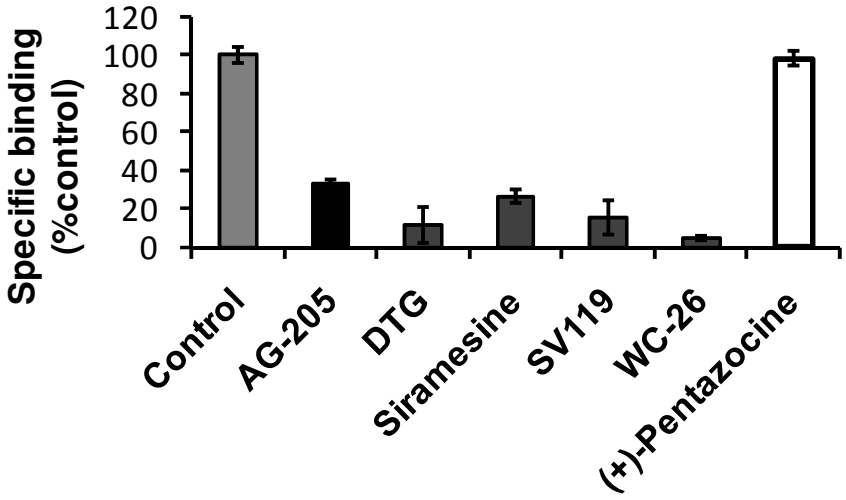
Supplementary Figure S2. Analytical HPLC chromatograph of WC-21. The purity of **WC-21** (>99%) was confirmed by HPLC.



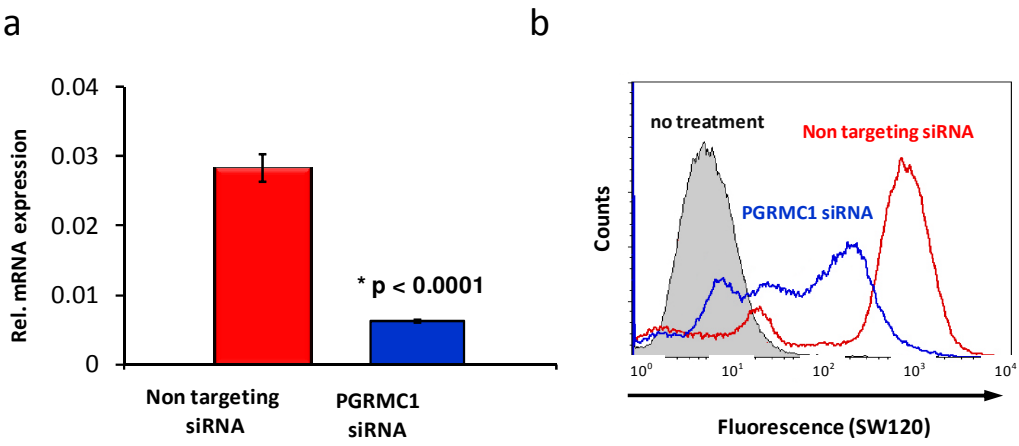
Supplementary Figure S3. WC-21 ESI/Mass Spectrometry. The molecular weight of WC-21 was verified by LCMS.



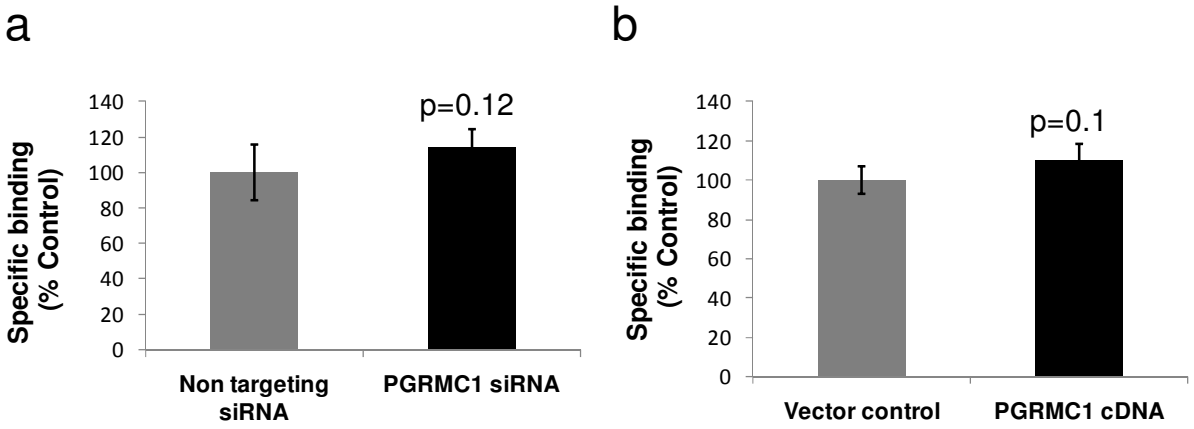
Supplementary Figure S4. Western blot analysis of the 24 kDa photoaffinity labeled protein; specific binding to sigma-2 receptors in hepatic membrane homogenates. Hepatic membranes were photo cross-linked after incubation with **WC-21** alone or together with the known, non-selective sigma-2 receptor ligands DTG and haloperidol or with a selective sigma-2 compound **RHM-1**. Western blot with a HRP-conjugated anti-FITC antibody shows a single protein band (sigma-2 receptor), which can be blocked by sigma-2 receptor ligands. Molecular weight of specifically labeled protein band, ~24 kD, was determined using the protein standards.



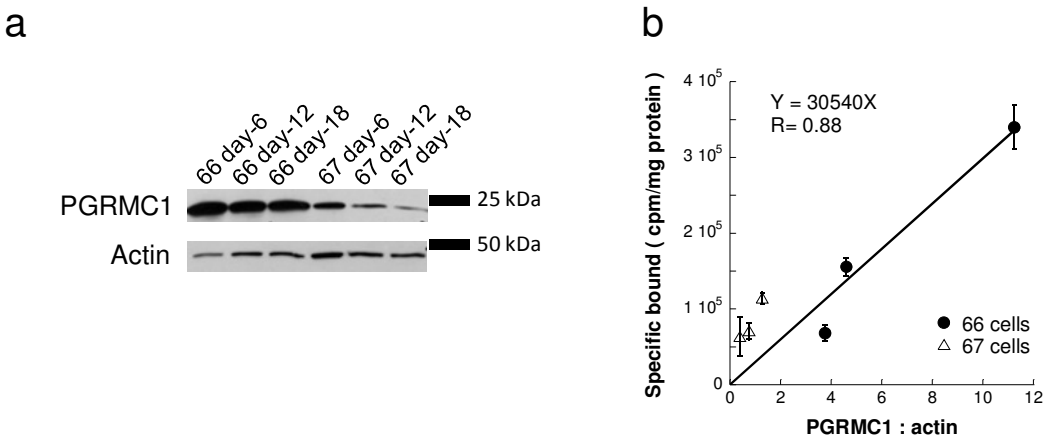
Supplementary Figure S5. Pharmacological profile of [^{125}I]RHM-4 at sigma-2 receptors in mouse mammary 66 tumor cells. Mouse mammary 66 tumor cell homogenates were incubated [^{125}I]**RHM-4** together with the PGRMC1 ligand AG-205, known sigma-2 receptor ligands DTG, **WC-26**, **SV119**, siramesine, or the sigma-1 receptor ligand (+)-pentazocine, [^{125}I]**RHM-4** binding was blocked by AG-205, DTG, **WC-26**, **SV119** and siramesine, but the binding was not blocked by (+)-pentazocine. Data represents means ± SEM of %control specific binding, n=2, sample in triplicate.



