Supplementary Information

Structural basis for CCR6 modulation by allosteric antagonists

David Jonathan Wasilko[1], Brian S. Gerstenberger[2], Kathleen A. Farley[1], Wei Li[3], Jennifer Alley[3], Mark E. Schnute[2], Ray J. Unwalla[2], Jorge Victorino[1], Kimberly K. Crouse[3], Ru Ding[3], Parag V. Sahasrabudhe[1], Fabien Vincent[1], Richard K. Frisbie[1], Alpay Dermenci[4], Andrew Flick[4], Chulho Choi[4], Gary Chinigo[4], James J. Mousseau[4], John I. Trujillo[4], Philippe Nuhant[2], Prolay Mondal[4], Vincent Lombardo[4], Daniel Lamb[5,6], Barbara J. Hogan[5,6], Gurdeep Singh Minhas[5], Elena Segala[5], Christine Oswald[5], Ian W. Windsor[1], Seungil Han[1], Mathieu Rappas[5,6], Robert M. Cooke[5], Matthew F. Calabrese[1], Gabriel Berstein[3], Atli Thorarensen[2], and Huixian Wu[1]*

[1]Discovery Sciences, Medicine Design, Pfizer Inc., Groton, CT 06340, USA

[2]Medicine Design, Pfizer Inc., Cambridge, MA 02139, USA

[3]Inflammation and Immunology Research, Pfizer Inc., Cambridge, MA 02139, USA

[4]Medicine Design, Pfizer Inc., Groton, CT 06340, USA

[5]Sosei Heptares, Steinmetz Building, Granta Park, Great Abington, Cambridge CB21 6DG, United Kingdom

[6]Current address: Nxera Pharma UK Limited, Steinmetz Building, Granta Park, Great Abington, Cambridge CB21 6DG, United Kingdom

*Correspondence to: huixian.wu@pfizer.com

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Supplementary Methods

Preparation of [^{3}H]-SQA1 and synthesis of different OXM analogues, OXM1, OXM2, OXM3, and OXM4.

Preparation of [^{3}H]-SQA1.

General synthetic conditions: Reagents and solvents were obtained from Aldrich and/or Alfa and were used without further purification. Solvents were commercial anhydrous grades and were used as received. All reactions were conducted with continuous magnetic stirring under an atmosphere of dry nitrogen unless otherwise specified. Chromatographic purifications were affected by medium pressure ("flash") chromatography on silica gel unless noted otherwise. Compounds were characterized by proton (1H) NMR spectra using Bruker spectrometers and are reported in parts per million (ppm) relative to the residual resonances of the deuterated solvent. All ^{13}C NMR spectra were proton decoupled.

(*S*)-*N*-((4-bromofuran-2-yl)methylene)-2-methylpropane-2-sulfinamide: To a solution of 4-bromo-2-furaldehyde (1.0 g, 5.7 mmol) in THF (29 ml) was added $Ti(OEt)_4$ (2.78 ml, 11.4 mmol) at ambient temperature. The mixture was stirred for 20 min and (*S*)-2-methyl-2-propanesulfinamide (727 mg, 6.0 mmol) was added and the resulting mixture was stirred for 16 h. The reaction was quenched with water (10 ml). The mixture was filtered, and the filter cake was washed with ethyl acetate (20 ml × 2). The combined filtrate was evaporated under reduced pressure to afford crude residue that was purified by column chromatography (90:10 heptanes:ethyl acetate to 100% ethyl acetate) to afford the title compound (1.2 g, 76%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28 (s, 9H), 7.06 (s, 1H), 7.65 (s, 1H), 8.35 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.6, 58.2, 102.2, 120.1, 144.7, 149.4, 151.2; LCMS (ESI): >99% (UV), $[M+H]^+$ m/z 278.1

(*S*)-*N*-((*R*)-1-(4-bromofuran-2-yl)allyl)-2-methylpropane-2-sulfinamide: To a solution of (*R*)-*N*-((4-bromofuran-2-yl)methylene)-2-methylpropane-2-sulfinamide (1.0 g, 3.6 mmol) in dichloromethane (20 ml) was added dropwise a solution of vinyl magnesium bromide (11.5 ml, 1.0 M) at -78 °C. The resulting solution was stirred at -78 °C for 1 h. The reaction was quenched by sat. aq. NH_4Cl (10 ml) at 0 °C. The resulting mixture was concentrated to afford crude residue which was purified by flash chromatography (90:10 heptanes:Ethyl acetate to 100% ethyl acetate) to afford the title compound (200 mg, 20%) as the minor diastereomeric product. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (s, 9H), 3.44 (d, J = 5.5 Hz, 1H), 4.97-5.00 (m, 1H), 5.29-5.41 (m, 2H), 6.05 (ddd, J = 17.1, 10.2, 6.6 Hz, 1H), 6.33 (s, 1H), 7.40 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.5, 56.0, 56.2, 100.1, 111.2, 118.6, 135.5, 140.8, 154.3; MS (ESI): $[M+H]^+$ m/z 307.0.

(*S*)-2-methyl-N-((*R*)-1-(4-(prop-1-en-2-yl)furan-2-yl)allyl)propane-2-sulfinamide: A mixture of (*S*)-N-((*R*)-1-(4-bromofuran-2-yl)allyl)-2-methylpropane-2-sulfinamide (200 mg, 0.65 mmol), potassium carbonate (271 mg, 1.63 mmol), isopropenylboronic acid pinacol ester (274 mg, 1.63 mol) and $Pd(dppf)Cl_2$ (38 mg, 0.05 mmol) in dioxane (17.8 ml) and water (4.0 ml) was degassed by bubbling N_2 gas through the solution for 10 min. The reaction was then refluxed (100 °C) for 5 h. After cooling to ambient temperature, water (10 ml) was added and the mixture was extracted

ethyl acetate (20 ml × 2). The organic layers were combined and evaporated under reduced pressure and then purified by column chromatography (90:10 heptanes:ethyl acetate to 100% ethyl acetate) to afford the title compound as an oil (70 mg, 40%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.19 (s, 9H), 2.00 (s, 3H), 3.46 (br s, 1H), 4.85-4.96 (m, 1H), 5.00 (br s, 1H), 5.19 (s, 1H), 5.27-5.35 (m, 1H), 5.40 (d, *J* = 17.2 Hz, 1H), 6.10 (ddd, *J* = 16.88, 10.24, 6.83 Hz, 1H), 6.44 (s, 1H), 7.38 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 20.9, 22.6, 55.6, 56.0, 105.5, 111.1, 118.7, 128.3, 134.7, 135.3, 138.9, 154.2.

(*R*)-1-(4-(propan-2-yl-1,2-t_2)furan-2-yl)propan-2,3-t_2-1-amine hydrochloride salt : To a solution of (*S*)-2-methyl-N-((*R*)-1-(4-(prop-1-en-2-yl)furan-2-yl)allyl)propane-2-sulfinamide (5 mg, 0.019 mmol) in THF (1 ml) in a tritiation flask was added $Pd(OH)_2/C$ (10 mg). The reaction was stirred under an atmosphere of tritium gas (5 Ci) for 3 h. The reaction was filtered, and the filtrate was evaporated under reduced pressure. The residue was repeatedly dissolved in ethanol and evaporated under reduced pressure to remove residual reagents. The resulting residue was dissolved in ethyl acetate (0.3 ml) and 12 M HCl (3 μl) was added. The resulting solution was stirred for 40 min. The reaction then concentrated under reduced pressure. The residue was repeatedly dissolved in ethanol and evaporated under reduced pressure to remove residual reagents to provide the title compound that was used in the next step without further purification.

4-((3,4-dioxo-2-(((1*R*)-1-(4-(propan-2-yl-1,2-t_2)furan-2-yl)propyl-2,3-t_2)amino)cyclobut-1-en-1-yl)amino)-3-hydroxy-*N*,*N*-dimethylpicolinamide ([^{3}H]-SQA1): To a solution of (*R*)-1-(4-(propan-2-yl-1,2-t_2)furan-2-yl)propan-2,3-t_2-1-amine hydrochloride salt (assumed 0.019 mmol) in ethanol (0.3 ml) was added diisopropylethylamine (30 μl) followed by 4-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)-3-hydroxy-N,N-dimethylpicolinamide (4.5 mg, 0.015 mmol). The reaction was stirred for 5 h at ambient temperature. The reaction was evaporated under reduced pressure and then initially purified by column chromatography (90:10 dichloromethane:methanol). The resulting material was further purified by HPLC (Gemini C18 25 × 1 cm with 0.1% trifluoracetic acid in water (v/v) as the mobile phase A; 0.1% trifluoracetic acid in acetonitrile (v/v) as the mobile phase B; 20% B to 100% B over 100 min at 3 ml/min flow rate) to provide the title compound which was dissolved in ethanol. The radiochemical purity was determined by high performance liquid chromatography and has a specific activity of 106Ci/mmol. The title compound was dispensed and stored as 8 × 2mCi packs as an ethanol solution at 10 mCi/ml. HPLC (Inertsil ODS3 5 μm 250 × 2.6 mm at 22 °C with 0.1% trifluoracetic acid in water (v/v) as the mobile phase A; 0.1% trifluoracetic acid in acetonitrile (v/v) as the mobile phase B; 20% B to 80% B over 20 min at 1 ml/min flow rate) RT 13.483 min; 97.5%; HRMS (ESI): $[M+H]^+$ m/z 435.2219.

Preparation of OXM1

(*R*)-*N*-(3-chloro-2-hydroxypropyl)-1-(4-chlorophenyl)cyclopropane-1-carboxamide: To a solution of 1-(4-chlorophenyl)-1-cyclopropanecarboxylic acid (2.00 g, 10.17 mmol) in DMF (68.5 ml) was added (*R*)-1-amino-3-chloropropan-2-ol (1.00 g, 6.85 mmol), HATU (3.91 g, 10.3 mol), and *N*-methyl morpholine (2.64 ml, 24.0 mmol). The reaction was stirred at ambient temperature

overnight. The reaction was quenched with saturated aqueous ammonium chloride solution and extracted with ethyl acetate. The organic layers were combined and washed with water (100 ml × 2) and saturated aqueous NaCl solution then was evaporated under reduced pressure. The crude residue was purified by column chromatography (90:10 heptanes:ethyl acetate to 100% ethyl acetate) to afford the title compound (1.61 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.05-1.11 (m, 2H), 1.62-1.65 (m, 2H), 3.27-3.34 (m, 1H), 3.81-3.87 (m, 2H), 5.69 (br s, 1H), 7.34-7.39 (m, 4H); MS (ESI): $[M+H]^+$ m/z 288.2.

(*R*)-1-(4-chlorophenyl)-N-(oxiran-2-ylmethyl)cyclopropane-1-carboxamide: A solution of (*R*)-N-(3-chloro-2-hydroxypropyl)-1-(4-chlorophenyl)cyclopropane-1-carboxamide (135 mg, 0.47 mmol) in THF (0.47 ml) in a screw cap vial was cooled to -40 °C. To the reaction vial was added potassium-*t*-butoxide (33.8 mg, 0.47 mmol) and the reaction was stirred for 10 min. The reaction was filtered through silica pad which was washed with 5% MeOH in DCM. The filtrate was concentrated under reduced pressure. The crude residue was purified by column chromatography (90:10 heptanes:ethyl acetate to 20:80) to afford the title compound (66 mg, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ1.00-1.08 (m, 2H), 1.61 (t, *J* = 3.4 Hz, 2H), 2.47 (dd, *J* = 4.7, 2.6 Hz, 1H), 2.73 (t, *J* = 4.3 Hz, 1H), 2.99-3.04 (m, 1H), 3.19-3.25 (m, 1H), 3.60-3.66 (m ,1H), 5.45 (s, 1H), 7.40-7.30 (m, 4H); MS (ESI): $[M+H]^+$ m/z 252.2.

(*R*)-1-(4-chlorophenyl)-N-((4-(2,3-dihydro-1H-inden-2-yl)-5-oxomorpholin-2-yl)methyl) cyclopropane-1-carboxamide (OXM1): To a solution of (*R*)-1-(4-chlorophenyl)-N-(oxiran-2-ylmethyl)cyclopropane-1-carboxamide (850 mg, 3.38 mmol) in toluene (6.75 ml) was added indan-2-amine (450 mg, 3.38 mmol) and the reaction was heated at 100 °C for 1 h. The reaction was then allowed to cool to ambient temperature to which was added triethylamine (0.57 ml, 4.05 mmol) and 2-chloroacetyl chloride (0.27 ml, 3.38 mmol). The reaction was stirred overnight at ambient temperature. To the reaction was added sodium methylate (205 in methanol, 2.32 ml, 10.1 mmol). The reaction was stirred for 1.5 h. The reaction was quenched with 1N HCl (1 ml) and diluted with dichloromethane. The organic layer was separated and the aqueous was extracted with dichloromethane (3×). The organic layers were combined and concentrated under reduced pressure. The crude residue was initially purified by column chromatography (heptanes:ethyl acetate). The resulting material was further purified by SFC (Phenomenex Bipenyl 5 μm 250 × 2.1 mm with CO_2 as the mobile phase A; methanol as the mobile phase B; 10% B over 100 min at 80 ml/min flow rate) to provide the title compound as a white solid (578 mg, 40%). HPLC (Kinetic C18 2.6 μm 100 × 3.0 mm with 0.1% formic acid in water (v/v) as the mobile phase A; 0.1% formic acid in acetonitrile (v/v) as the mobile phase B; 5% B for 0.5 min then to 100% B over 4 min and hold for 1.5 min at 0.75 ml/min flow rate) RT 5.173 min; >99%; MS (ESI): $[M+H]^+$ m/z 425.2; Chiral HPLC (Chiral Tech AD-H 5 μm 250 × 4.6 mm with CO_2 as the mobile phase A; 0.2% ammonia in methanol (v/v) as the mobile phase B; 5% B for 1.0 min then to 60% B over 8 min and hold for 0.5 min at 3.0 ml/min flow rate) RT 8.699 min; >99 EE ((*S*)-1-(4-chlorophenyl)-*N*-((4-(2,3-dihydro-1H-inden-2-yl)-5-oxomorpholin-2-yl)methyl)cyclopropane-1-carboxamide prepared from (*R*)-1-amino-3-chloropropan-2-ol RT = 7.557 min); ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 0.89-0.95 (m, 2H), 1.23-1.29 (m, 2H), 2.85-2.90 (m, 1H), 2.94-3.00 (m, 2H), 3.05-3.17 (m, 4H),

3.72-3.79 (m, 1H), 4.07 (dd, *J* = 8.0 8.0 Hz, 2H), 5.27-5.34 (m, 1H), 6.85-6.91 (m, 1H), 7.18-7.27 (m, 6H), 7.30-7.35 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 14.7, 14.8, 29.5, 34.5, 35.7, 41.1, 43.4, 51.8, 66.8, 71.5, 124.2, 124.3, 126.6, 126.6, 128.5, 131.9, 138.7, 140.9, 140.9, 165.3, 172.3. HRMS (ESI/QTOF) m/z: $[M+H]^+$ Calcd for $C_{24}H_{25}ClN_2O_3$ 425.1627; Found 425.1624; $[\alpha]_D^{23}$ (*c* 0.003, MeOH) = -33.3.

Preparation of OXM2.

***tert*-butyl (*S*)-(3-(bicyclo[1.1.1]pentan-1-ylamino)-2-hydroxypropyl)carbamate:** To a solution of bicyclo[1.1.1]pentan-1-amine (16.68 g, 139.4 mmol) in isopropanol (500 ml) was added DIPEA (18 g, 139.3 mmol) followed by a solution of *tert*-butyl (R)-(oxiran-2-ylmethyl)carbamate (24 g, 138.6 mmol) in isopropanol (200 ml) at room temperature. The reaction was stirred for 16 h and then concentrated under reduced pressure. The crude residue was purified by column chromatography (2:8 ethyl acetate in petroleum ether to 100% ethyl acetate) twice to provide the title compound as a yellow solid (7.6 g, 21%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 9H), 1.70 – 1.80 (m, 6H), 2.48 – 2.53 (m, 1H), 2.68 – 2.72 (m, 1H), 3.05 – 3.11 (m, 1H), 3.26 – 3.32 (m, 1H), 3.66 – 3.73 (m ,1H), 4.98 (br S, 1H).

***tert*-butyl (*R*)-((4-(bicyclo[1.1.1]pentan-1-yl)-5-oxomorpholin-2-yl)methyl)carbamate:** To a solution of *tert*-butyl (R)-(3-(N-(bicyclo[1.1.1]pentan-1-yl)-2-chloroacetamido)-2-hydroxypropyl)carbamate (7.7 g, 23.1 mmol) in THF (300 ml) at 5 °C was added NaH (60% in mineral oil, 3.7 g, 92.5 mmol) in portions. The reaction was then stirred at room temperature for 5 h. The reaction was cooled to 5 °C and quenched with saturate aqueous NH_4Cl (50 ml) and water (50 ml). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (50 ml × 2). The combined organic layers were concentrated under reduced pressure. The residue was purified by column chromatography (petroleum ether to 50% ethyl acetate in petroleum ether) to provide the title compound as a white solid (4.34 g, 63%). LCMS (Waters Xbridge C180 30 × 2.1 mm with 0.05% ammonia hydroxide in water (v/v) as the mobile phase A; acetonitrile as the mobile phase B; 0% B to 95% over 0.6 min then 95% B for 0.8 min) RT 2.510 min; >95%; MS (ESI): $[M+H-56]^+$ 241.2; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 9H), 2.14 - 2.19 (m, 6H), 2.48 (s, 1H), 3.14 - 3.20 (m, 3H), 3.43 - 3.46 (m, 1H), 3.75 – 3.78 (m, 1H), 4.03-4.20 (m, 2 H), 4.91 (br s, 1H).