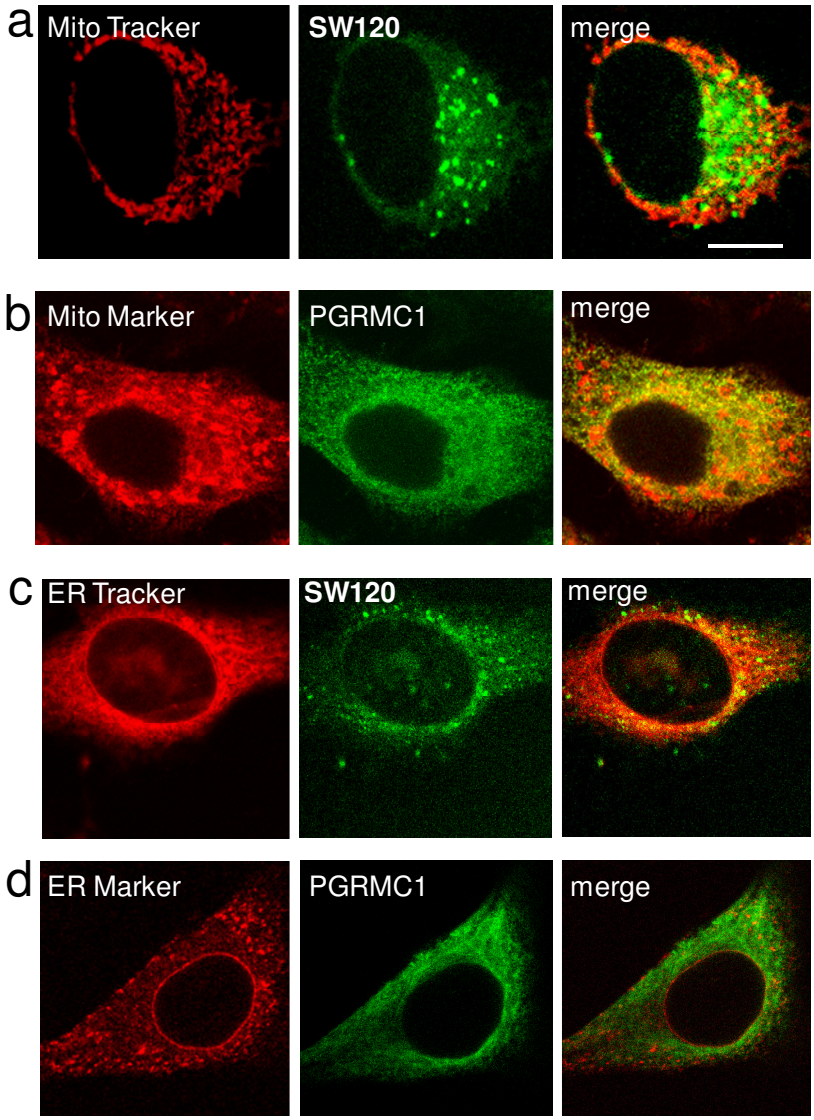
Supplementary Figure S6. Correlation between the sigma-2 receptor binding and PGRMC1 expression levels in human HEK 293T cells. HEK 293T cells were treated with PGRMC1-specific siRNA; a nontargetting scrambled siRNA was used as a control. **a**, Quantitative real-time RT-PCR was performed to assess downregulation of the target mRNA. **b**, cells treated with PGRMC1-specific siRNA showed a large reduction in binding of the fluorophore-containing sigma-2 ligand, **SW120**. Data represents one of the two replicates, sample in triplicate.



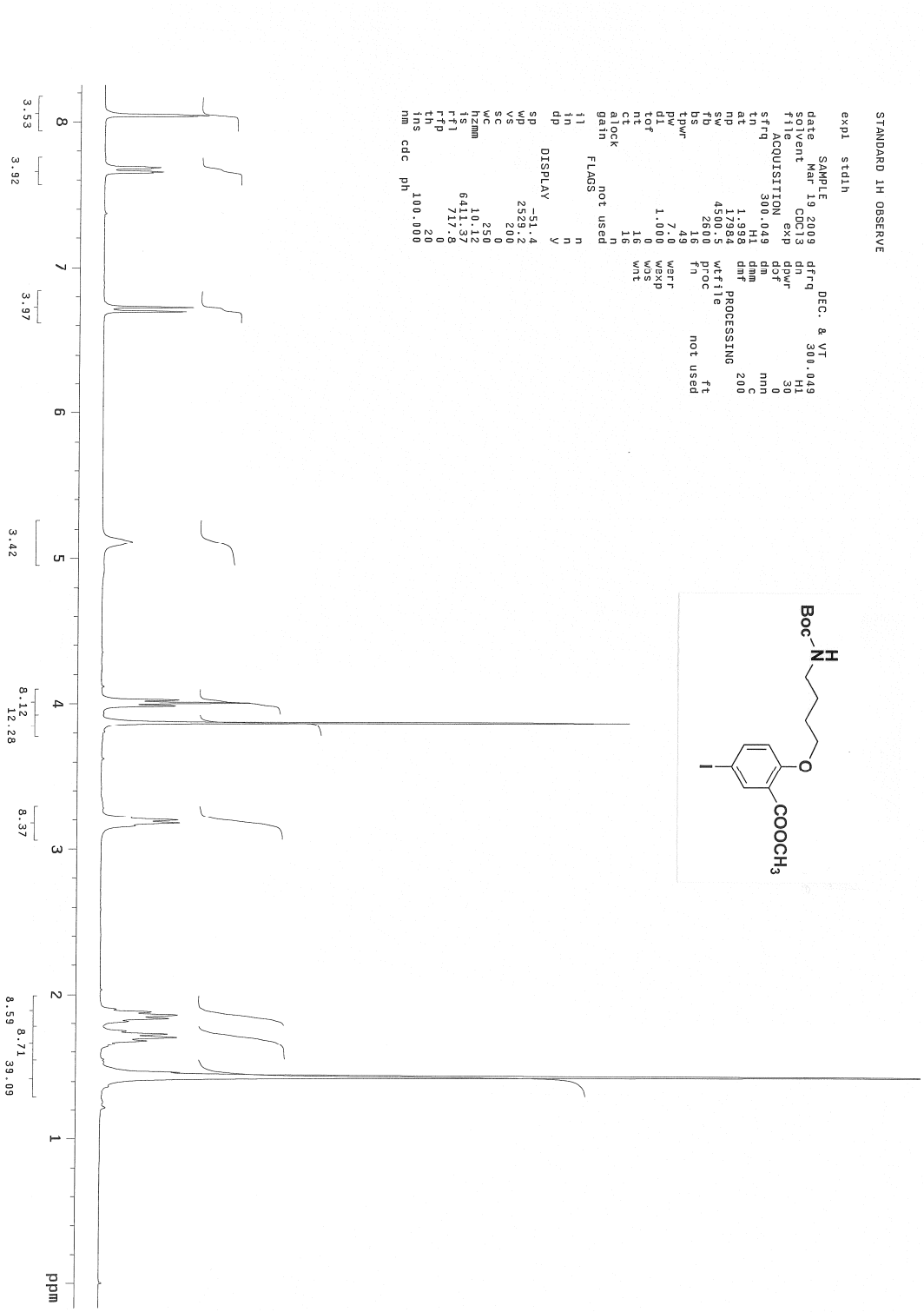
Supplementary Figure S7. Sigma-1 receptor binding activity assayed using [^{3}H](+)-pentazocine in nontargeting siRNA and PGRMC1 siRNA treated cells; and in cells transfected with vector and PGRMC1 cDNA. The bar graphs show that no significant changes in sigma-1 receptor binding activity were observed for the PGRMC1 specific siRNA treated (**a**) or the PGRMC1 cDNA transfected (**b**) HeLa cells relative to nontargeting siRNA treated or the vector transfected controls. Data represents means ± SEM of %control specific binding, n=3, sample in triplicate.