(*R*)-6-(aminomethyl)-4-(bicyclo[1.1.1]pentan-1-yl)morpholin-3-one: To a solution of *tert*-butyl (*R*)-((4-(bicyclo[1.1.1]pentan-1-yl)-5-oxomorpholin-2-yl)methyl)carbamate (100 mg, 0.34 mmol) in methanol (3 ml) was added HCl (4 M in dioxane, 1 ml) at room temperature. The reaction was stirred for 3 h. The reaction was concentrated under reduced pressure to provide the title compound as a yellow oil (80 mg, 100%) which was used without further purification as the presumed HCl salt. LCMS (Waters Xbridge C18 30 × 2.1 mm with 0.05% NH_3 in water (v/v) as the mobile phase A; acetonitrile as the mobile phase B; 0% B to 95% over 0.6 min then 95% B for 0.8 min) RT 0.732 min; 86%; MS (ESI): $[M+H]^+$ m/z 197.1.

(*R*)-N-((4-(bicyclo[1.1.1]pentan-1-yl)-5-oxomorpholin-2-yl)methyl)-1-(4-(trifluoromethyl) phenyl)cyclopropane-1-carboxamide (OXM2): To a solution of (*R*)-6-(aminomethyl)-4-

(bicyclo[1.1.1]pentan-1-yl)morpholin-3-one (1.50 g, 6.44 mmol) in dichloromethane (50 ml) was added 1-(4-trifluoromethyl)cyclopropane-1-carboxylic acid (Aldrich, 1.48 g, 6.44 mmol), triethylamine (2.61 g, 25.8 mmol), and HATU (2.94 mg, 7.73 mmol) at 15 °C. The reaction was allowed to warm to room temperature and stir for 16 h. The reaction mixture was poured into water (15 ml) and extracted with ethyl acetate (50 ml × 3). The combined organic concentrated under reduced pressure.

The residue was first purified by column chromatography (petroleum ether to 33% ethyl acetate in petroleum ether) then purified by HPLC (Phenomenex Gemini C18 10 μm 250 × 50 mm with 0.5% ammonia hydroxide in water (v/v) as the mobile phase A; acetonitrile as the mobile phase B; 35% B to 55% B over 15 min at 110 ml/min flow rate) to provide the title compound as a white solid (1.39 g, 50%). LCMS (Waters Xselect CSH C18 30 × 2.1 mm with 10 mM ammonium acetate in water as the mobile phase A; 10 mM acetonitrile as the mobile phase B; 0% B for 0.6 min then to 100% over 3.4 min) RT 3.098 min; 100%; MS (ESI): $[M+H]^+$ m/z 409.2; Chiral HPLC (Chiralcel OD-3 150×4.6 mm 3um with CO_2 as the mobile phase A; 0.05% diethylamine in ethanol as the mobile phase B; 5% to 40% B over 5 mins, 2.5 ml/min) RT 2.705 min; >99 EE.

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.09 - 1.11 (m, 2H), 1.63 – 1.67 (m, 2H), 2.13 – 2.18 (m, 6H), 2.48 (s, 1H), 3.02 – 3.17 (m, 3H), 3.54 - 3.56 (m, 1H), 3.68 – 3.71 (m, 1H), 3.93 – 4.12 (m, 2H), 5.54 – 5.57 (m, 1H), 7.54 (d, *J* = 8.0 Hz, 2H), 7.66 (d, *J* = 8.0 Hz, 2H). ^{19}F NMR (400 MHz, $CDCl_3$) δ - 62.65 (s). ^{13}C NMR (151 MHz, DMSO-d_6) δ 14.7, 14.8, 23.4, 30.2, 41.2, 46.1, 51.6, 53.0, 66.8, 71.5, 121.6, 123.4, 125.2, 125.3, 125.3, 125.3, 125.4, 127.0, 127.3, 127.5, 127.7, 127.9, 130.6, 144.8, 144.8, 166.2, 171.9.

Preparation of OXM3

***tert*-butyl (3-(benzyl(2,3-dihydro-1H-inden-2-yl)amino)-2-hydroxypropyl)carbamate:** To a solution of N-benzyl-2,3-dihydro-1H-inden-2-amine (22.3 g, 100 mmol) in methanol was added *tert*-butyl (oxiran-2-ylmethyl)carbamate (17.3 g, 100 mmol) in methanol. The reaction mixture was refluxed for 2 h, then cooled, and concentrated under reduced pressure. The residue was co-evaporated with dioxane to give the title compound as a yellow oil (30g, 60%) which was used without further purification.

***tert*-butyl (3-((2,3-dihydro-1H-inden-2-yl)amino)-2-hydroxypropyl)carbamate:** To a solution of *tert*-butyl (3-(benzyl(2,3-dihydro-1H-inden-2-yl)amino)-2-hydroxypropyl)carbamate (5.8 g, 15 mmol) in ethyl acetate (100 ml) was added 10% Pd/C (1 g). The mixture was hydrogenated under an atmosphere of hydrogen gas for 2 h. The reaction mixture was filtered through celite. The filtrate was concentrated under reduced pressure to provide the title compound (3g, 80%) as a brown oil and used without further purification.

***tert*-butyl (3-(2-chloro-N-(2,3-dihydro-1H-inden-2-yl)acetamido)-2-hydroxypropyl) carbamate:** To a solution of *tert*-butyl (3-((2,3-dihydro-1H-inden-2-yl)amino)-2-hydroxypropyl) carbamate (30.6 g, 100 mmol) in THF/DMF (1:1) was added diisoprylethylamine (5 ml) and the reaction mixture was cooled to -60 ºC. To the cooled reaction mixture chloroacetyl chloride was added dropwise under an atmosphere of argon gas. The reaction was stirred overnight, purified

by column chromatography on silica gel, and concentrated under reduced pressure to provide the title compound (30g, 80%) as a yellow oil.

***tert*-butyl ((4-(2,3-dihydro-1H-inden-2-yl)-5-oxomorpholin-2-yl)methyl)carbamate:** A solution of *tert*-butyl (3-(2-chloro-N-(2,3-dihydro-1H-inden-2-yl)acetamido)-2-hydroxypropyl)carbamate (38 g, 100 mmol) in DMF (300 ml) was cooled and NaH (60% in mineral oil, 5g, 120 mmol) was added in portions. The reaction mixture was allowed to warm to room temperature and stirred for 2 h. Water and ethyl acetate was added and the resulting mixture was concentrated under reduced pressure. The resulting reside was diluted with water and ether. The organic layer was separated, and the aqueous layer was extracted with ether. The combined organic layer was washed with water and brine, then filtered through Na_2SO_4. The filtrate was evaporated under reduced pressure and the residue was crystallized with ether with hexanes to provide the title compound (30 g, 60%) as a white solid.

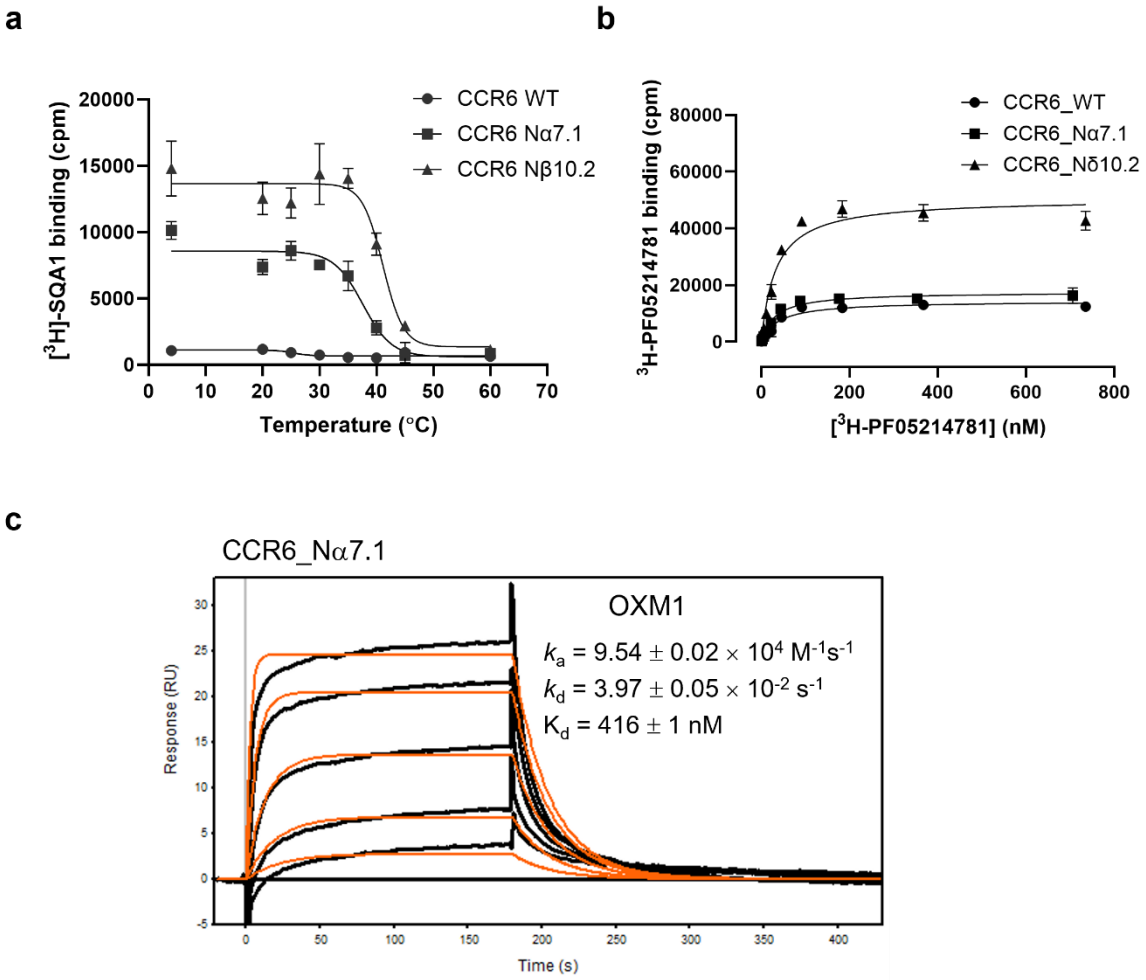
6-(aminomethyl)-4-(2,3-dihydro-1H-inden-2-yl)morpholin-3-one: To a solution of *tert*-butyl ((4-(2,3-dihydro-1H-inden-2-yl)-5-oxomorpholin-2-yl)methyl)carbamate (34.6 g, 100 mmol) in dichloromethane (500 ml) was added trifluoroacetic acid. The reaction mixture was stirred for 24 h and then evaporated under reduced pressure. The residue was dissolved in water and made alkaline with saturated K_2CO_3. The mixture was extracted with chloroform. The organic layer was dried and evaporated to provide the title compound (20g, 75%).

2-(4-chlorophenyl)-*N*-((4-(2,3-dihydro-1H-inden-2-yl)-5-oxomorpholin-2-yl)methyl)-2-methylpropanamide (OXM3): To a solution of 6-(aminomethyl)-4-(2,3-dihydro-1H-inden-2-yl)morpholin-3-one (100 mg, 0.41 mmol) in DCM (6.0 ml) at 0 °C was added 2-(4-chlorophenyl)-2-methylpropanoic acid (81 mg, 0.41 mmol), triethylamine (0.113 ml, 0.81 mmol), and HATU (232 mg, 0.61 mmol). The reaction was stirred for 16 h at 15 °C. The reaction mixture was quenched the addition of aqueous potassium bicarbonate (20 ml). The layers were separated and the aqueous was washed with ethyl acetate (40 ml × 3). The organic layers combined and were dried with sodium sulfate, filtered, and concentrated under reduced pressure. The crude residue was purified by HPLC (DuraShell 5 μm 150 × 25 mm with 0.05% ammonia hydroxide in water (v/v) as the mobile phase A; acetonitrile as the mobile phase B; 46% B to 76% B over 10 min at 25 ml/min flow rate) to provide the title compound as a white solid (48 mg, 28%). LCMS (Waters Xselect CSH C18 5 μm 50 × 2.1 mm with 5% 10 mM ammonia acetate in water (v/v) as the mobile phase A; acetonitrile as the mobile phase B; 0% B for 0.6 min to 100% B over 3.4 min at 0.8 ml/min flow rate) RT 3.197 min; >99%; MS (ESI): $[M+H]^+$ m/z 427.4; HRMS (ESI/QTOF) m/z: $[M+H]^+$ Calcd for $C_{24}H_{27}ClN_2O_3$ 427.1783; Found 427.1778: ^{1}H NMR (400 MHz, (CD_3OD) δ 1.47 (s, 6H), 2.87-2.93 (m, 1H), 3.00-3.07 (m, 2H), 3.09-3.23 (m, 3H), 3.26 (d, *J* = 6.0 Hz, 2 H), 3.75-3.81 (m, 1H), 4.10 (d, *J* = 16.6 Hz, 1H), 4.21 (d, *J* = 16.6 Hz, 1H), 5.36-5.44 (m, 1H), 7.14-7.18 (m, 2H), 7.22-7.25 (m, 2H), 7.26-7.30 (m, 4 H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 14.7, 14.8, 23.4, 30.2, 41.2, 46.1, 51.6, 53.0, 66.8, 71.5, 121.6, 123.4, 125.2, 125.3, 125.3, 125.3, 125.4, 127.0, 127.3, 127.5, 127.7, 127.9, 130.6, 144.8, 144.8, 166.2, 171.9.

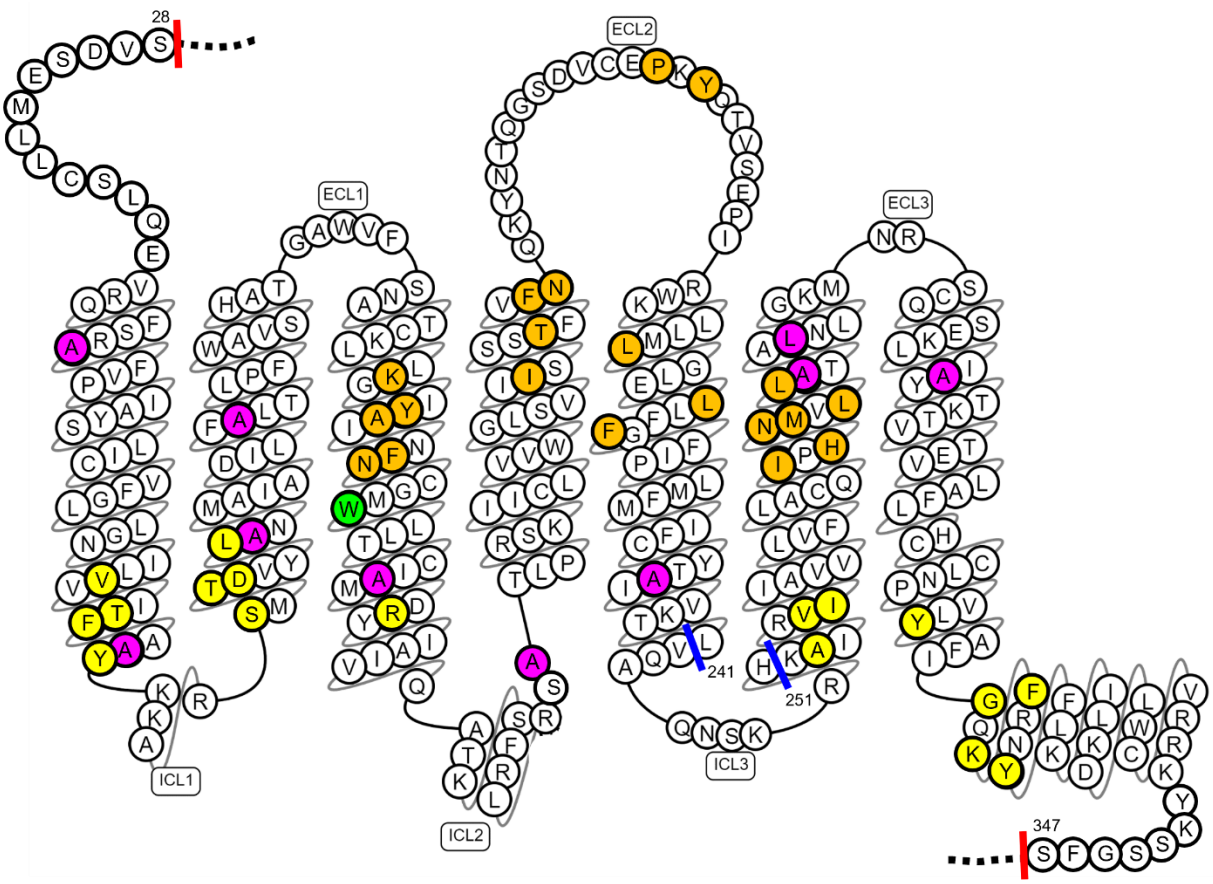
Preparation of OXM4

***N*-((4-(2,3-dihydro-1*H*-inden-2-yl)-5-oxomorpholin-2-yl)methyl)-1-phenylcyclopropane-1-carboxamide (OXM4)**: To a solution of 1-phenylcyclopropane-1-carboxylic acid (19.4 mg, 120 μm) in DCM (~0.1 ml) in a reaction vial was added 6-(aminomethyl)-4-(2,3-dihydro-1H-inden-2-yl)morpholin-3-one (24 mg, 0.1 mmol) in DCM (800 μL) and diisopropylethylamine (~50 μL, 300 mmol). A 0.55 M stock solution of HOPO and EDCI in DCM was separately prepared of which 200 ml (110 μmol) was added to the reaction. The reaction vial was capped and agitated at 50 °C for 16 h. The reaction was concentrated under reduced pressure. The crude residue was purified by HPLC (DuraShell 5 μm 150 × 25 mm with 0.225% formic acid in water (v/v) as the mobile phase A; acetonitrile as the mobile phase B; 9% B to 59% B over 12 min at 35 ml/min flow rate) to provide the title compound as a white solid (10 mg, 26%). LCMS (Waters Xselect CSH C18 5 μm 50 × 2.1 mm with 0.0375% trifluoroacetic acid in water (v/v) as the mobile phase A; 0.01875% trifluoracetic acid in acetonitrile as the mobile phase B; 1% B to 5% over 0.6 min then to 100% B over 3.4 min at 0.8 ml/min flow rate) RT 3.056 min; >99%; MS (ESI): $[M+H]^+$ m/z 391; ^{1}H NMR (600 MHz, $(CD_3)_2SO$) δ 0.93 (d, *J* = 3.1 Hz, 2H), 1.30–1.21 (m, 2H), 2.86 (dd, *J* = 16.4, 6.4 Hz, 1H), 3.01 – 2.92 (m, 3H), 3.06 (dd, *J* = 16.4, 8.6 Hz, 1H), 3.13 – 3.09 (m, 1H), 3.14 (t, *J* = 6.1 Hz, 2H), 3.81 – 3.69 (m, 1H), 4.14 – 3.96 (m, 2H), 5.30 (tt, *J* = 8.5, 6.5 Hz, 1H), 6.71 (t, *J* = 6.0 Hz, 1H), 7.21 – 7.16 (m, 2H), 7.28 – 7.22 (m, 5H), 7.34 – 7.28 (m, 2H); ^{13}C NMR (151 MHz, DMSO) δ 14.6, 14.7, 30.2, 34.5, 35.7, 36.0, 41.1, 43.4, 44.9, 51.9, 66.8, 71.6, 124.2, 124.3, 126.6, 126.6, 126.7, 127.3, 128.6, 130.0, 139.8, 140.9, 140.9, 165.4, 172.7.