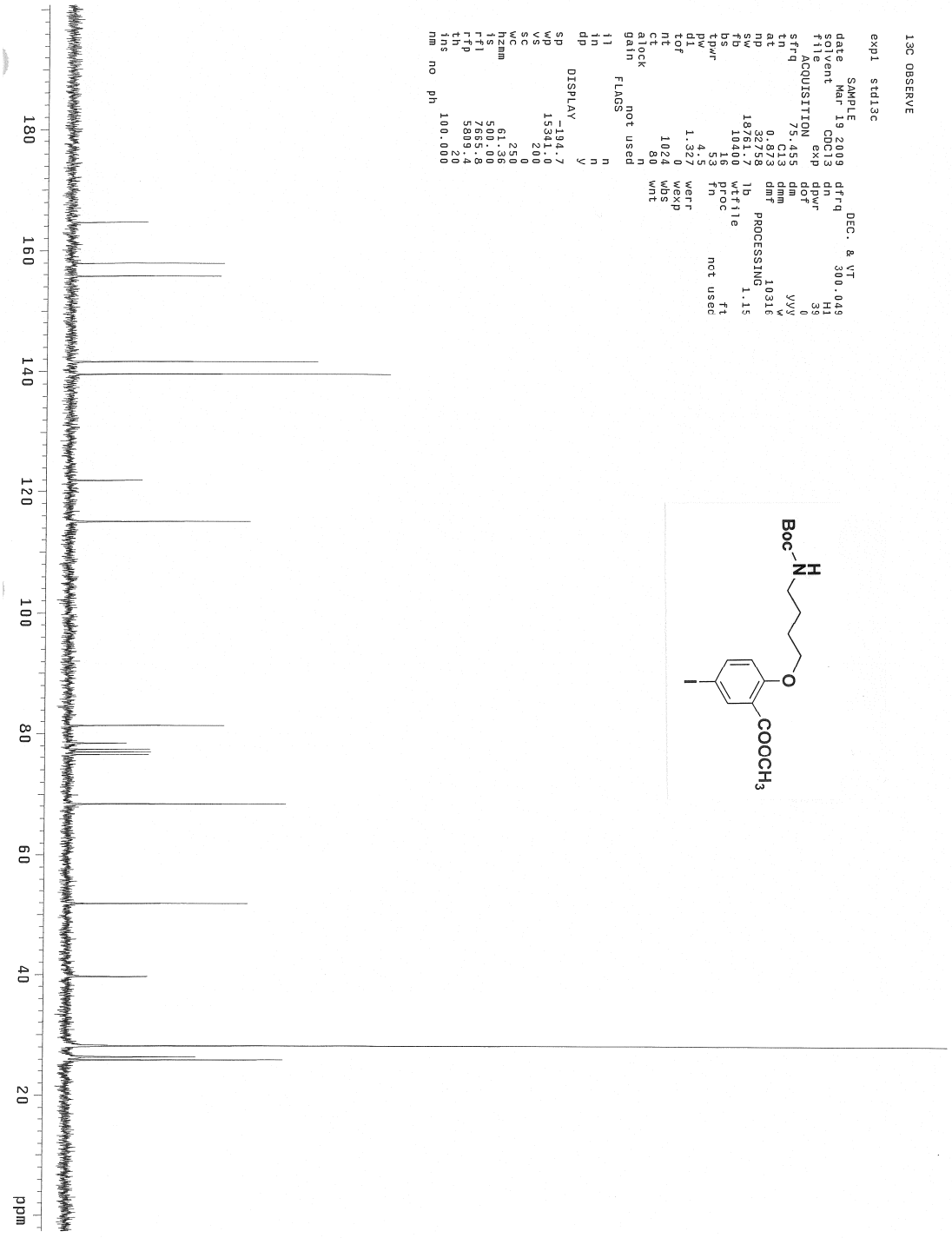
Supplementary Figure S8. Correlation between the sigma-2 receptor binding of [^{125}I]RHM-4 and PGRMC1 protein expression in mouse mammary adenocarcinoma 66 and 67 cells. a, Typical Western blot of PGRMC1 protein expression in lysates of mouse mammary cancer cell lines 66 and 67 after 6 (proliferative cells), 12 (early quiescent cells) and 18 (late quiescent cells) days in culture; actin is the loading control. **b,** Correlation (R=0.88) between [^{125}I]**RHM-4** binding and the PGRMC1: actin ratio. Data represents means ± SEM [^{125}I]**RHM-4** specific binding of two replicates, sample in triplicate.



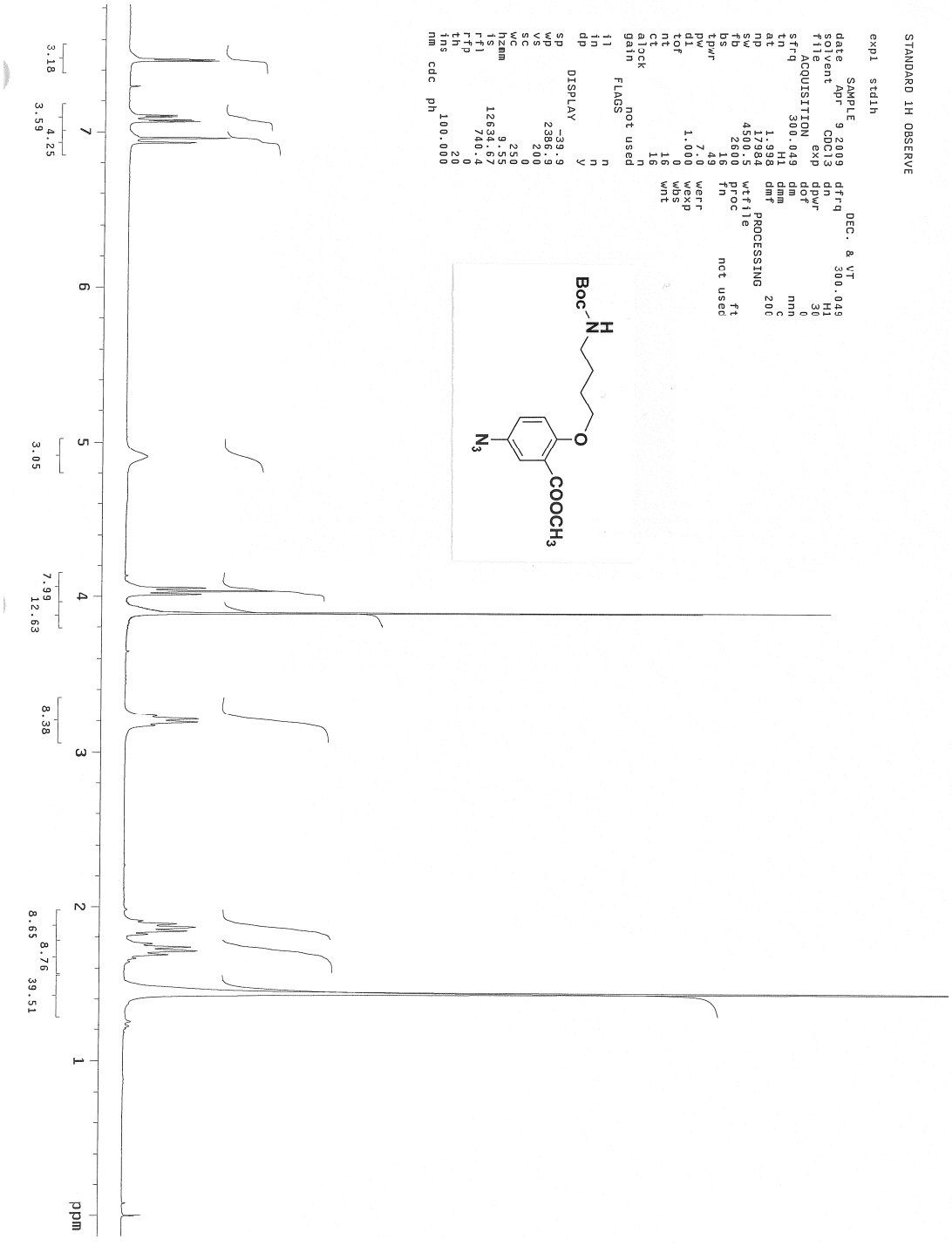
Supplementary Figure S9. Intracellular localization of PGRMC1 labeled with a fluorescent anti-PGRMC1 antibody, and sigma-2 receptors labeled with SW120 in HeLa cells using confocal microscopy, high magnification images. Sigma-2 receptors labeled with **SW120** and PGRMC1 partially colocalized with mitochondria trackers/markers (**a, b**) and endoplasmic reticulum trackers/markers (**c, d**) in HeLa cells. For imaging sigma-2 receptors, live cells were incubated with **SW120** and either MitoTracker Red CMXRos or ER-Tracker Red and then imaged by confocal microscopy. For imaging PRGMC1, cells were fixed and incubated with goat anti-PGRMC1 antibody followed by staining with Mito-marker (anti-COX IV antibody) or ER-marker (anti-GRP78 BiP antibody). Fluorescent labeled HeLa cells were then coverslipped and imaged by confocal microscopy. Scale bar represents 10 μm.



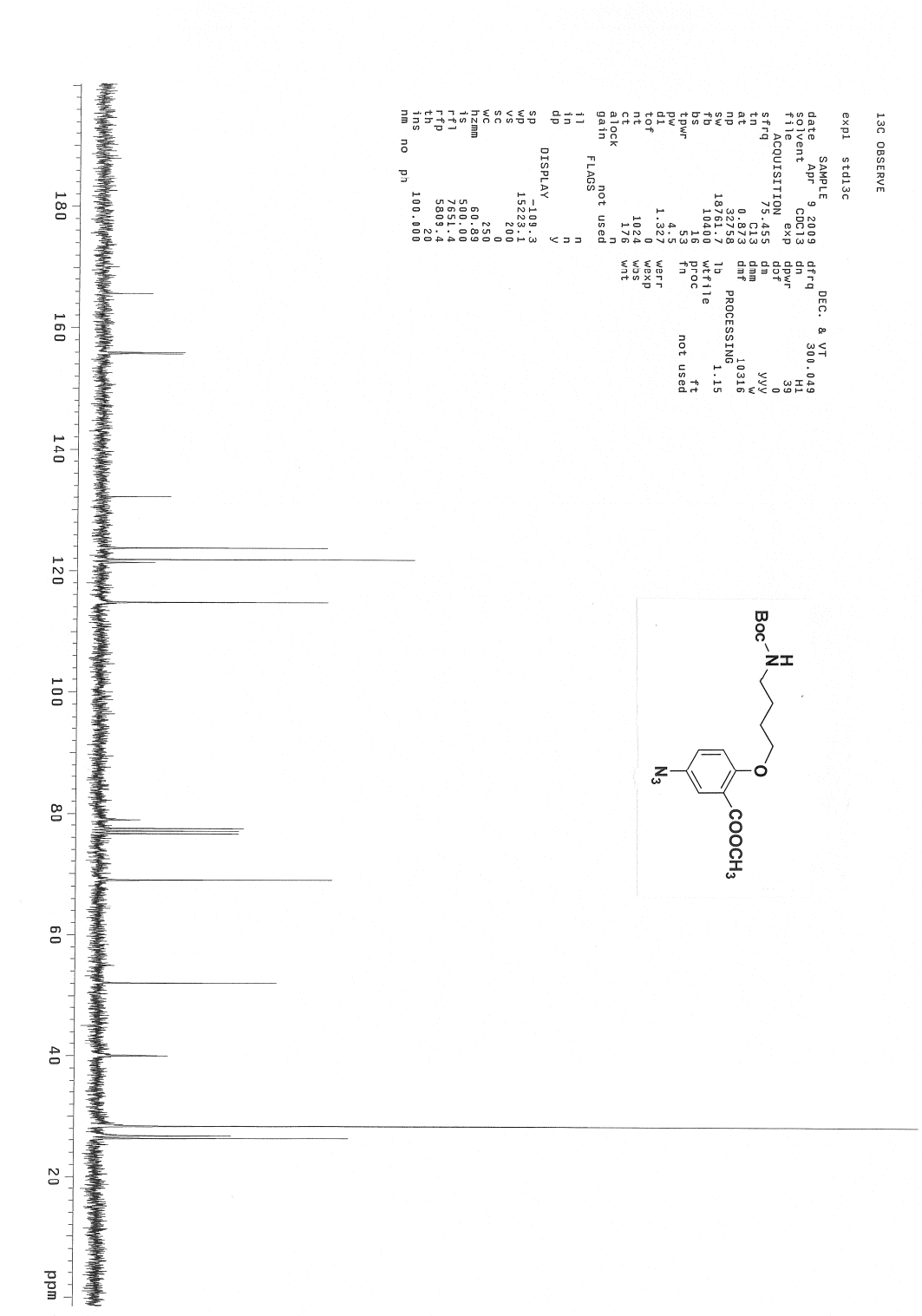
Supplementary Figure S10. 300 MHz ^{1}H NMR ($CDCl_3$) spectrum of compound 2.



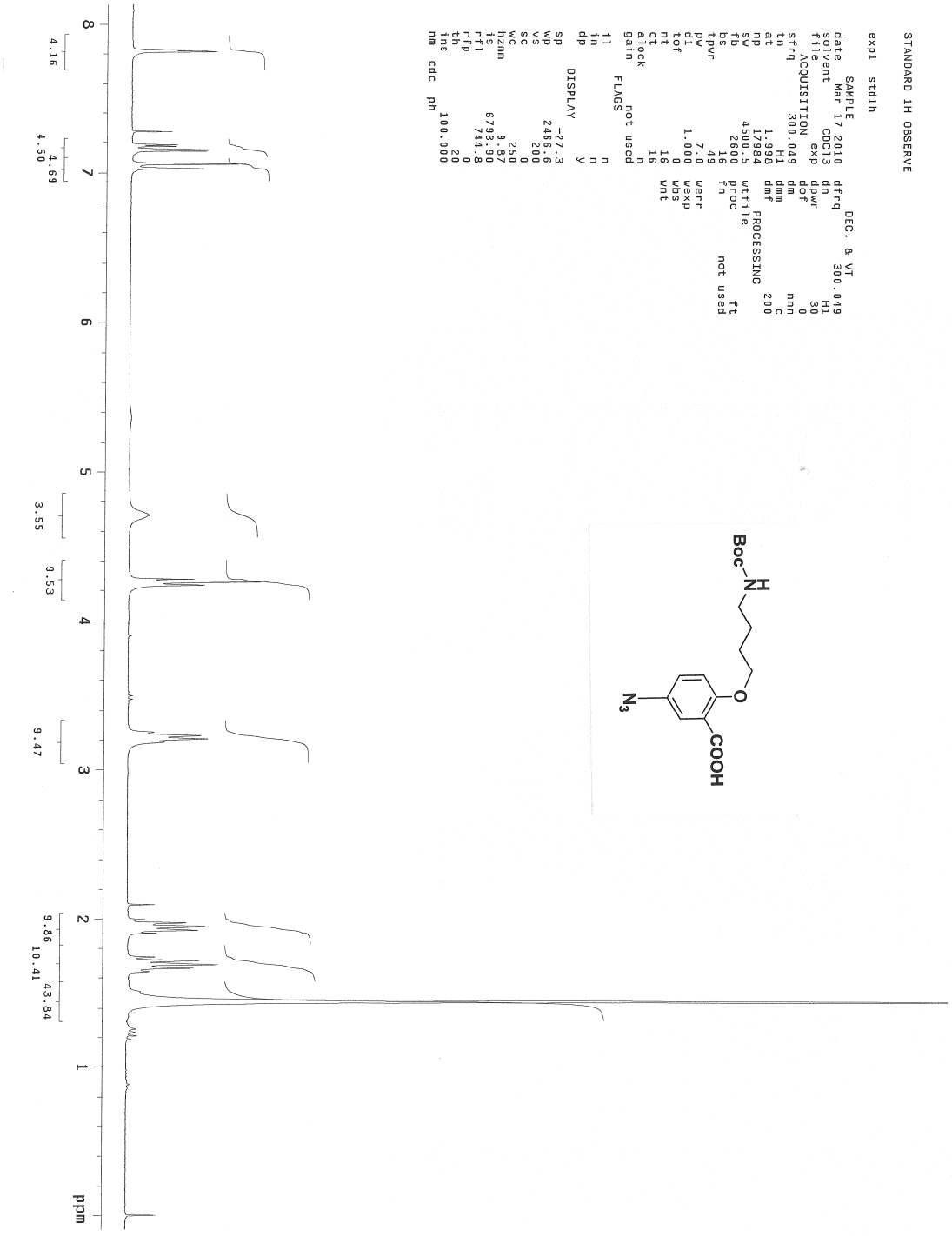
Supplementary Figure S11. 75 MHz ^{13}C NMR ($CDCl_3$) spectrum of compound 2.



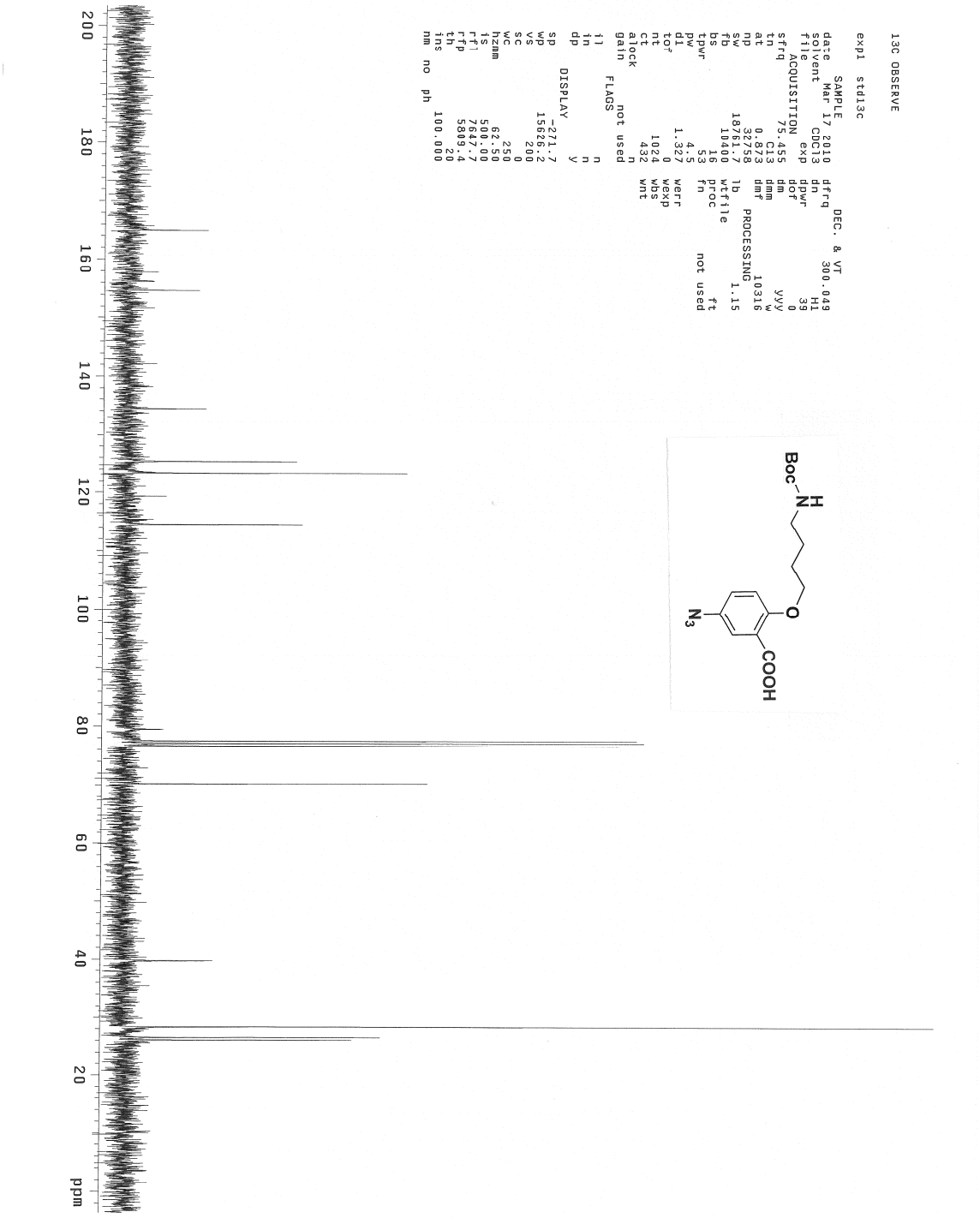
Supplementary Figure S12. 300 MHz ^{1}H NMR ($CDCl_3$) spectrum of compound 3.