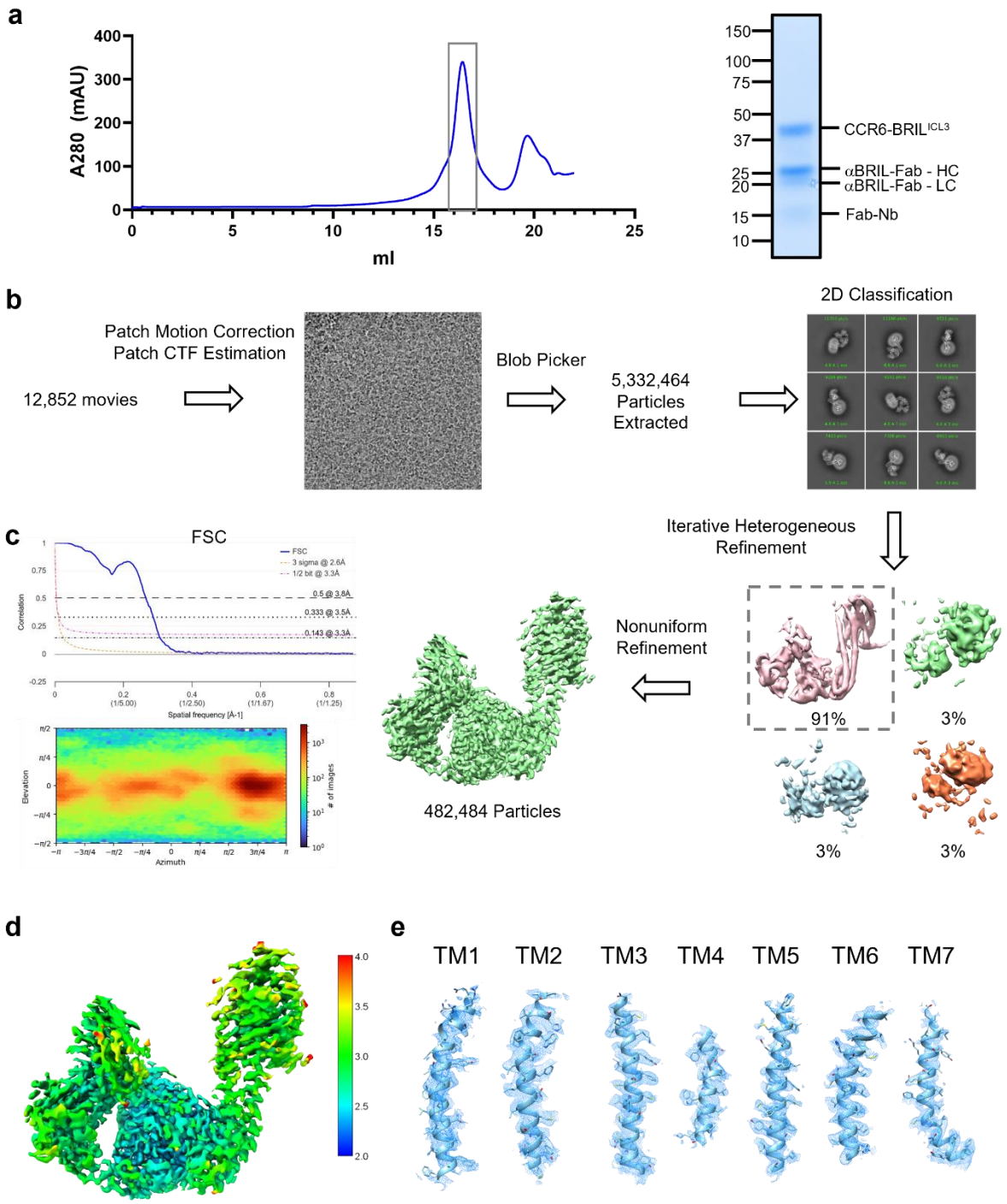
Supplementary Figures



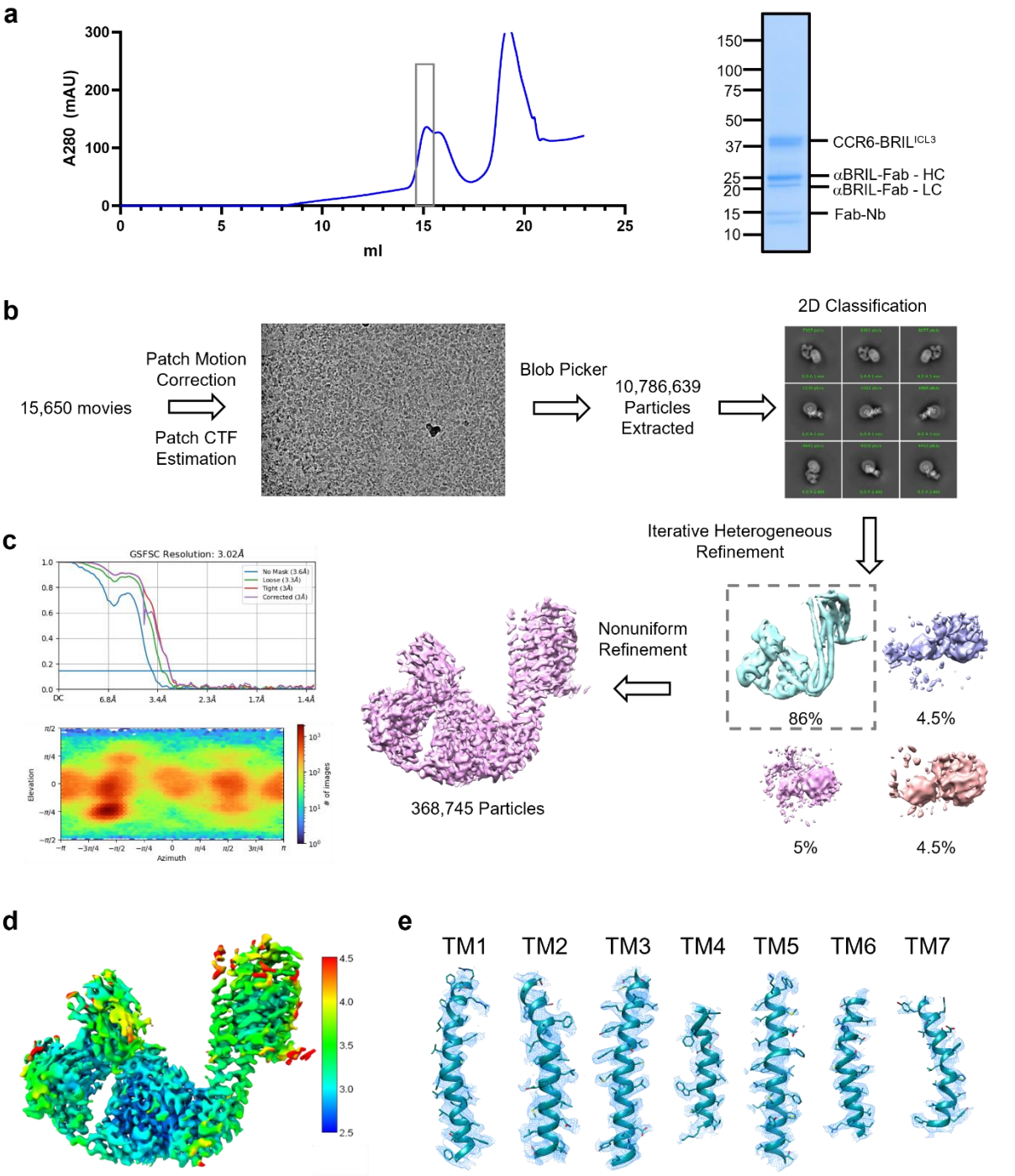
Supplementary Figure 1. Thermostability and characterization of CCR6 constructs. a, Thermostability of human WT and thermostabilized CCR6 constructs in DM with 5 mM $MgCl_2$ are shown. The individual temperature data points are shown in the format of mean ± s.d. derived from n = 3 independent measurements. WT and thermostabilized CCR6 produced a mean T_m of 25.2 ± 3.7 °C (WT), 37.6 ± 0.7 °C (Nα7.1), and 41.1 ± 0.4 °C (Nβ10.2). **b**, Saturation binding curves of [^{3}H]-SQA1 to the membranes of HEK293 cells that transiently expressed human WT or thermostabilized CCR6 constructs. Data plotted with average un-transfected control subtracted in the format of mean ± s.d. derived from n = 4 independent measurements. There was no significant difference in the affinity of [^{3}H]-SQA1 at WT (Kd = 39.2 ± 4.1 nM), Nα7.1 (Kd = 30.7 ± 1.2 nM), or Nβ10.2 (Kd = 32.8 ± 2.5 nM) CCR6 (n = 4 independent experiments). **c**, SPR sensorgrams of OXM1 binding to purified protein of thermostabilized CCR6. A concentration series of OXM1 consisting of 6 three-fold dilutions from 10 to 0.014 μM are shown in black curves (experimental data), fitting using a model of 1:1 binding (orange curves). Data plotted with average non-specific binding control subtracted. Kd in mean ± s.d. and binding kinetics derived from n = 3 independent repeats are shown in the figure. Source data are provided as a Source Data file.



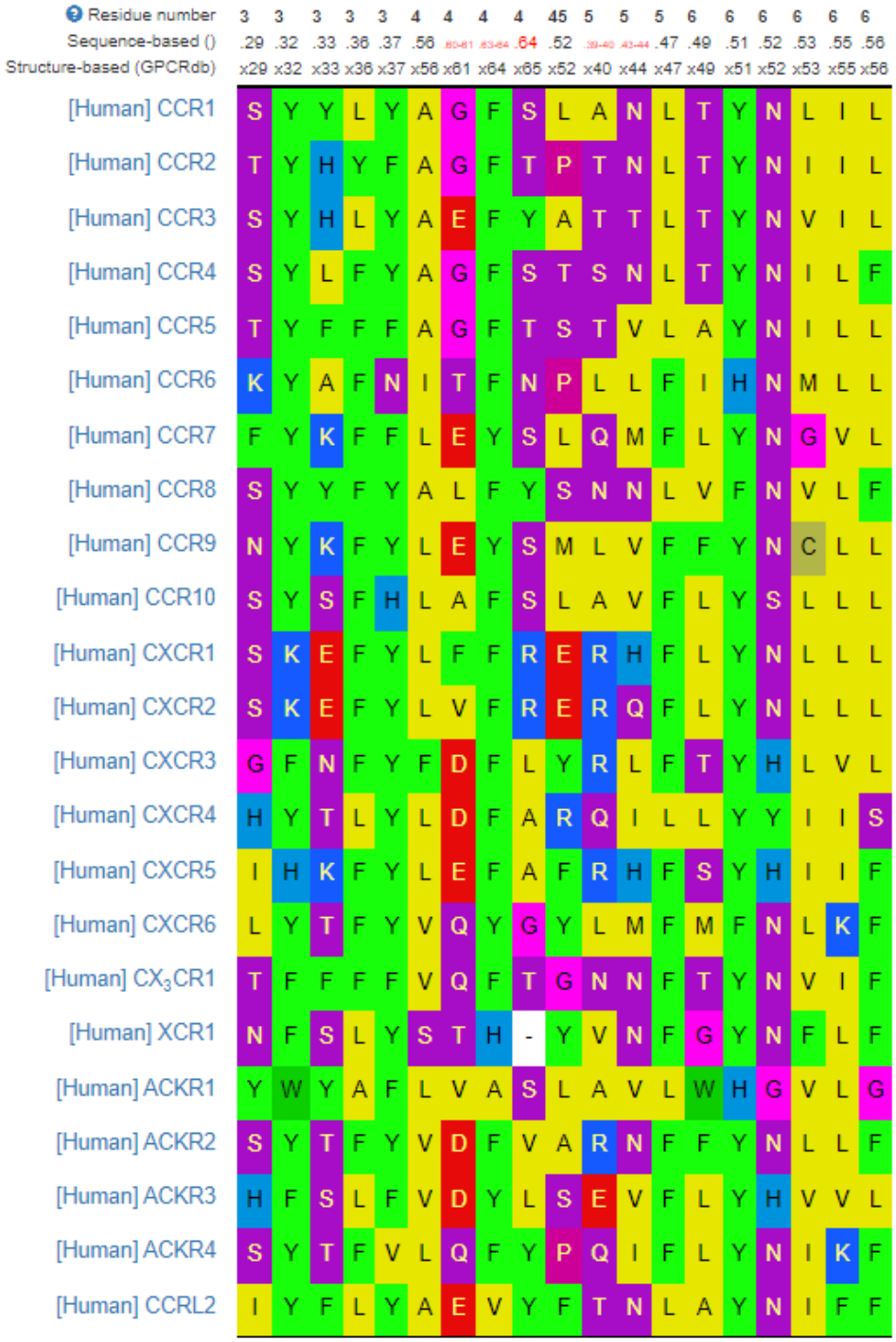
Supplementary Figure 2. Snakeplot of the thermostabilized CCR6 constructs. The thermostabilizing substituations, L47$^{1.33}$A, F73$^{1.59}$A, L86$^{2.44}$A, V96$^{2.54}$A, S140$^{3.47}$A, R159A, F236$^{5.60}$A, V276$^{6.57}$A, A279$^{6.60}$L, and G295$^{7.32}$A are highlighted in magenta. The L134$^{3.41}$W mutation is highlighted in green. Residues interacting with OXM or SQA analogues are highlighted in orange or yellow, repectively. Deletions at ICL3 where the seqeunce of BRIL was inserted are highlighted by blue lines. The termini truncations are highlighted by red lines. The truncated N- and C-termini (Δ1-27, Δ348-374) are not included in the schematic representation. Instead, they are replaced by black dashes.



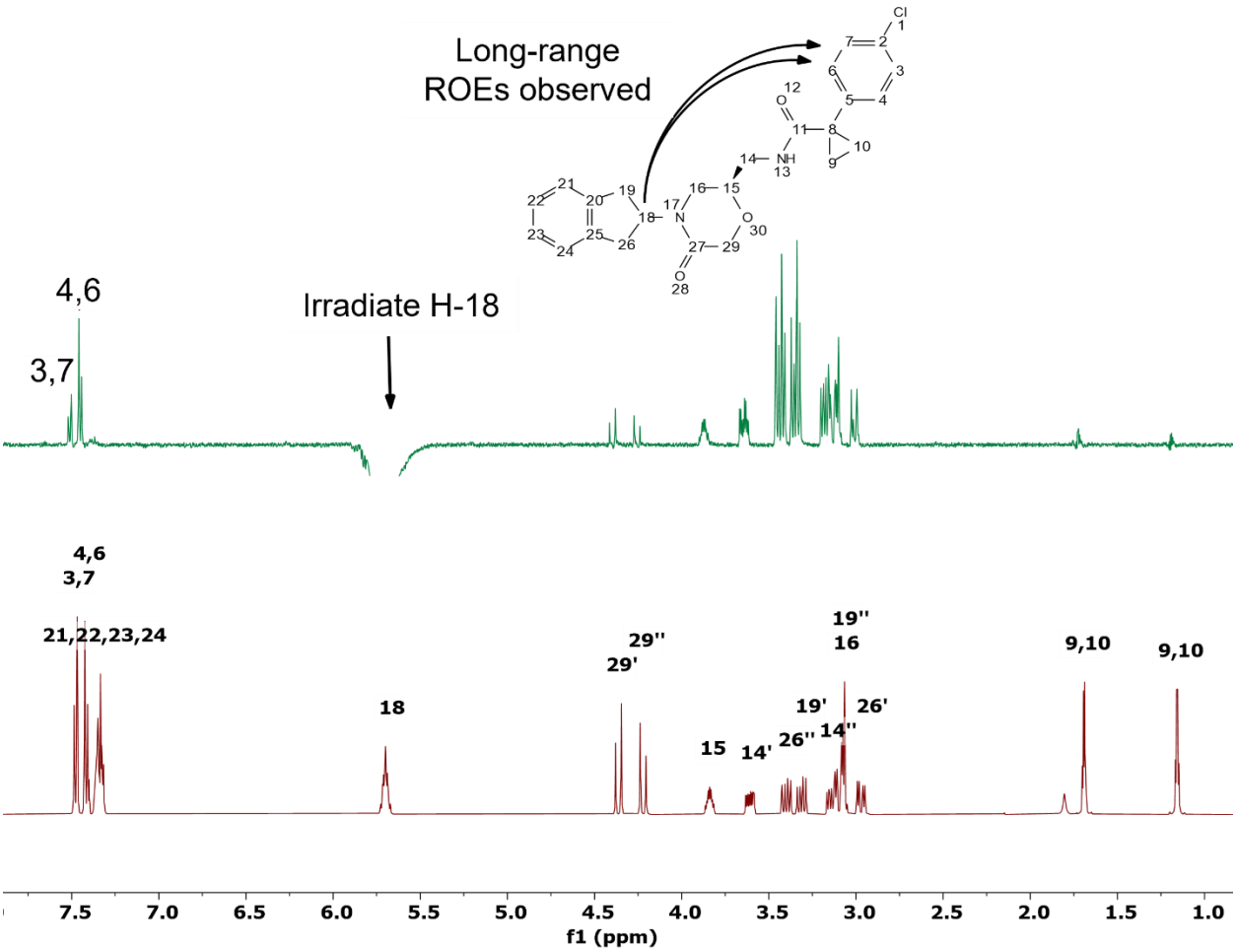
Supplementary Figure 3. Cryo-EM data processing workflow for CCR6/SQA1/OXM1. a, SEC profile of CCR6/SQA1/OXM1 complex purification. Fractions used in cryo-EM study are highlighted by the grey box and the corresponding SDS-PAGE image is shown. The uncropped SDS-PAGE image is supplied at the end of the Supplementary Information file. **b**, Data processing workflow. **c**, Fourier shell correlation curves from gold-standard refinement and particle angular distribution. **d**, Cryo-EM map colored by local resolution. **e**, CCR6 TM helices fitted into the cryo-EM density map. Source data are provided as a Source Data file.



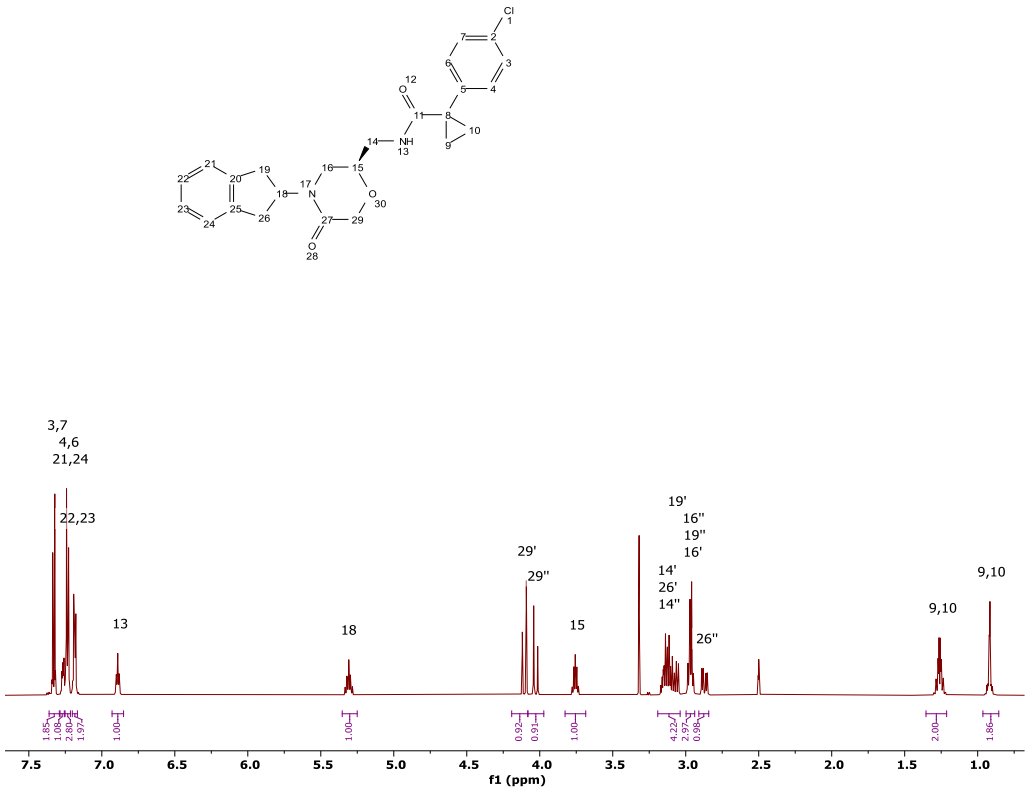
Supplementary Figure 4. Cryo-EM data processing workflow for CCR6/SQA1/OXM2. a, SEC profile of CCR6/SQA1/OXM2 complex purification. Fractions used in cryo-EM study are highlighted by the grey box and the corresponding SDS-PAGE image is shown. The uncropped SDS-PAGE image is supplied at the end of the Supplementary Information file. **b**, Data processing workflow. **c**, Fourier shell correlation curves from gold-standard refinement and particle angular distribution. **d**, Cryo-EM map colored by local resolution. **e.** CCR6 TM helices fitted into the cryo-EM density map. Source data are provided as a Source Data file.



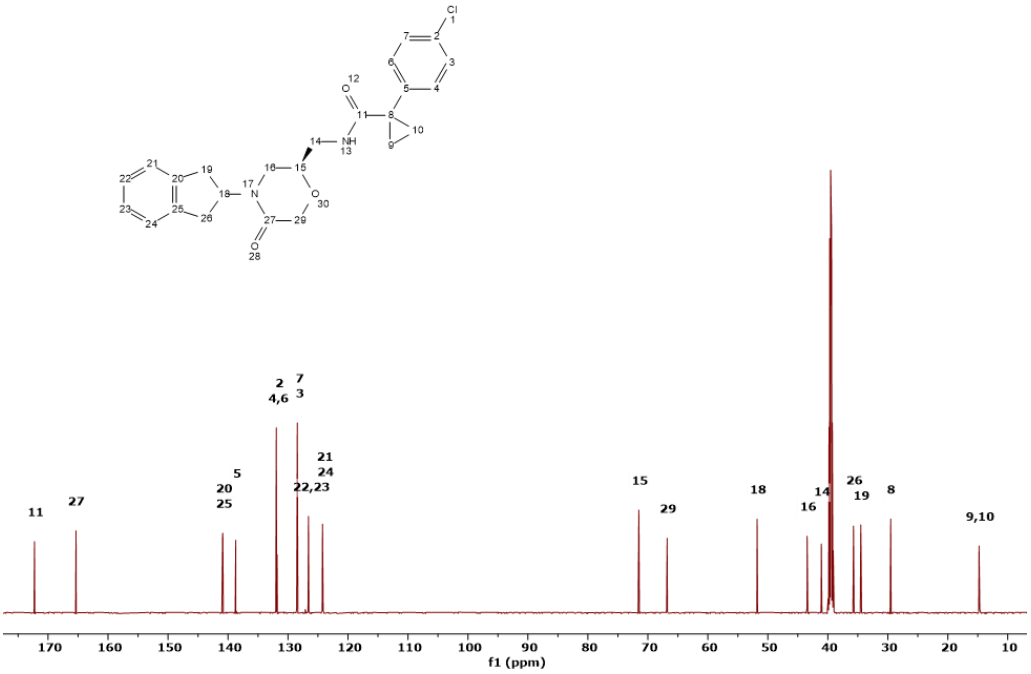
Supplementary Figure 5. Structure-based sequence alignment of human chemokine receptors at the OXM binding pocket. The sequence alignment was performed using servers from www.GPCRdb.org[55].



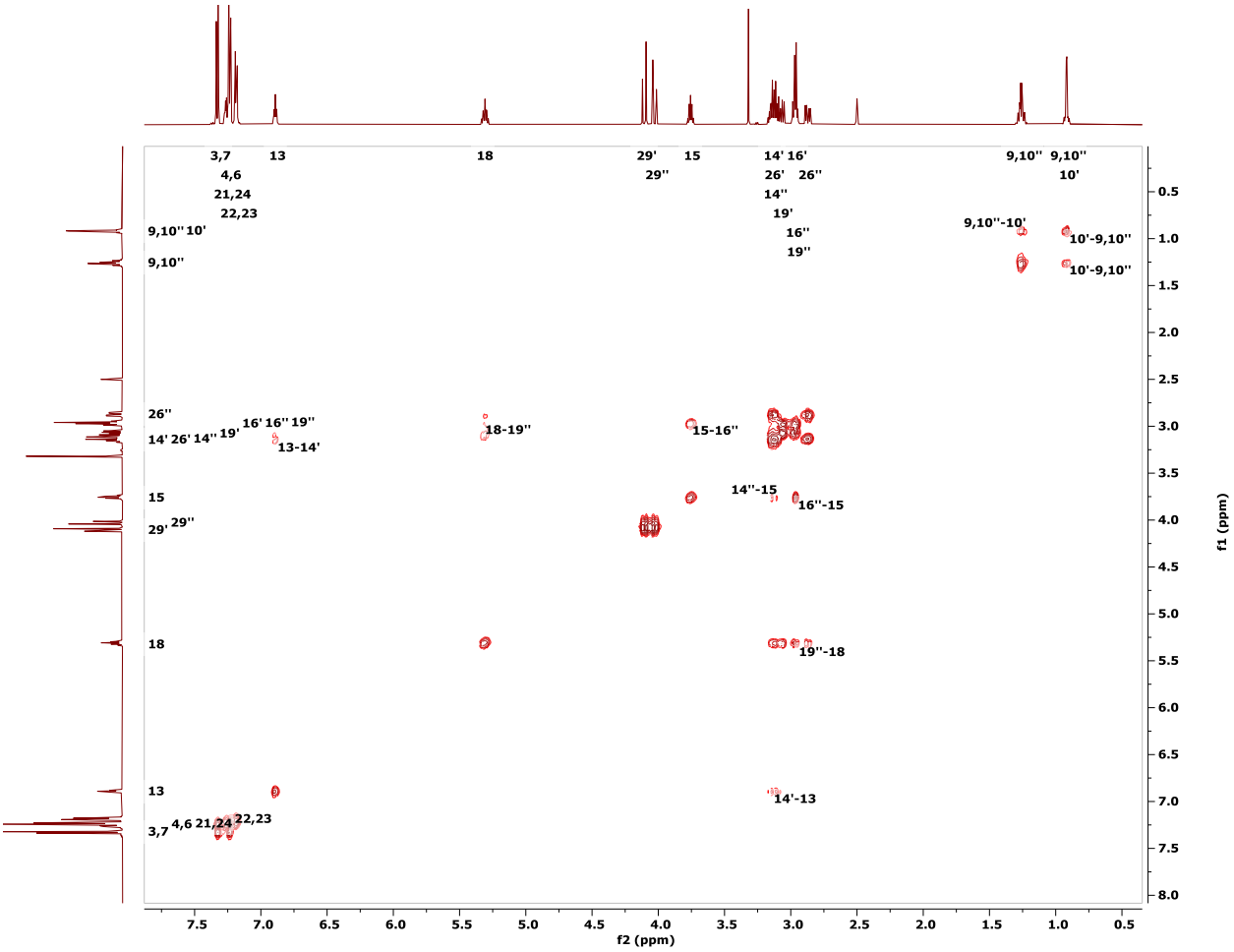
Supplementary Figure 6. Rotating-frame Overhauser Enhancement signals (ROEs) observed for OXM1 in $CDCl_3$. The observation of long-range ROEs from H-18 to H-4/H-6 and to H3/H-7 suggests that OXM1 adopts a U-shaped conformation in solution.



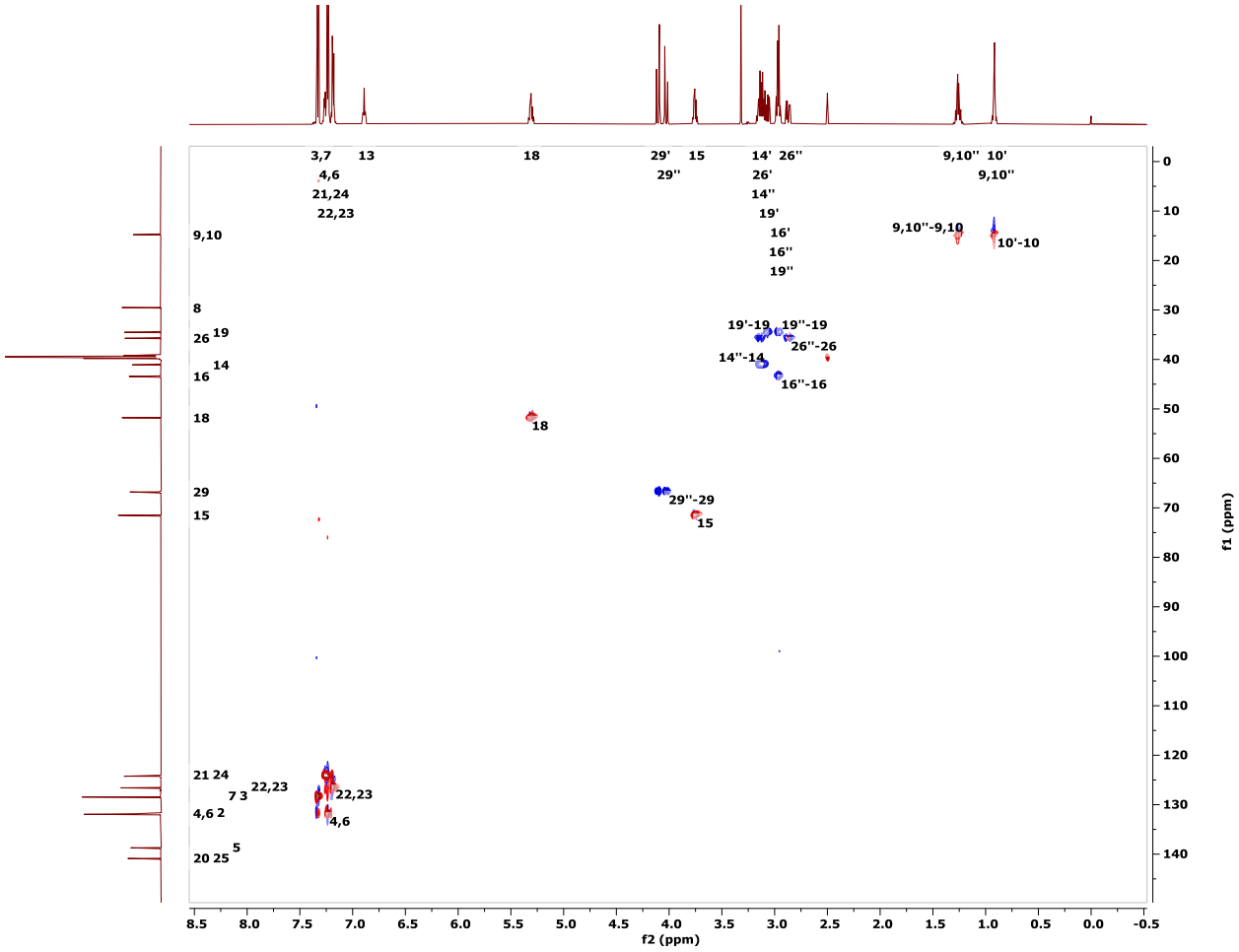
Supplementary Figure 7. 600.1 MHz ^{1}H spectrum of OXM1 in DMSO-d$_6$.



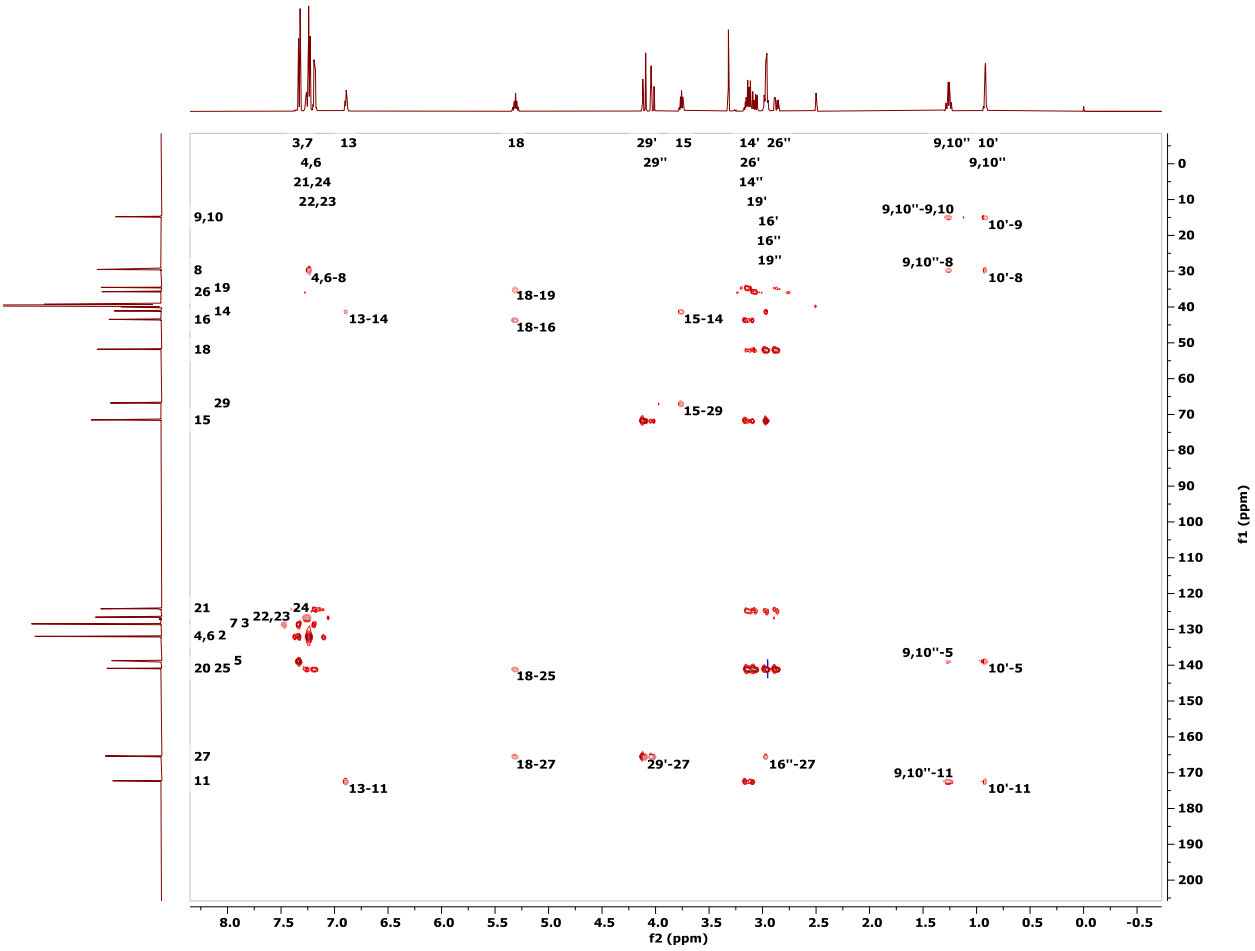
Supplementary Figure 8. 150.9 MHz ^{13}C spectrum of OXM1 in DMSO-d$_6$.