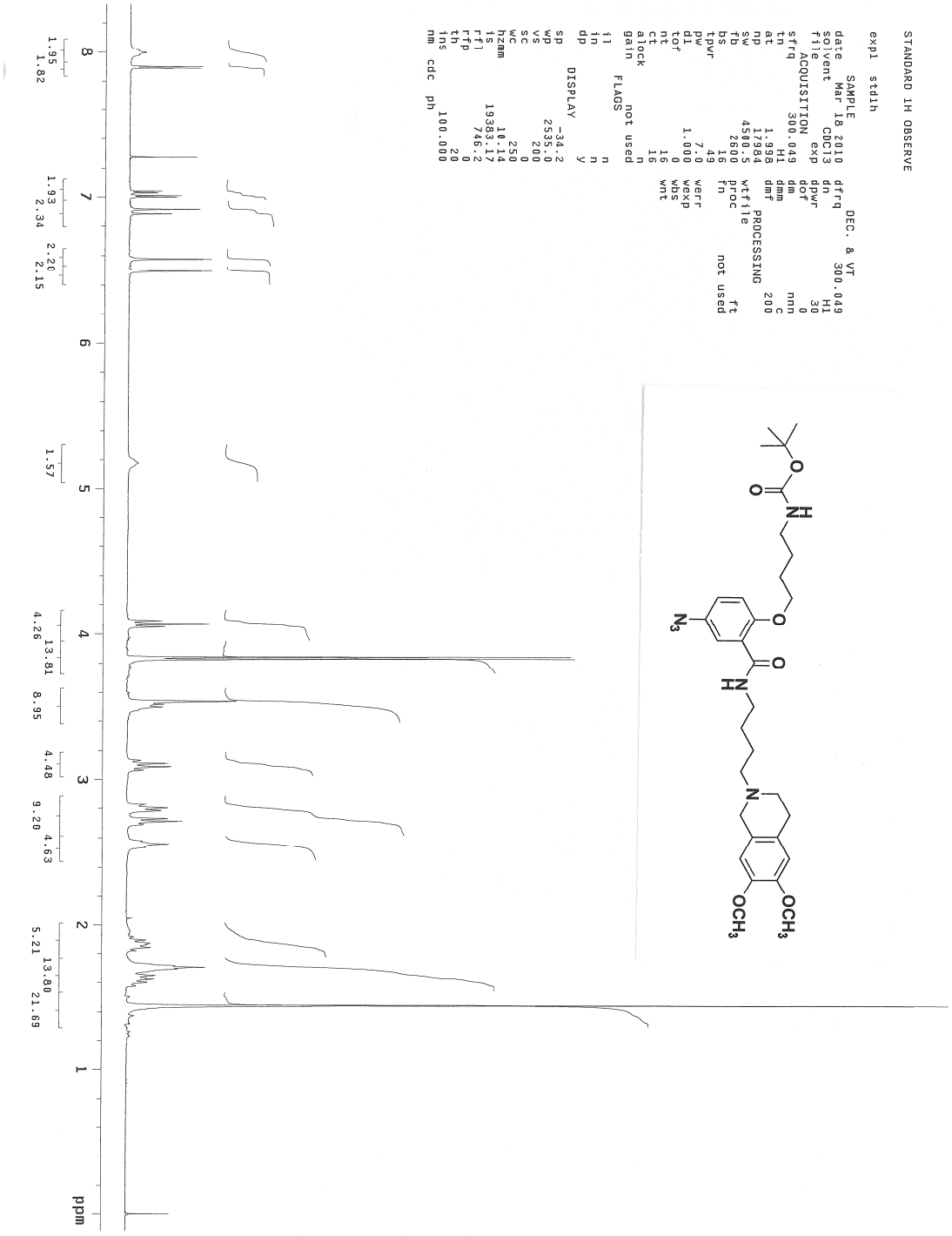
Supplementary Figure S13. 75 MHz ^{13}C NMR ($CDCl_3$) spectrum of compound 3.



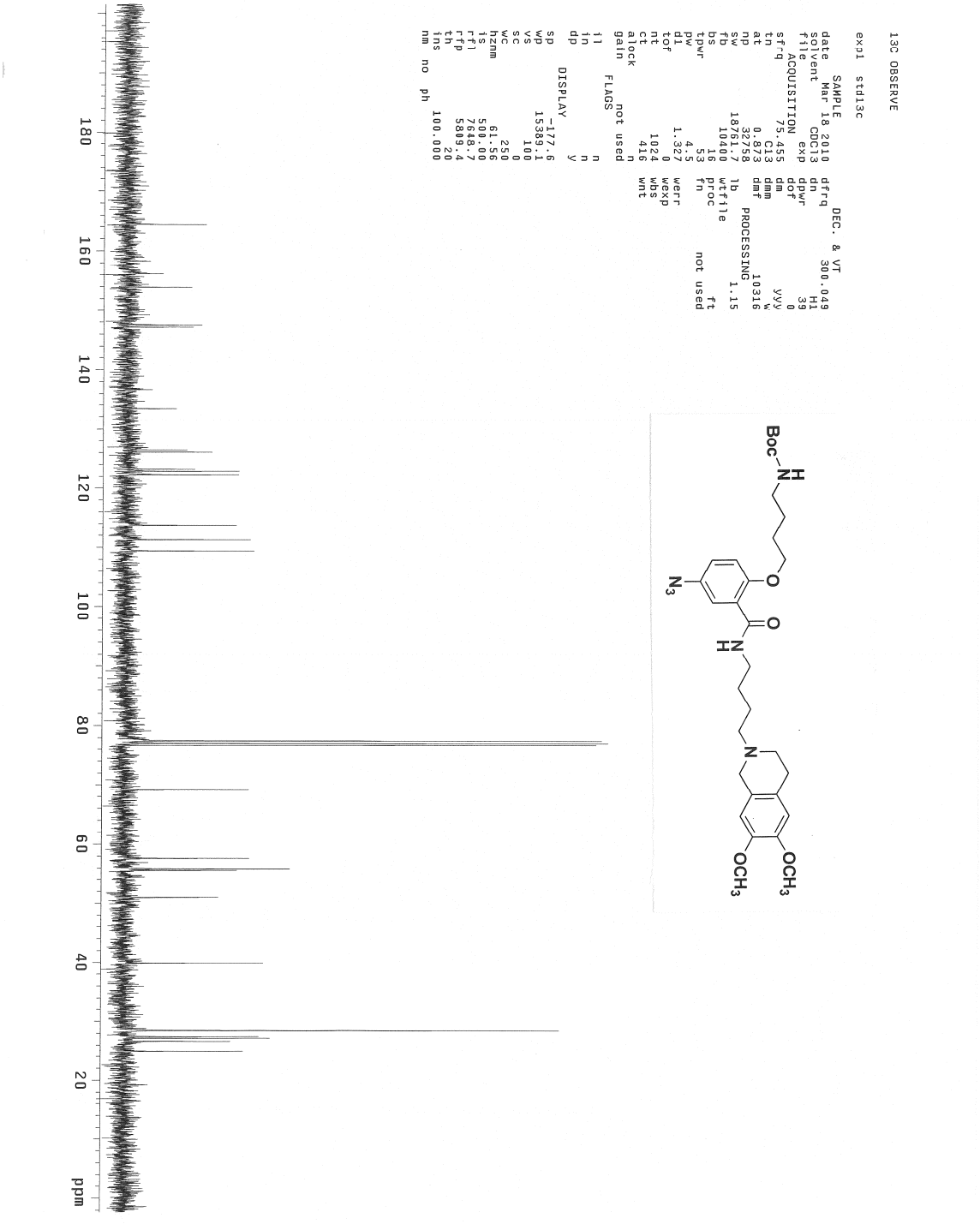
Supplementary Figure S14. 300 MHz ^{1}H NMR ($CDCl_3$) spectrum of compound 4.