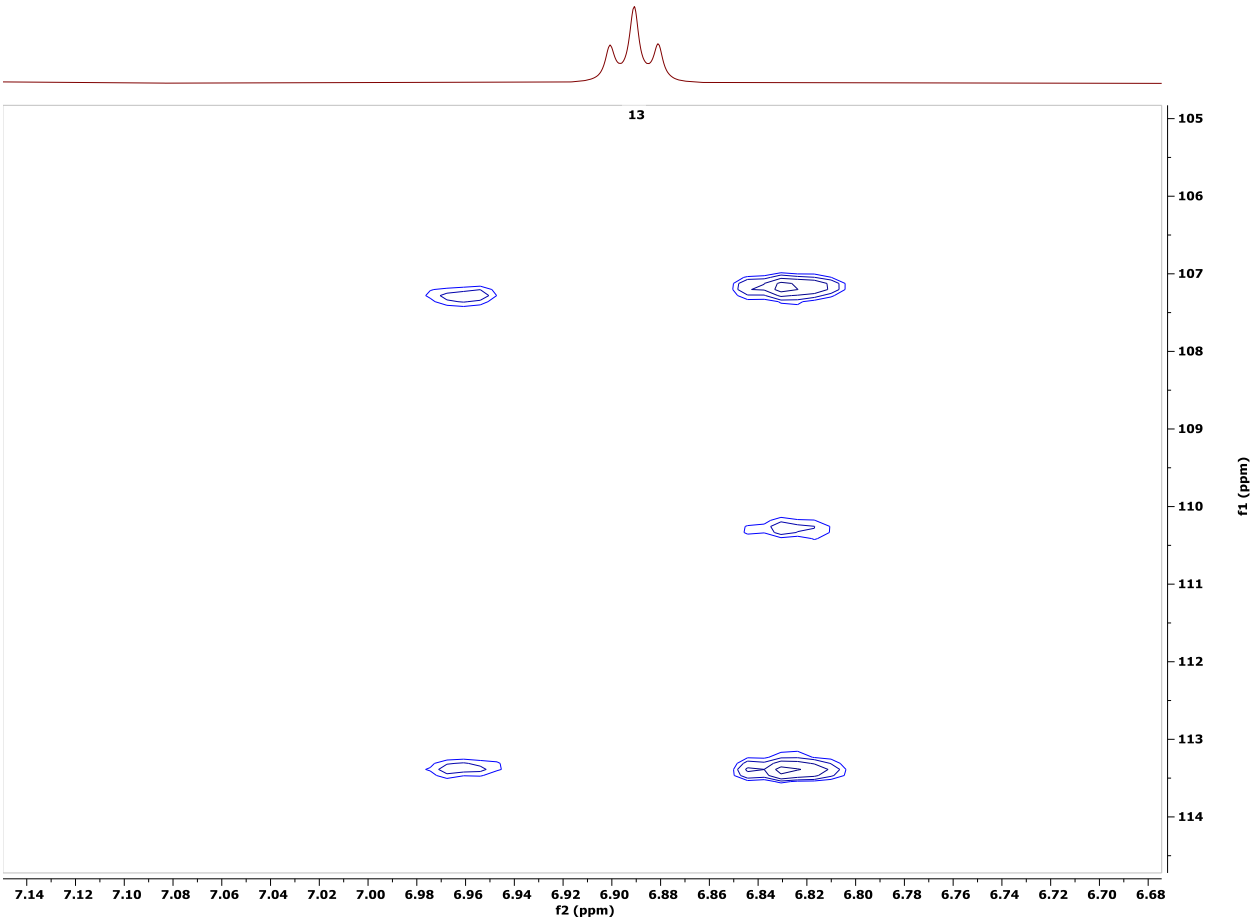
Supplementary Figure 9. 600.1 MHz COSY spectrum of OXM1 in DMSO-d_6.



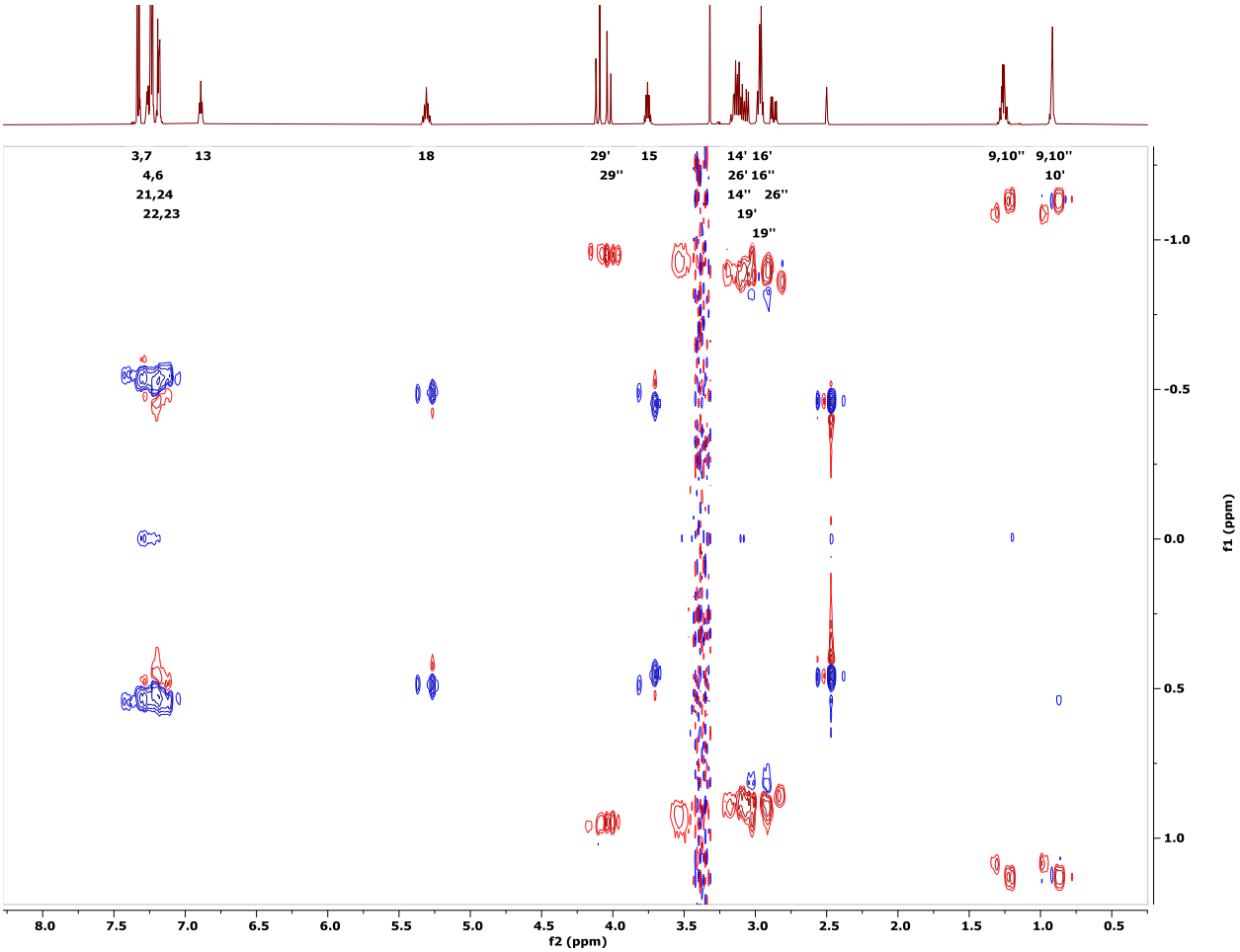
Supplementary Figure 10. 600.1 MHz HC-HSQC spectrum of OXM1 in DMSO-d_6.



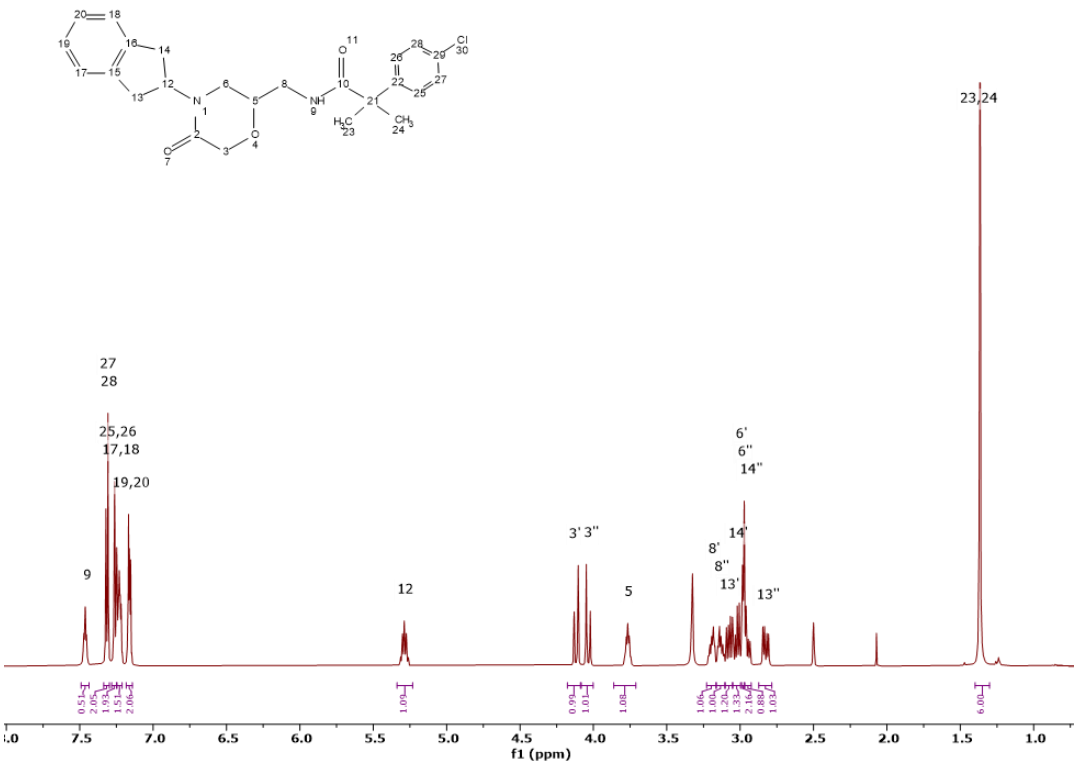
Supplementary Figure 11. 600.1 MHz HC-HMBC spectrum of OXM1 in DMSO-d_6.



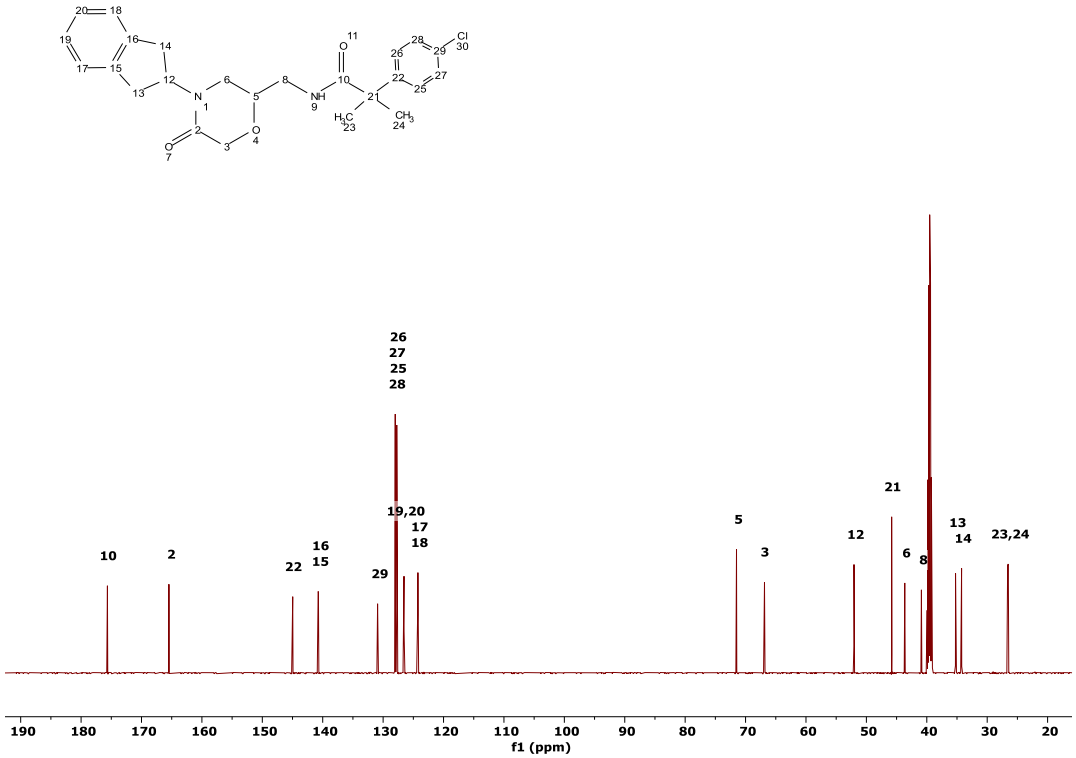
Supplementary Figure 12. 600.1 MHz JSBHN-HSQC spectrum of OXM1 in poly-HEMA gel in DMSO-d_6.



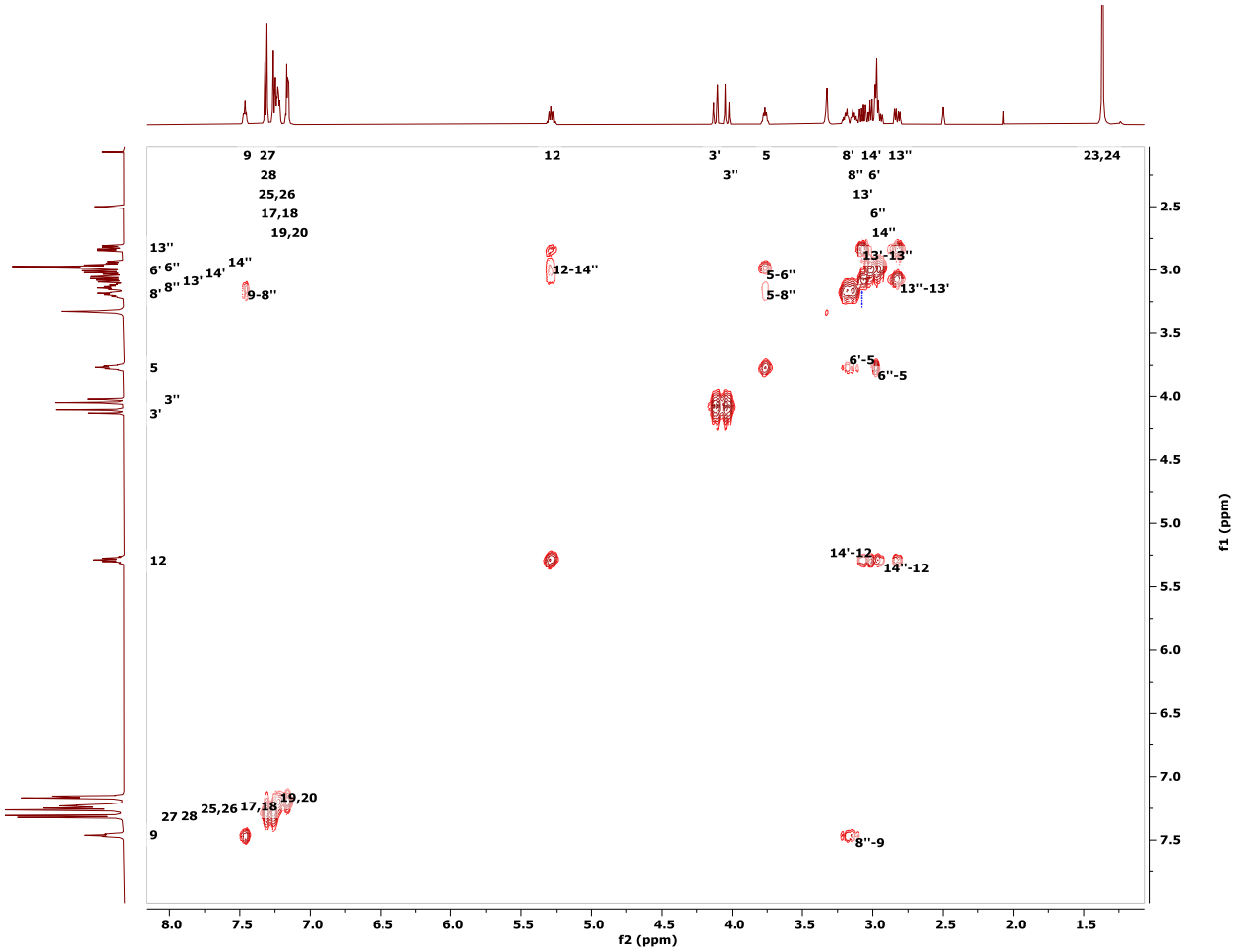
Supplementary Figure 13. 600.1 MHz JSBHC-HSQC spectrum of OXM1 in poly-HEMA gel in DMSO-d_6.



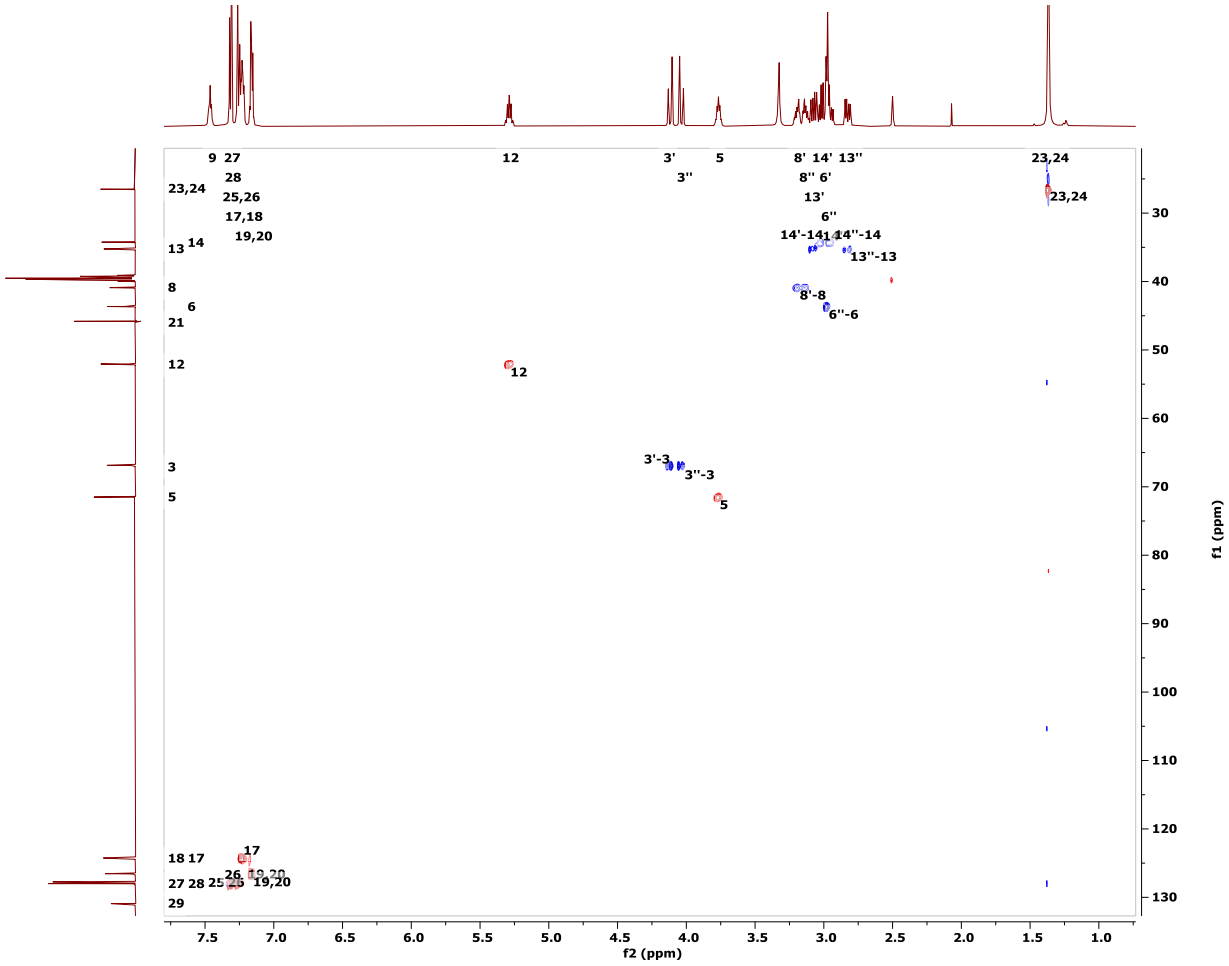
Supplementary Figure 14. 600.1 MHz ^{1}H spectrum of OXM3 in DMSO-d$_6$.



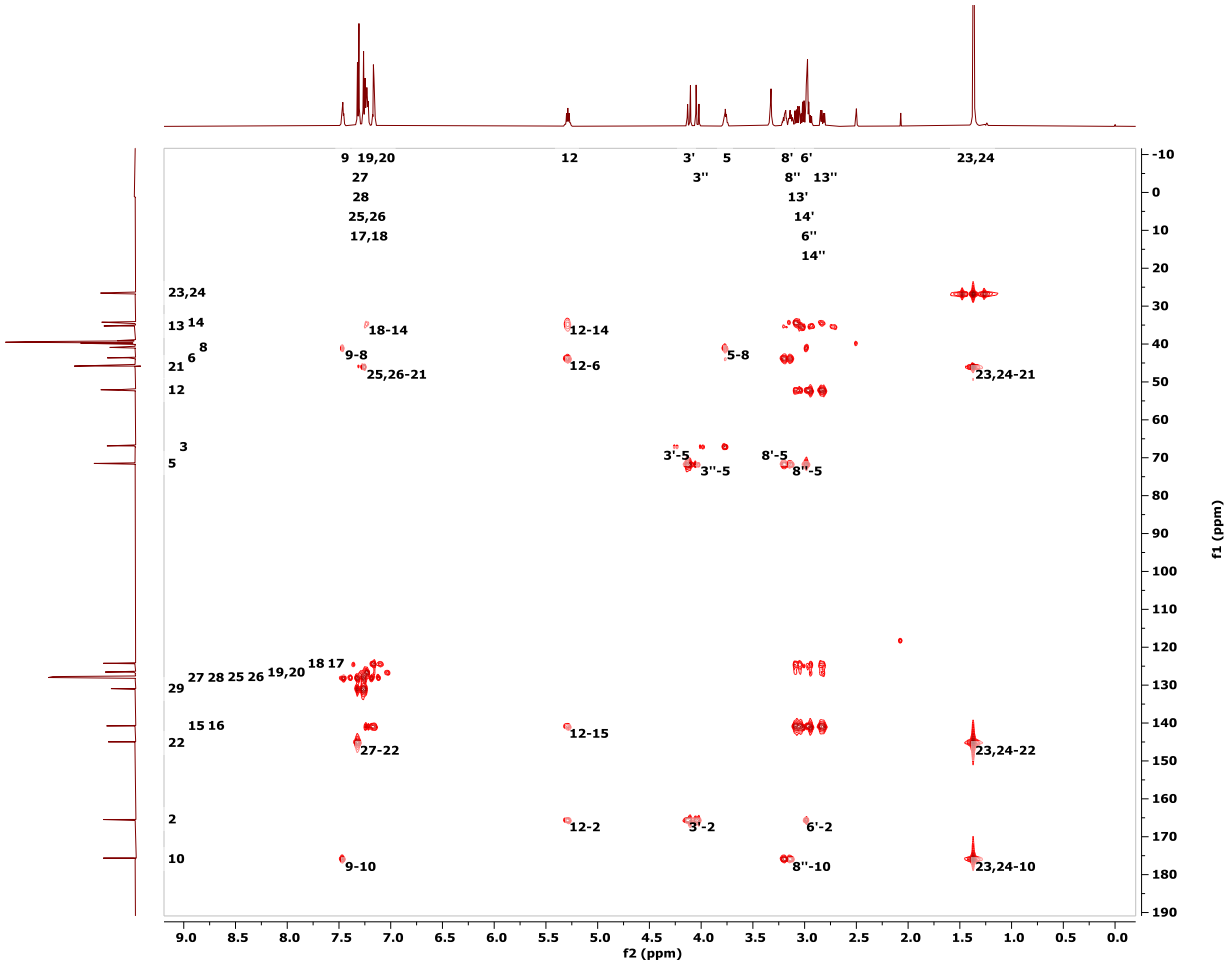
Supplementary Figure 15. 150.9 MHz ^{13}C spectrum of OXM3 in DMSO-d$_6$.



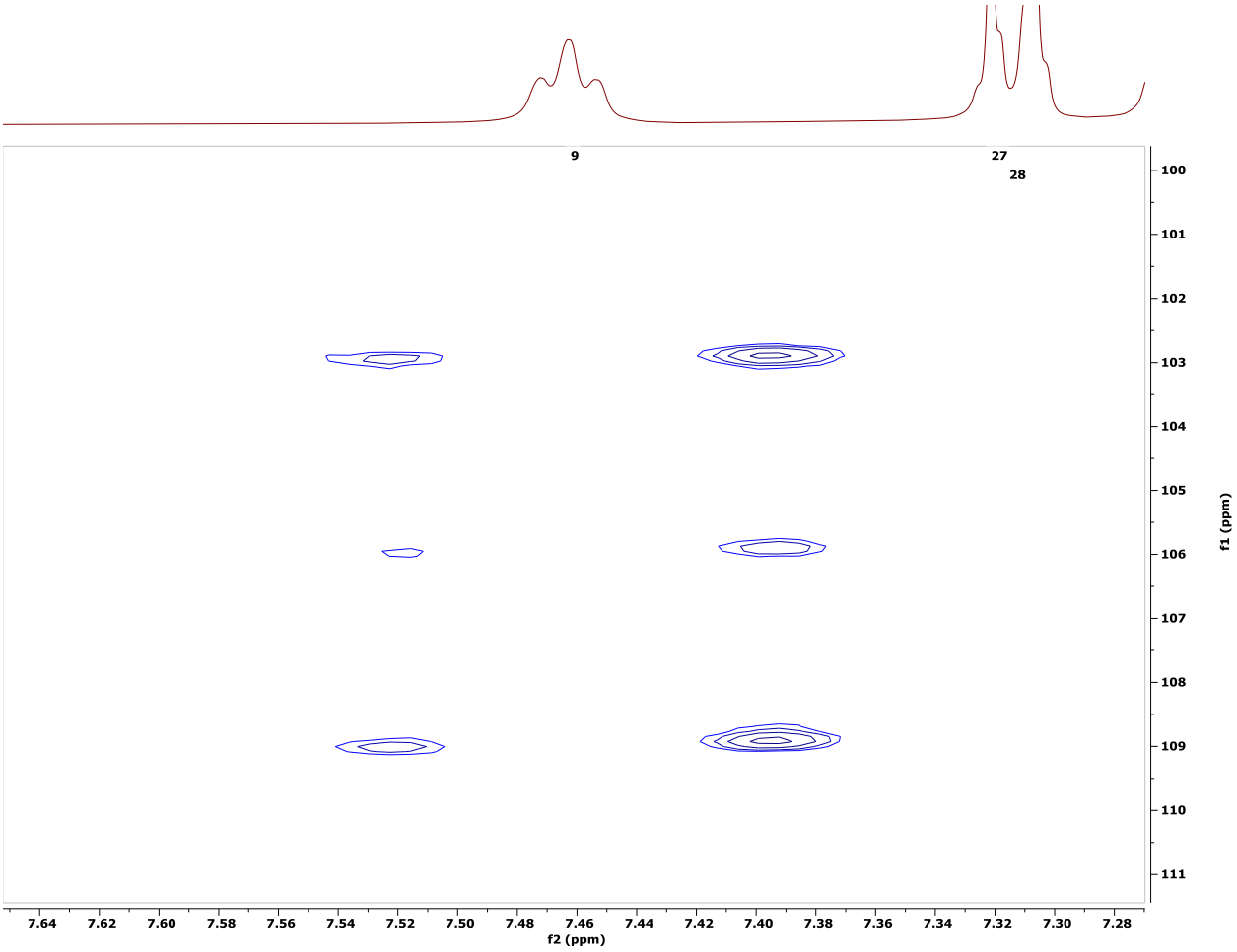
Supplementary Figure 16. 600.1 MHz COSY spectrum of OXM3 in DMSO-d_6.



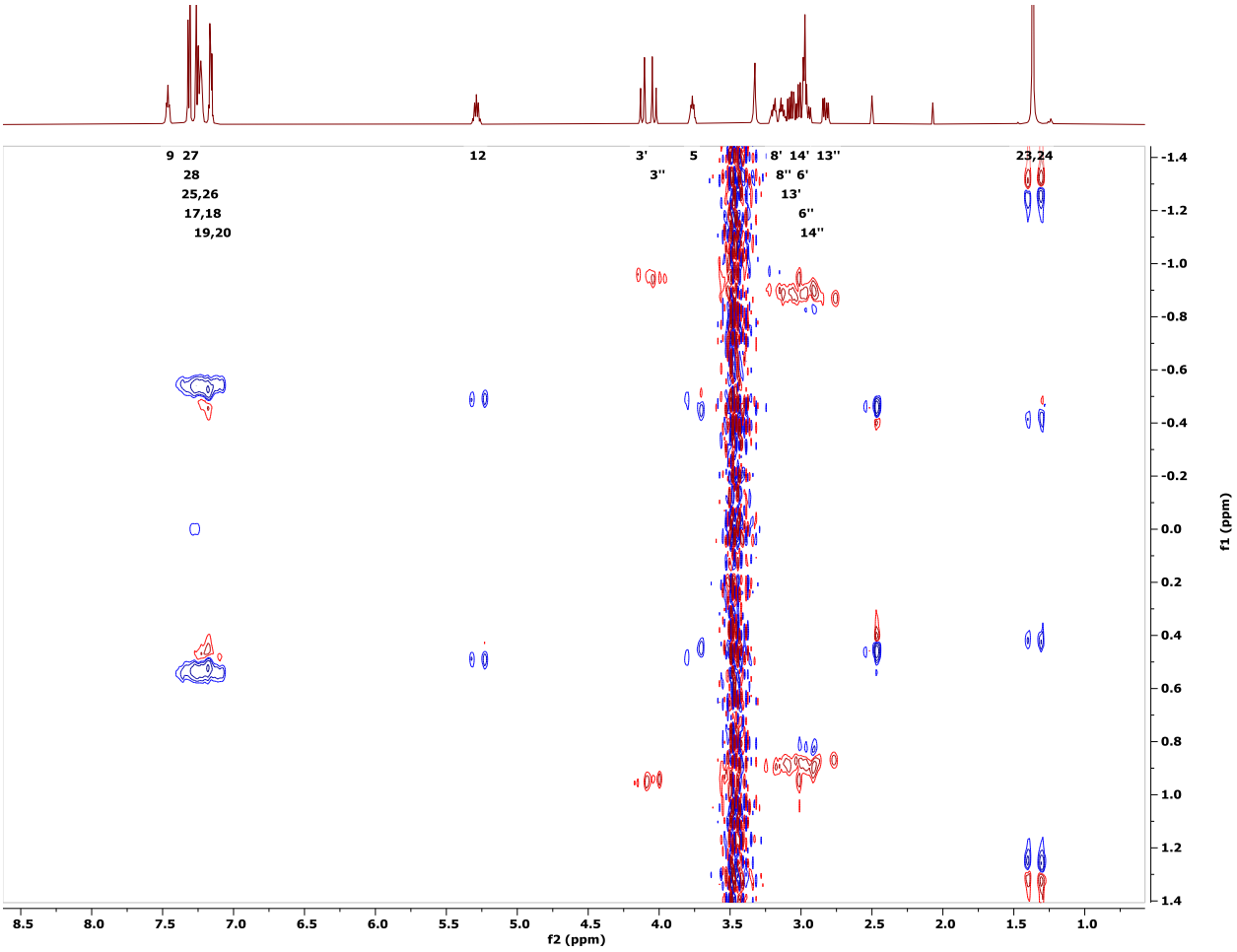
Supplementary Figure 17. 600.1 MHz HC-HSQC spectrum of OXM3 in DMSO-d_6.



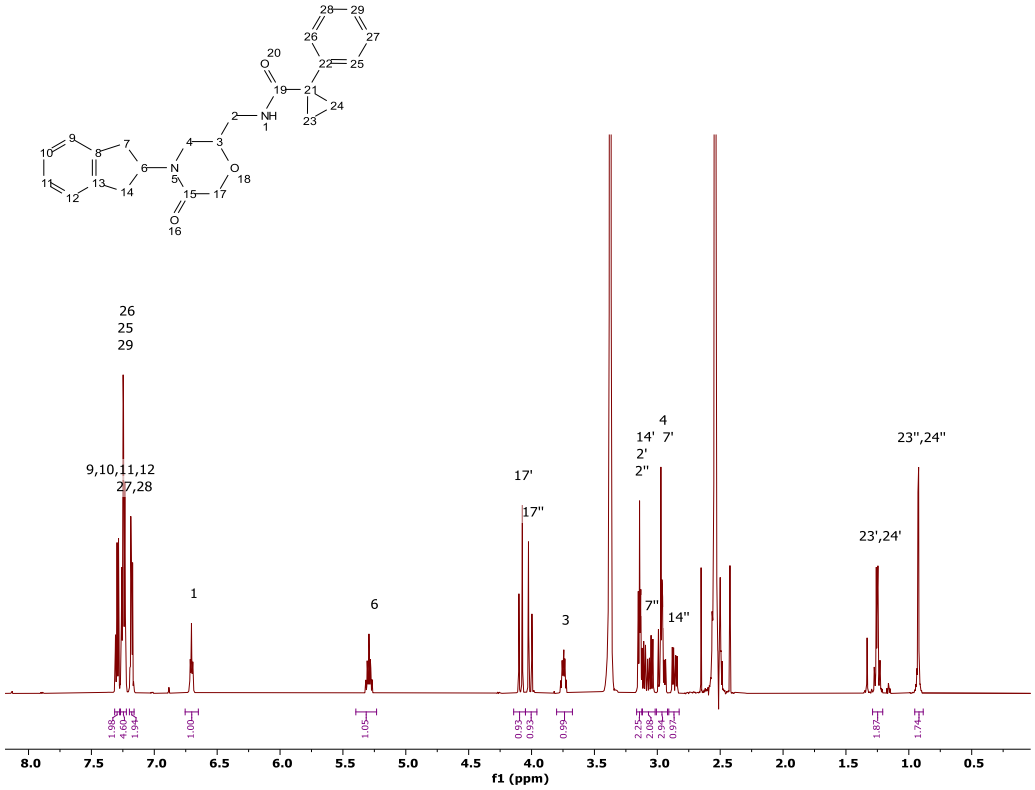
Supplementary Figure 18. 600.1 MHz HC-HMBC spectrum of OXM3 in DMSO-d6.