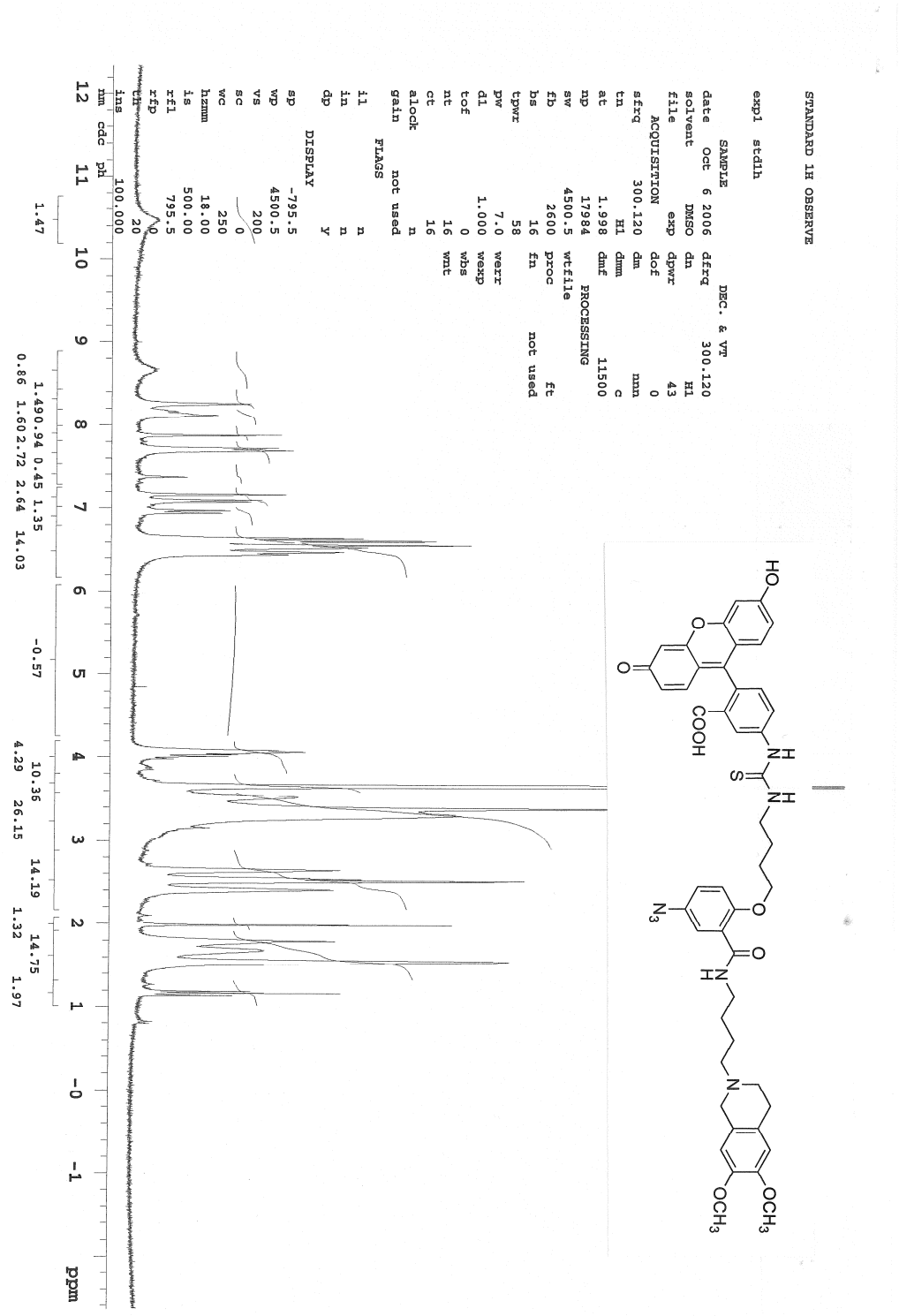
Supplementary Figure S15. 75 MHz ^{13}C NMR ($CDCl_3$) spectrum of compound 4.



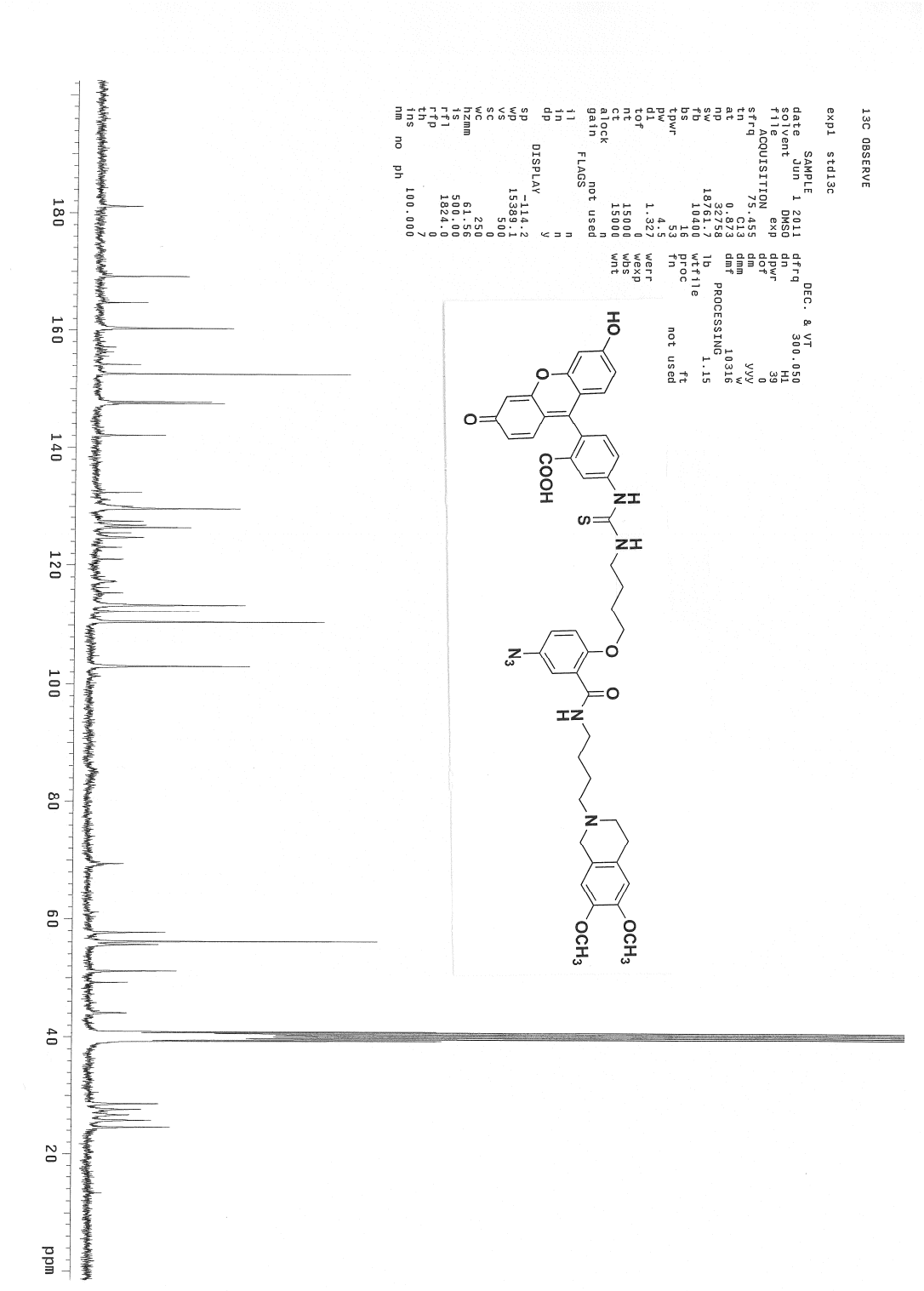
Supplementary Figure S16. 300 MHz ^{1}H NMR ($CDCl_3$) spectrum of compound 5.



Supplementary Figure S17. 75 MHz ^{13}C NMR ($CDCl_3$) spectrum of compound 5.



Supplementary Figure S18. 300 MHz ^{1}H NMR spectrum (DMSO-d_6) of compound WC-21.



Supplementary Figure S19. 75 MHz ^{13}C NMR (DMSO-d_6) spectrum of compound WC-21.

Supplementary Table S1. PCR primers used for fragment amplification of PGRMC1.