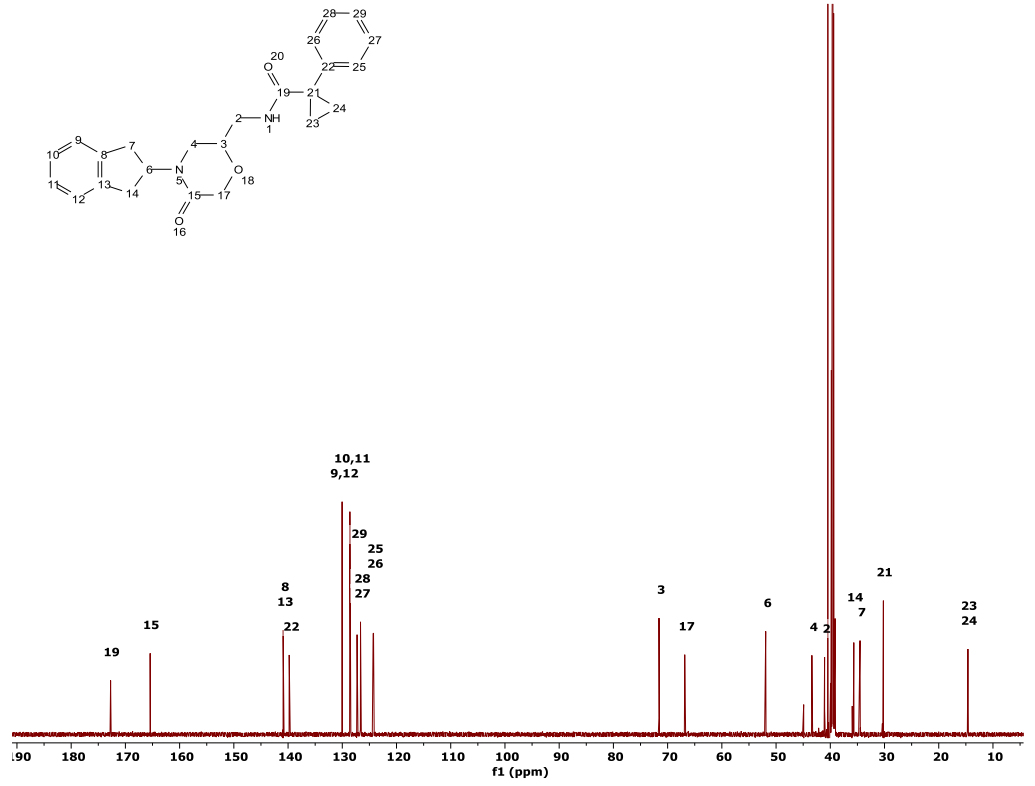
Supplementary Figure 19. 600.1 MHz JSBHN-HSQC spectrum of OXM3 in poly-HEMA gel in DMSO-d_6.



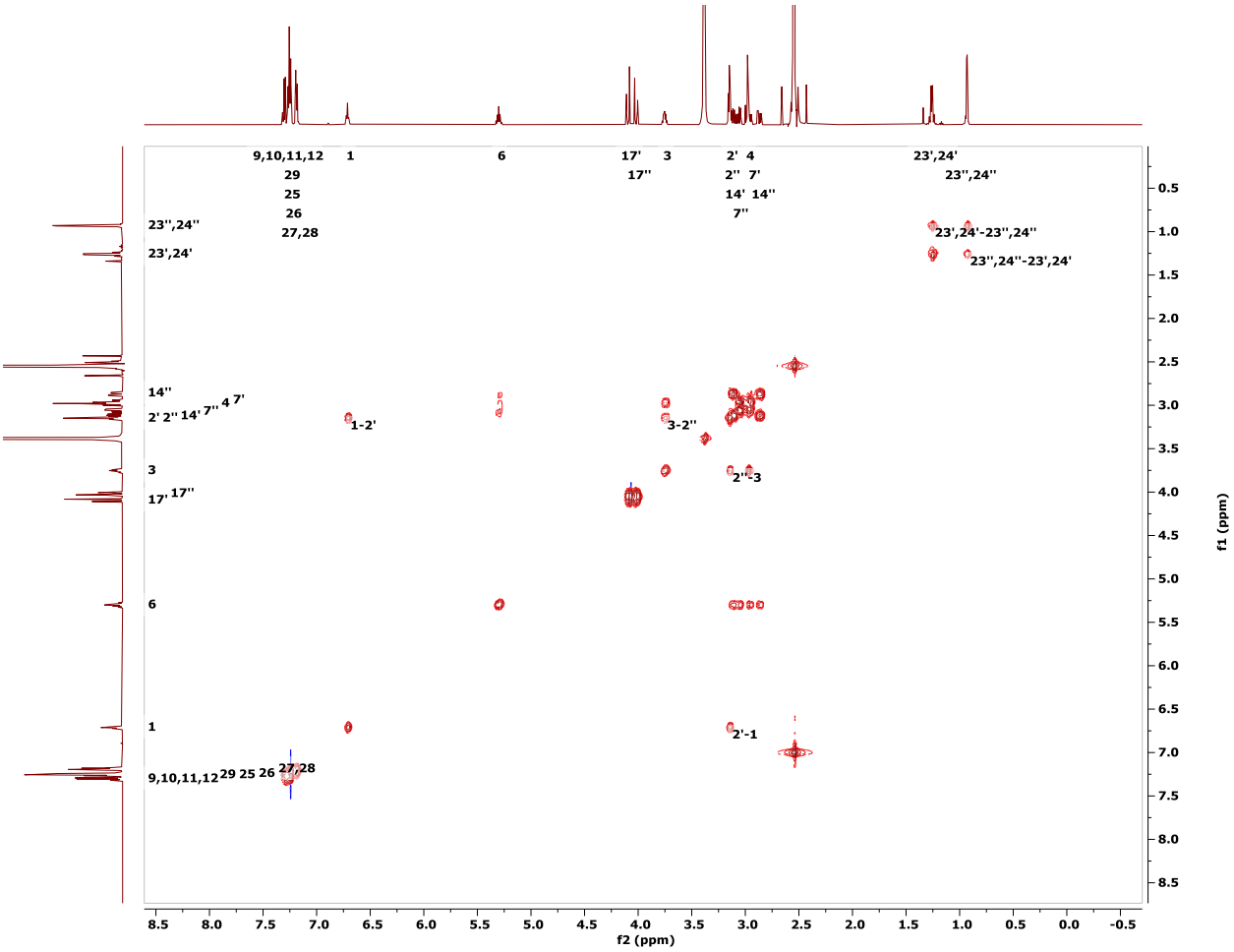
Supplementary Figure 20. 600.1 MHz JSBHC-HSQC spectrum of OXM3 in poly-HEMA gel in DMSO-d_6.



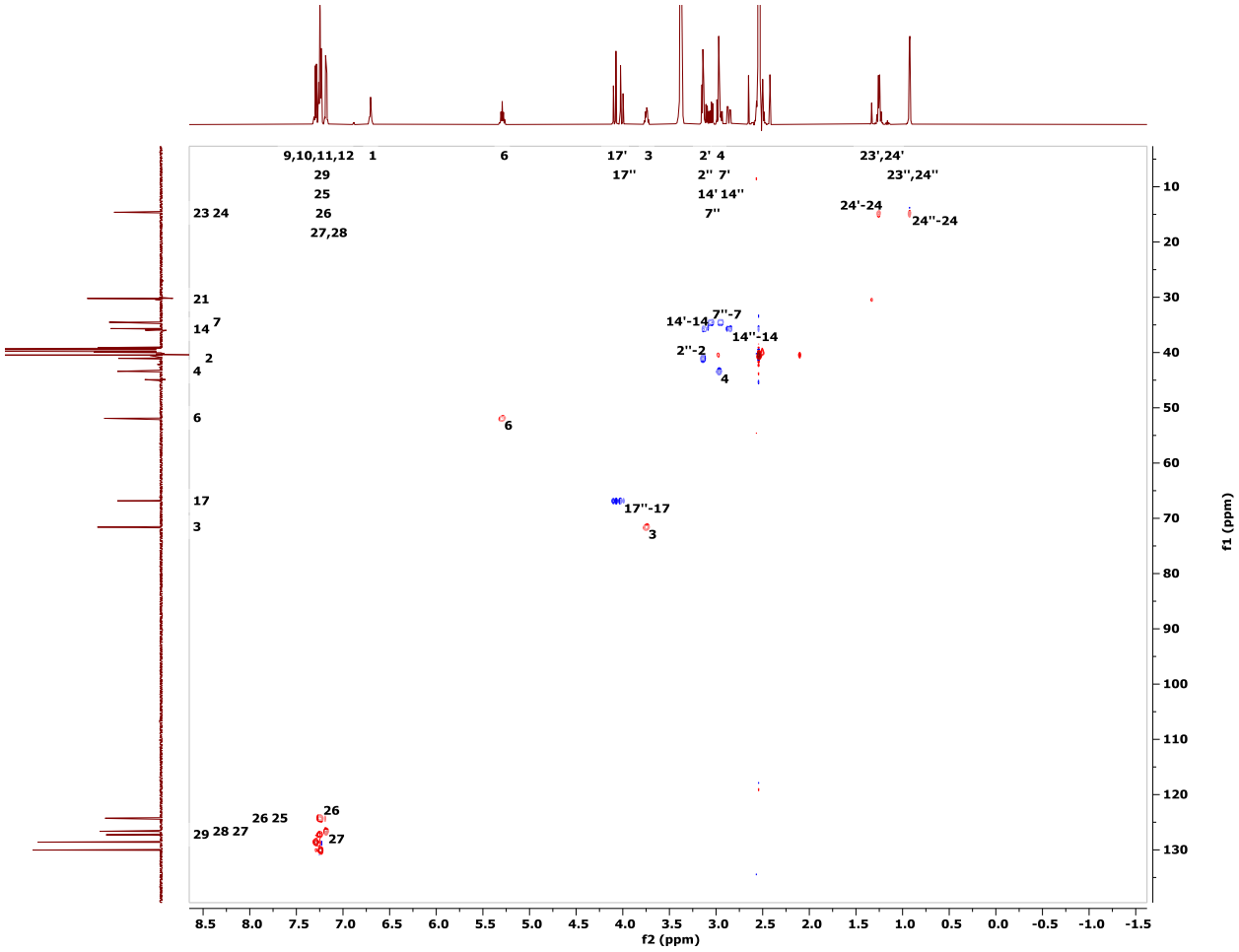
Supplementary Figure 21. 600.1 MHz ^{1}H spectrum of OXM4 in DMSO-d$_6$.



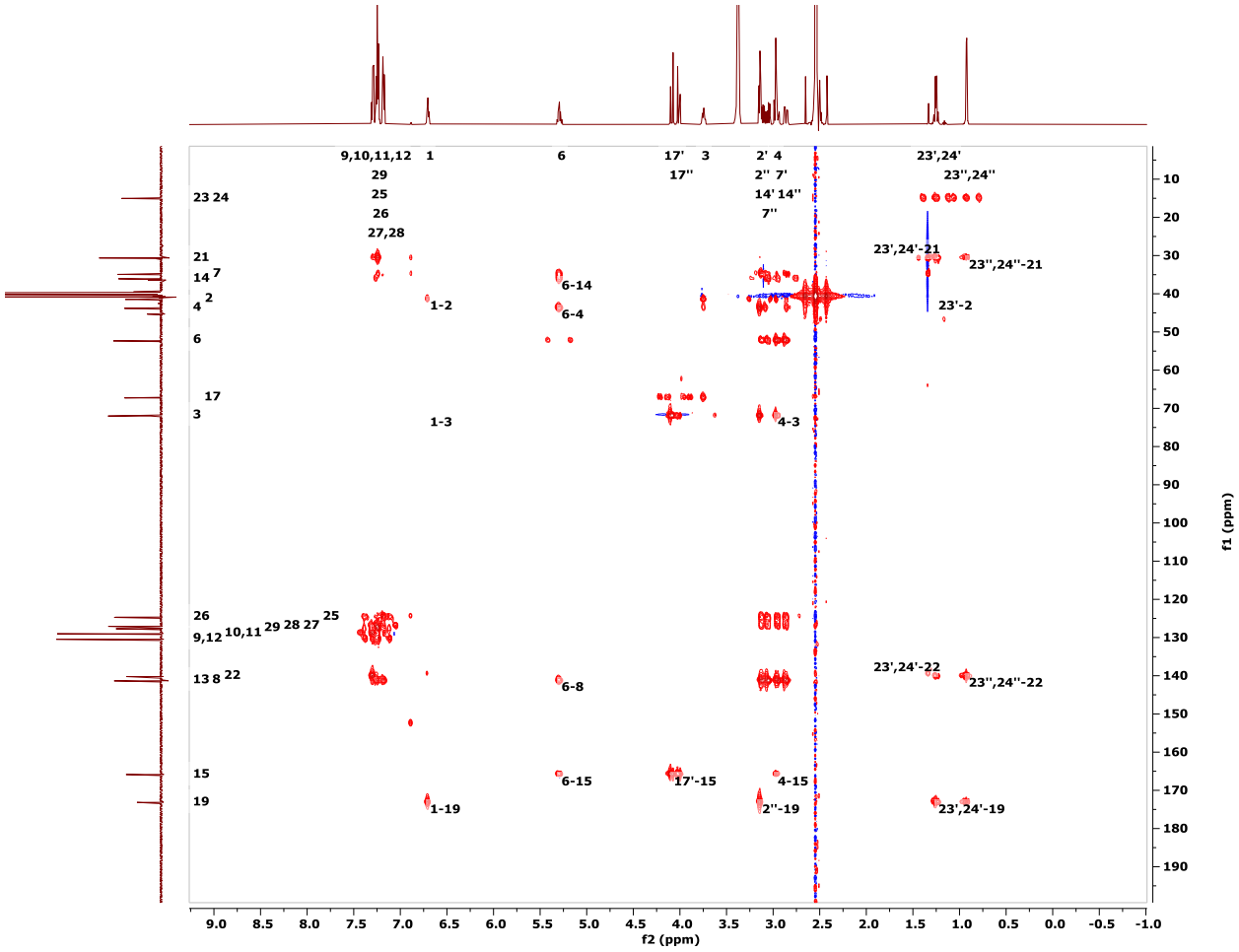
Supplementary Figure 22. 150.9 MHz ^{13}C spectrum of OXM4 in DMSO-d$_6$.



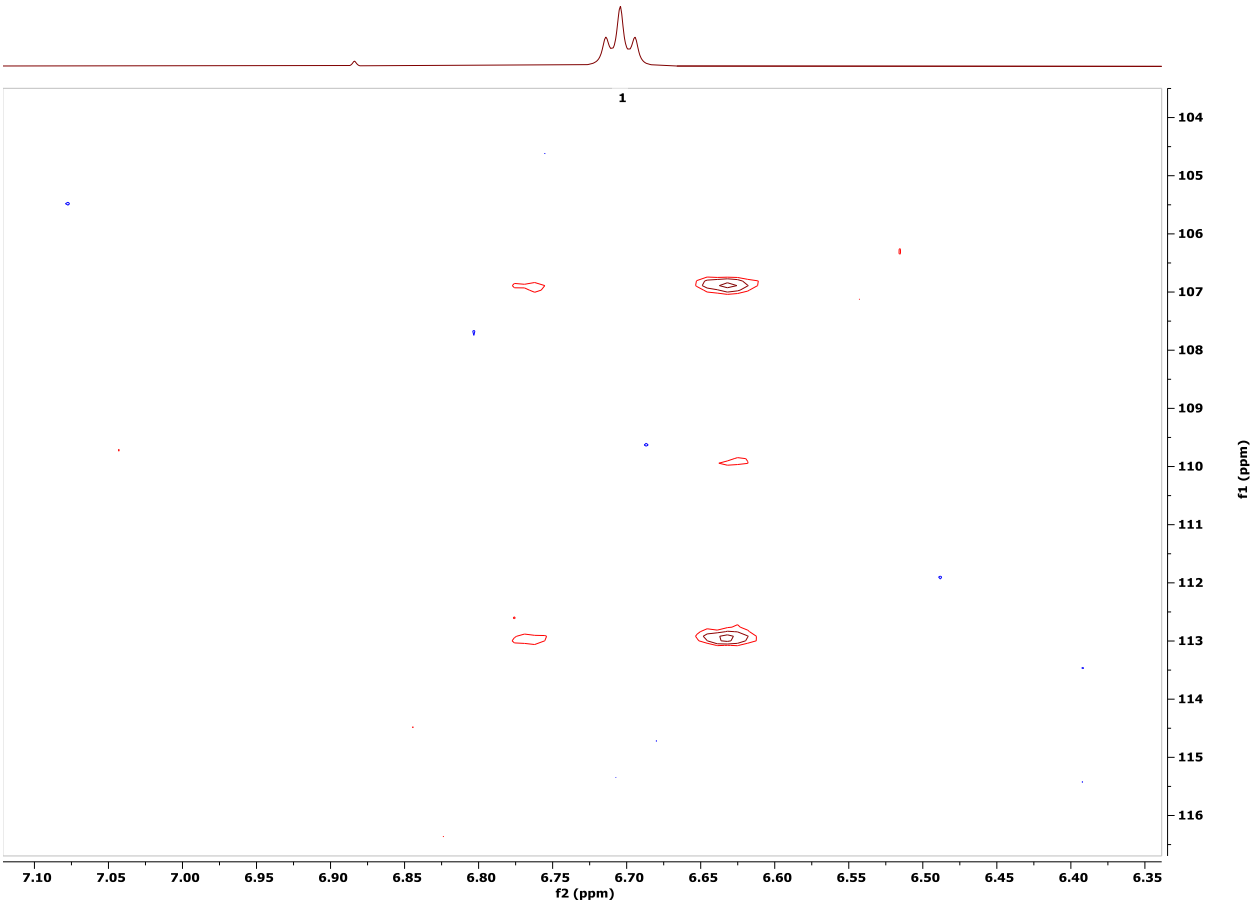
Supplementary Figure 23. 600.1 MHz COSY spectrum of OXM4 in DMSO-d_6.