Forward primer	Reverse primer
ACCAAAGGCCGCAAATTC	ACCAAAGGCCGCAAATTC

Supplementary Methods

Receptor binding assay

The receptor binding assay was performed with a filtration procedure as previously described [20]. Competitive binding with [^{3}H]**RHM-1** was used to measure the sigma-2 receptor binding affinity of **WC-21**, **RHM-4** and **SW120** in rat liver membrane. [^{3}H](+)-pentazocine was used to determine the sigma-1 receptor binding affinity of **WC-21**, **RHM-4** and **SW120** in guinea pig brain membrane (Figs. 1a, 2a inset, 4a). Briefly, rat liver (sigma-2 receptor assay) or guinea pig brain (sigma-1 receptor assay) membrane homogenates (~300 μg protein) were diluted with 50 mM Tris-HCl, pH 8.0 and incubated with either 1 nM [^{3}H]**RHM-1** (80 Ci/mmol~5 nM; sigma-2 receptor assay) or [^{3}H](+)-pentazocine (34.9 Ci/mmol; sigma-1 receptor assay) in a total volume of 150 μL in 96 well plates at 25 °C. The concentrations of **WC-21**, **RHM-4** and **SW120** ranged from 0.1 nM to 10 μM. After incubation for 1 h, the reactions were terminated by the addition of 150 μL of cold wash buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) using a 96 channel pipette, and the samples were harvested and filtered rapidly into a 96 well fiberglass filter plate (Millipore, Billerica, MA, USA) that had been presoaked with 200 μL of 50 mM Tris-HCl, at pH 8.0 for 1 h. Each filter was washed three times with 200 μL of ice-cold wash buffer, and the bound radioactivity quantified using a Wallac 1450 MicroBeta liquid scintillation counter (Perkin Elmer, Boston, MA, USA). Nonspecific binding was determined from samples in the presence of 10 μM haloperidol.

To determine the pharmacological profile of [^{125}I]**RHM-4** at sigma-2 receptors in the human HeLa and mouse mammary 66 cells, cell homogenates (~100 μg protein) were incubated with 0.5 nM of [^{125}I]**RHM-4** together with the PGRMC1 ligand AG-205 (10 μM), known sigma-2 receptor ligands DTG (1 μM), **WC-26** (100 nM), **SV119** (100 nM), and siramesine (100 nM), or with the sigma-1 receptor ligand (+)-pentazocine (100 nM). The competitive [^{125}I]**RHM-4** binding assay was carried out using the filtration procedure described above; the filters were punched out and the radioactivity of the bound protein on each filter was quantified via a Packard gamma counter (Perkin Elmer, Boston, MA, USA).

Direct binding assays were performed as above with 3.5 nM [^{125}I]**RHM-4** but without the competing ligands to determine the sigma-2 receptor binding activity in HeLa cell membranes (~100 μg protein) prepared from either PGRMC1 knockdown cells treated with PGRMC-1 siRNA or from control cells treated with nontargeting siRNA. Direct binding assays were also performed for the PGRMC1 overexpressed HeLa cells treated with PGRMC1-cDNA and for vector treated control cells. The radioligand [^{3}H](+)-pentazocine (8 nM) was used for binding assays to measure the sigma-1 receptor binding activity in those HeLa cells.

Western blot analysis of PGRMC1

Human HeLa and mouse mammary 66 tumor cells were lysed in radioimmunoprecipitation assay buffer [50 mM Tris, 150 mM sodium chloride, 1.0 mM EDTA, 1% Nonidet P40, and 0.25% SDS (pH 7.0)], supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor cocktail 1 (Sigma Chemical Co., St. Louis, MO, USA). The cells were sonicated briefly, centrifuged at 13,000 × g for 20 min at 4 °C, and the supernatant collected. The protein concentration was determined using a Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Lysates containing 30 μg of protein were run on a 12% acrylamide gel and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The PVDF membrane was incubated with 5% nonfat dry milk for 1 h at room temperature, then overnight with a rabbit anti-PGRMC1 antibody (1:600) at 4 °C, and finally with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) at a 1:3,000 dilution. Actin was identified using an anti-β-actin antibody (Cell Signaling Technology, Danvers, MA, USA) at a 1:1,000 dilution and horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody. The SuperSignal West Pico Chemiluminescent Substrate assay kit (Pierce Biotechnology, Inc. Rockford, IL, USA) was used to detect the secondary antibody. Quantitative analysis was performed using ImageJ software (National Institute of Health, USA), and the integrated intensity of the protein band determined.

Real time PCR for PGRMC1 mRNA expression

HEK 293T cells were grown to 70-80% confluence in 6-well plates. 5 μL of the Fugene-6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) was mixed in 100 μL of Opti-MEM. An appropriate amount of the siRNA (Dharmacon, Inc., Chicago, IL, USA) was combined with Opti-MEM to create a final concentration of 200 nM in 500 μL of Opti-MEM. After 72 h cells were treated with 50 nM of SW-120 for 30 min, assayed by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA), and the data analyzed with the CellQuest software (BD Biosciences, San Jose, CA, USA).

Real-time quantitative PCR was performed to validate mRNA expression knockdown. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The amount of RNA was measured by spectrophotometer. RNA (2 μg) was transcribed into cDNA using SupersScript™ III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Real-time quantitative PCR was performed in triplicate with PGRMC1-specific primers (TaqMan® Gene Expression Assay, Hs00198499_m1, amplicon size 54; forward primer 5'-ACCAAAGGCCGCAAATTC-3', reverse primer 5'-TCCAGCAAAGACCCCATAC-3', Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's directions. Fragment amplification was performed on an ABI Prism™ 7500 Sequence Detector. The expression of PGRMC1 was standardized relative to the expression of β-Actin.

Synthesis of the novel photoaffinity probe WC-21

All chemicals were obtained from standard commercial sources and used without further purification. All reactions were carried out by standard air-free and moisture-free techniques under an inert argon atmosphere with dry solvents unless otherwise stated. Flash column chromatography was conducted using Scientific Adsorbents, Inc. silica gel, 60Å, "40 Micron Flash" (32-63 μm). Melting points were determined using MEL-TEMP 3.0 apparatus and are uncorrected. Routine ^{1}H and ^{13}C NMR spectra were recorded at 300 MHz on a Varian Mercury-VX spectrometer. All chemical shifts were reported as a part per million (ppm) downfield from tetramethylsilane (TMS). All coupling constants (*J*) are given in Hertz (Hz). Splitting patterns are typically described as follows: s, singlet; d, doublet; t, triplet; m, multiplet. ESI/MS was

performed on a Waters ZQ 4000 single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) LC-MS interface. High performance liquid chromatography (HPLC) was performed with an ultraviolet detector operating at 290 nm. Alltech Econosil C18 250 × 20 mm preparative column and Altech Econosil C18 250 × 4.6 mm analytical column were used for purification and analysis respectively.

Methyl 2-(4-(tert-butoxycarbonylamino)butoxy)-5-iodobenzoate (2) A mixture of 4-(Boc-amino)butyl bromide (2.50 g, 9.92 mmol), methyl 5-iodosalicylate **1** (3.11 g, 11.19 mmol), and potassium carbonate (2.00 g, 14.47 mmol) in acetone (75 mL) was stirred at reflux for 8 h. The acetone was evaporated under reduced pressure, then ethyl acetate (100 mL) was added, and the mixture was washed with water (50 mL × 2), saturated NaCl (50 mL) and dried over Na_2SO_4. After evaporation of the ethyl acetate under reduced pressure, the crude pruduct was purified by flash column chromatography with hexane: ether (1:1) to afford 2.34 g (53%) of **2** as a white solid, mp 76.3-76.9 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04 (d, *J* = 1.8 Hz, 1H, Ar-*H*), 7.67 (dd, *J* = 8.7 Hz, 2.1 Hz, 1H, Ar-*H*), 6.71 (d, *J* = 8.7 Hz, 1H, Ar-*H*), 5.11 (br, 1H, N-*H*), 4.00 (t, *J* = 6.0 Hz, 2H, OC*H*$_2$), 3.87 (s, 3H, OC*H*$_3$), 3.19 (m, 2H, NH-C*H*$_2$), 1.86 (m, 2H, OCH$_2$C*H*$_2$), 1.71 (m, 2H, NHCH$_2$C*H*$_2$), 1.43 (s, 9H, tBu-C*H*$_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.8, 158.0, 155.8, 141.6, 139.6, 121.9, 115.1, 81.3, 78.4, 68.3, 51.8, 39.6, 28.1, 26.3, 25.8.

Methyl 5-azido-2-(4-(tert-butoxycarbonylamino)butoxy)benzoate (3) A mixture of **2** (900 mg, 2.0 mmol), sodium azide (390 mg, 6.0 mmol), *L*-proline (46 mg, 0.4 mmol), NaOH (16 mg, 0.4 mmol), and CuI (38 mg, 0.2 mmol) in DMSO (12 mL) was heated to 60 °C for 8 h. Ethyl acetate (75 mL) was added to the cooled reaction mixture which was washed with water (50 mL × 2), saturated NaCl (50 mL), and dried over Na_2SO_4. After evaporation of the ethyl acetate under reduced pressure, the crude pruduct was purified by flash column chromatography with dichloromethane: hexane: ether (3:3:1) to afford 714 mg (98%) of **3** as a white solid, mp 58.1-58.7 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.47 (d, *J* = 3.0 Hz, 1H, Ar-*H*), 7.09 (dd, *J* = 9.0 Hz, 3.0 Hz, 1H, Ar-*H*), 6.94 (d, *J* = 8.7 Hz, 1H, Ar-*H*), 4.90 (s, 1H, N-*H*), 4.03 (t, *J* = 5.1 Hz, 2H, OC*H*$_2$), 3.89 (s, 3H, OC*H*$_3$), 3.20 (m, 2H, NH-C*H*$_2$), 1.87 (m, 2H, OCH$_2$C*H*$_2$), 1.72 (m, 2H, NHCH$_2$C*H*$_2$), 1.43 (s, 9H, tBu-C*H*$_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.6, 156.0, 155.7, 132.1, 123.7, 121.8, 121.3, 114.7, 78.8, 69.0, 52.0, 39.9, 28.3, 26.6, 26.2.

5-Azido-2-(4-(tert-butoxycarbonylamino)butoxy)benzoic acid (4) A mixture of **3** (714 mg, 1.96 mmol) and lithium hydroxide (56 mg, 2.33 mmol) in methanol (10 mL) and water (2 mL) was stirred overnight. The methanol was evaporated under reduced pressure, water (10 mL) was added, and impurities were extracted with ethyl acetate (50 mL). The mother liquor was then acidified with HCl (1:1) to pH=2, then extracted with ethyl acetate (50 mL); the ethyl acetate solution was washed with water (30 mL), saturated NaCl (30 mL), and dried over Na_2SO_4. After evaporation of the ethyl acetate under reduced pressure, the product was dried overnight to afford 651 mg (95%) of **4** as a white solid, mp 113.5-114.1 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82 (d, *J* = 2.7 Hz, 1H, Ar-*H*), 7.16 (dd, *J* = 8.9 Hz, 2.7 Hz, 1H, Ar-*H*), 7.04 (d, *J* = 8.7 Hz, 1H, Ar-*H*), 4.71 (s, 1H, N-*H*), 4.25 (t, *J* = 6.6 Hz, 2H, OCH_2), 3.22 (m, 2H, NH-CH_2), 1.95 (m, 2H, OCH_2CH_2), 1.69 (m, 2H, $NHCH_2CH_2$), 1.44 (s, 9H, tBu-CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.9, 154.6, 134.3, 125.3, 123.3, 119.2, 114.4, 79.4, 70.2, 39.3, 28.3, 26.5, 26.1.

***tert*-Butyl 4-(4-azido-2-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butylcarbamoyl)phenoxy)butylcarbamate (5)** Into a solution of **4** (651 mg, 1.86 mmol) and 4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butan-1-amine (590 mg, 2.23 mmol) in dichloromethane (25 mL) was added *N,N'*-dicyclohexylcarbodiimide (DCC) (575 mg, 2.79 mmol) and hydroxybenzotriazole (HOBt) (251 mg, 1.86 mmol) at 0 °C. The mixture was stirred overnight, then the dichloromethane was evaporated under reduced pressure. Ethyl acetate (75 mL) was added, and the mixture was washed with saturated $NaHCO_3$ (50 mL), water (50 mL), and saturated NaCl (50 mL), then dried over Na_2SO_4. After evaporation of the ethyl acetate under reduced pressure, the crude product was purified by flash column chromatography with ethyl acetate: methanol (10:1) to afford 670 mg (60%) of **5** as a white solid, mp 88.1-88.7 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.00 (s, 1H, CON-*H*), 7.90 (d, *J* = 3.0 Hz, 1H, Ar-*H*), 7.02 (dd, *J* = 8.7 Hz, 3.0 Hz, 1H, Ar-*H*), 6.90 (d, *J* = 8.7 Hz, 1H, Ar-*H*), 6.58 (s, 1H, Ar-*H*), 6.49 (s, 1H, Ar-*H*), 5.18 (s, 1H, BocN-*H*), 4.07 (t, *J* = 6.3 Hz, 2H, OCH_2), 3.84 (s, 3H, CH_3), 3.83 (s, 3H, CH_3), 3.54 (s, 2H, Ar-CH_2N), 3.52 (m, 2H, CONHCH_2), 3.09 (m, 2H, BocNHCH_2), 2.80 (t, *J* = 7.2 Hz, 2H, NCH_2), 2.71 (t, *J* = 5.1 Hz, 2H, Ar-CH_2), 2.56 (m, 2H, NCH_2), 1.86 (m, 2H, OCH_2CH_2), 1.70 (m, 4H, NCH_2CH_2), 1.62 (m, 2H, $NHCH_2CH_2$), 1.44 (s, 9H, tBu-CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.3, 156.1, 156.0, 153.9, 147.4, 147.1, 133.3, 126.4, 126.0, 123.2, 122.8, 122.2, 113.7, 111.2, 109.4, 69.1, 57.6, 55.8, 55.6, 51.0, 39.8, 28.4, 27.4, 27.1, 26.6, 24.8.

5-(3-(4-(4-Azido-2-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butylcarbamoyl)phenoxy)butyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (WC-21) A solution of **5** (297 mg, 0.5 mmol) in dichloromethane (5 mL) was added trifluoroacetic acid (TFA) (5 mL) at 0 °C. The mixture was stirred 3 h at 0 °C. The solvent was evaporated under reduced pressure, then triethylamine (TEA) (1 mL) and fluorescein thioisocyanate (FITC) (195 mg, 0.5 mmol) were added to a solution of the TFA salt in dimethylformamide (10 mL) and the mixture was stirred overnight. Ethyl acetate (100 mL) was added, the mixture was washed with water (50 mL x 3), saturated NaCl (50 mL), and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the crude product was purified with ethyl acetate: methanol (3:1) to afford 211 mg (48%) of **WC-21** as an orange solid, which was further purified by HPLC, mp 158.1-159.7 °C. LCMS: Calcd for Chemical Formula: $C_{47}H_{48}N_7O_9S$ (M+H), Exact Mass: 886.3, Found: 886.3. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.47 (s, 1H, COO*H*), 8.68 (s, 1H, N*H*), 8.25 (s, 1H, O*H*), 8.11 (m, 1H, Ar-*H*), 7.87 (d, J = 2.1 Hz, 1H, Ar-*H*), 7.70 (d, J = 8.4 Hz, 2H, Ar-*H*), 7.16 (s, 1H, N*H*), 7.08 (d, J = 8.1 Hz, 1H, Ar-*H*), 6.95 (d, J = 8.4 Hz, 1H, Ar-*H*), 6.64-6.44 (m, 9H, Ar-*H,* N*H*), 4.06 (m, 2H, OCH_2), 3.65 (s, 3H, CH_3), 3.63 (s, 3H, CH_3), 3.51 (s, 2H, Ar-CH_2N), 3.29 (m, 2H, CONHCH_2), 2.63 (m, 2H, ArCH_2), 2.48 (m, 4H, NCH_2), 2.39 (m, 2H, NCH_2), 1.78(m, 2H, OCH$_2$CH_2), 1.68 (m, 2H, NCH$_2$CH_2), 1.54 (m, 4H, NCH$_2$CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.1, 169.2, 164.7, 160.4, 154.2, 152.6, 147.8, 147.5, 142.1, 132.3, 129.6, 127.4, 126.7, 126.3, 125.4, 124.7, 123.0, 121.0, 115.4, 113.3, 112.3, 110.5, 102.9, 69.4, 57.7, 56.0, 55.5, 51.1, 49.2, 44.0, 28.6, 27.6, 26.7, 25.8, 24.5.

Preparative HPLC purification of WC-21

290 nm UV, Preparative column: Alltech Econosil C18 250×20mm,

Mobile phase: A: 40% Acetonitrile, 60% water, 0.1% TFA

B: 60% Acetonitrile, 40% water, 0.1% TFA

0----------2 min----------32min

A(100%)--------A(100%)----------B(100%)

10mL/min-------20mL/min

Analytical HPLC chromatograph of WC-21

290 nm UV, Analytical column: HPLC-Alltech Econosil C18 250×4.6mm, 10μ,

Mobile phase: 60% Acetonitrile, 40% water, 0.1% TFA, 2 ml/min