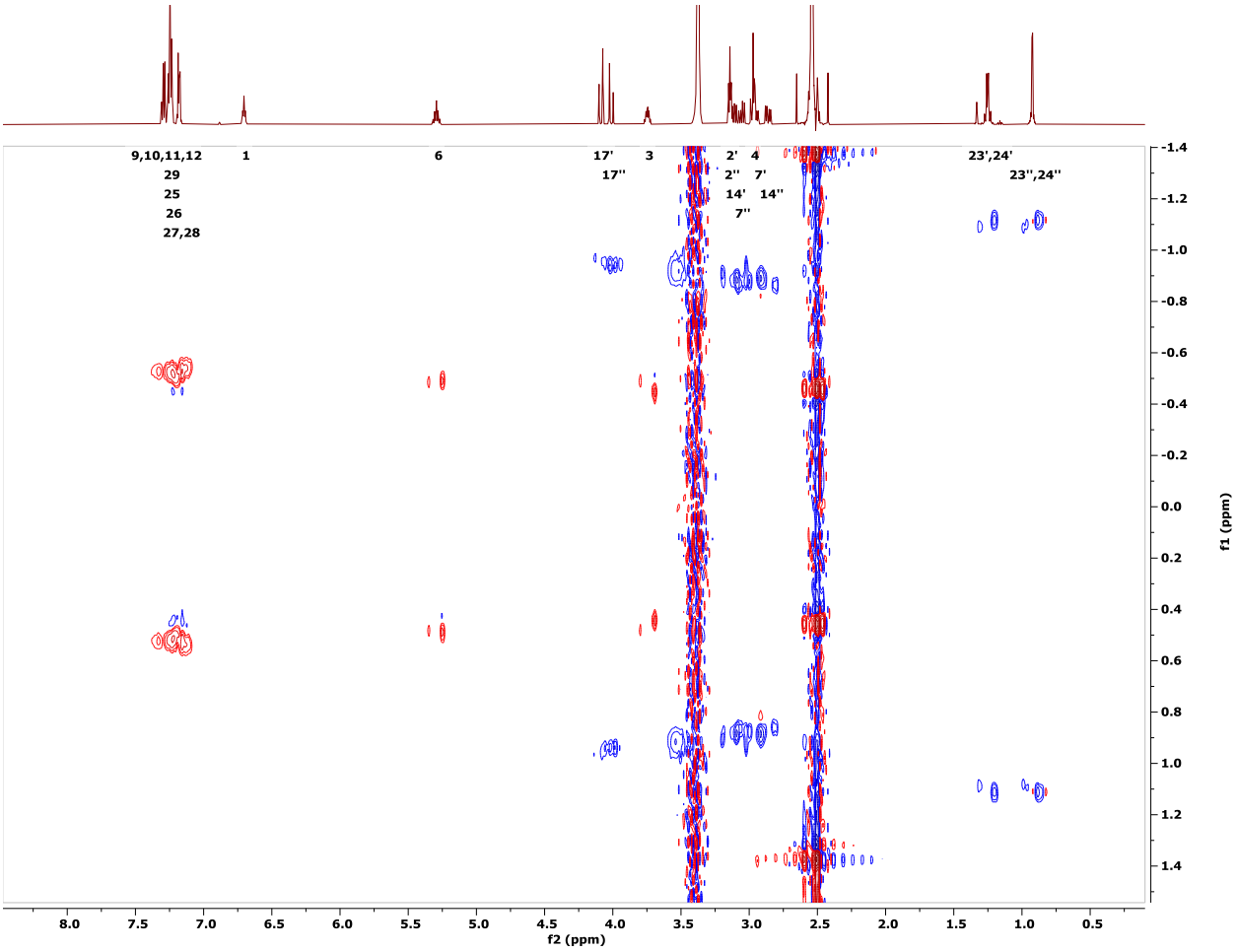
Supplementary Figure 24. 600.1 MHz HC-HSQC spectrum of OXM4 in DMSO-d6.



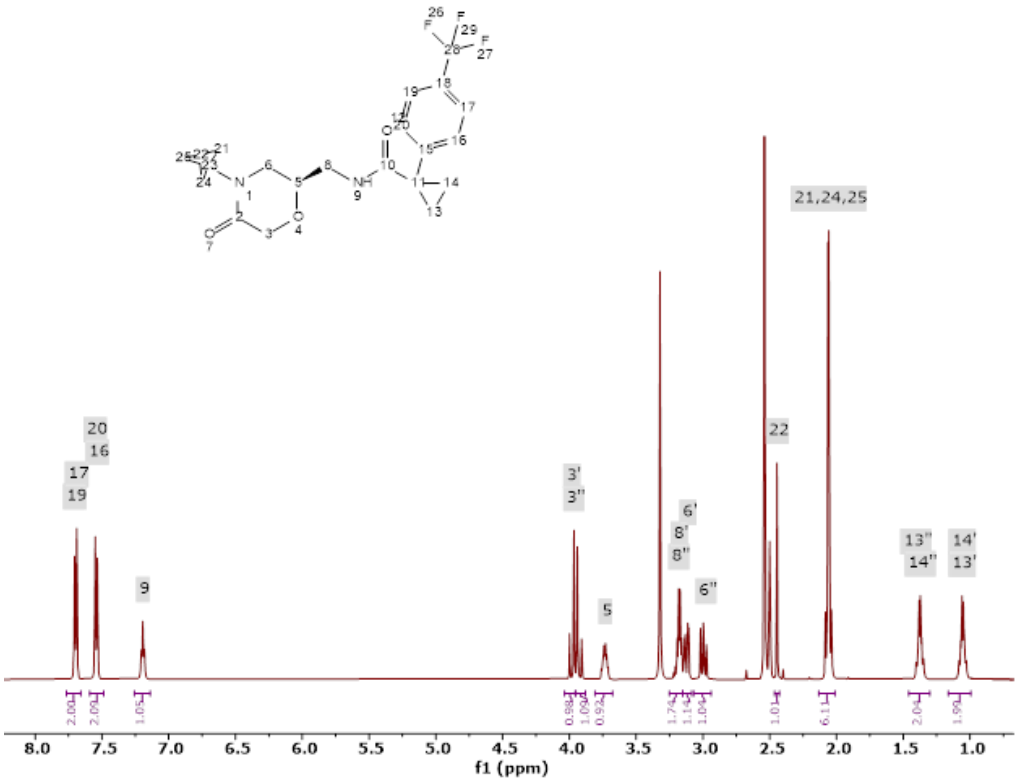
Supplementary Figure 25. 600.1 MHz HC-HMBC spectrum of OXM4 in DMSO-d6.



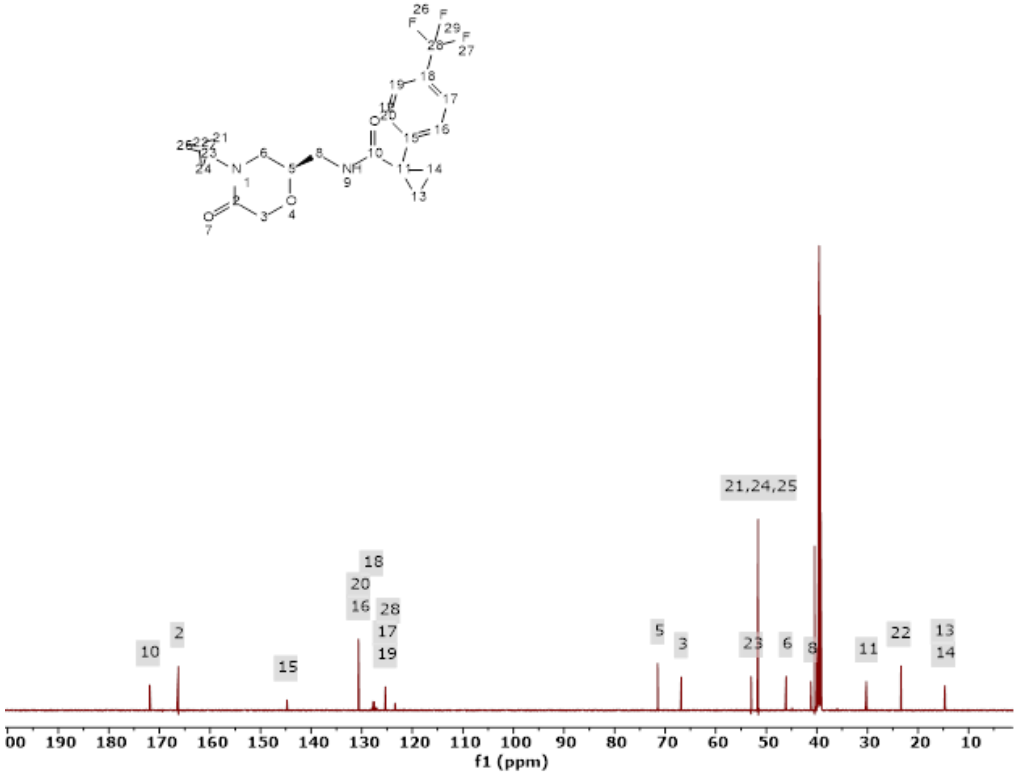
Supplementary Figure 26. 600.1 MHz JSBHN-HSQC spectrum of OXM4 in poly-HEMA gel in DMSO-d_6.



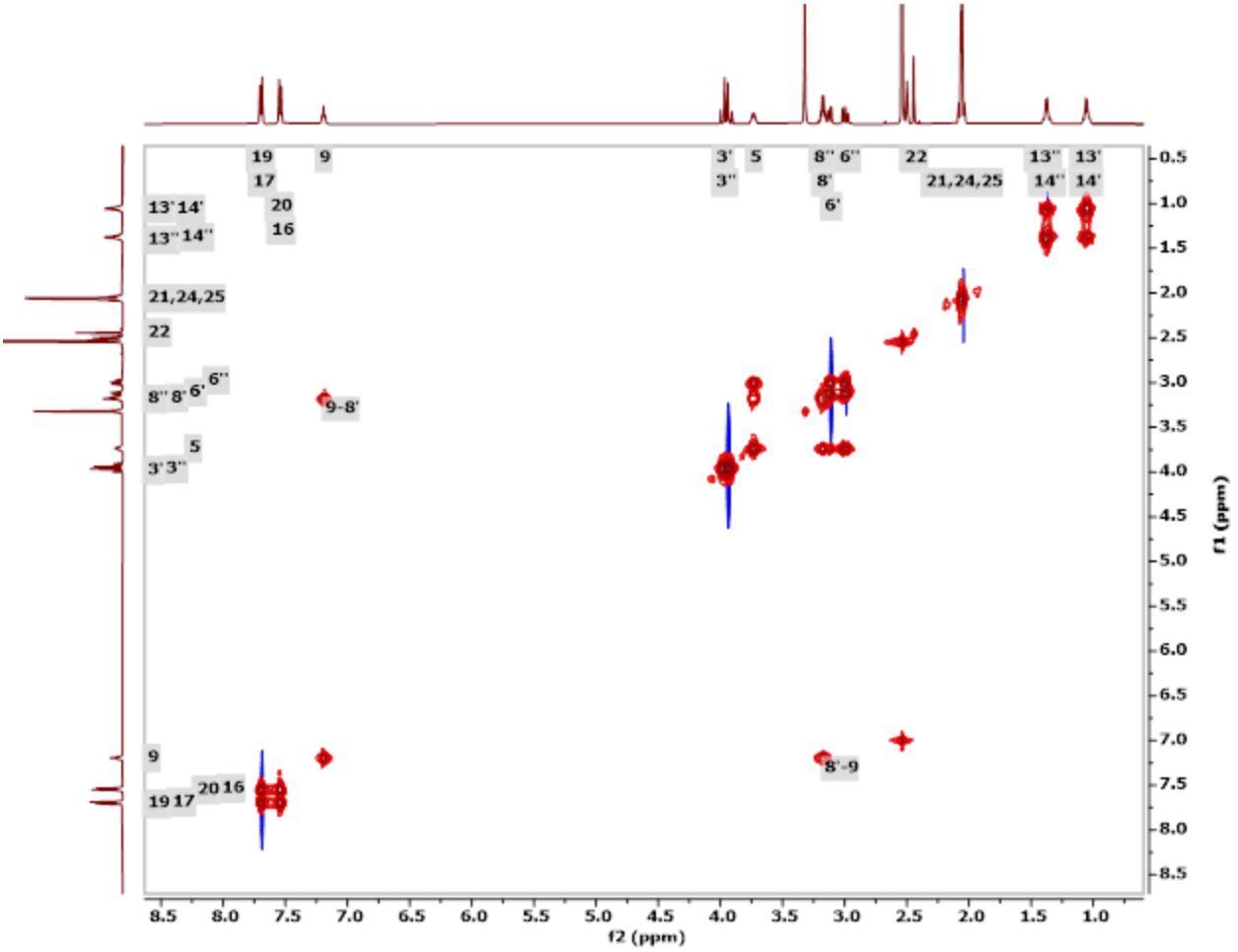
Supplementary Figure 27. 600.1 MHz JSBHC-HSQC spectrum of OXM4 in poly-HEMA gel in DMSO-d_6.



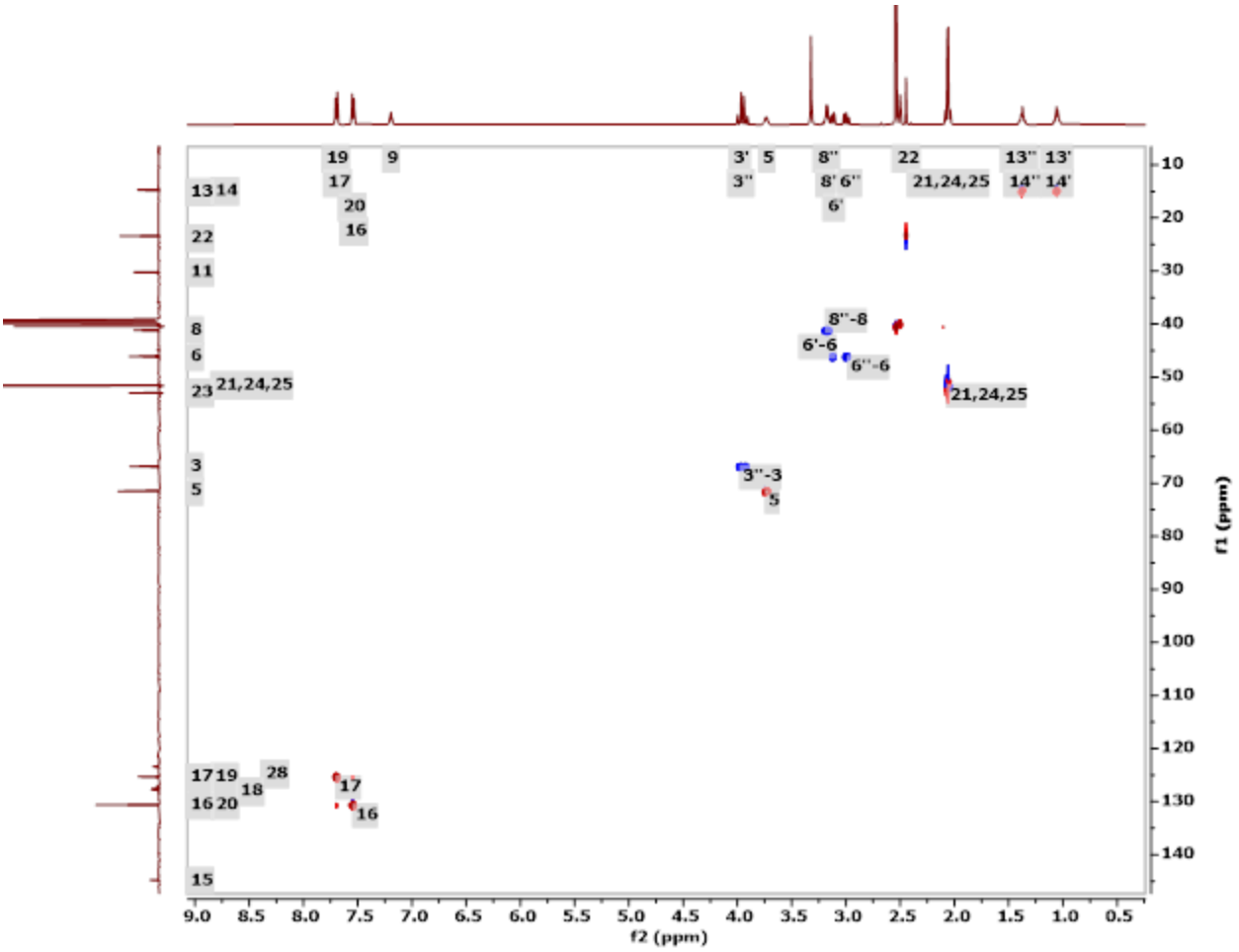
Supplementary Figure 28. 600.1 MHz ^{1}H spectrum of OXM2 in DMSO-d$_6$.



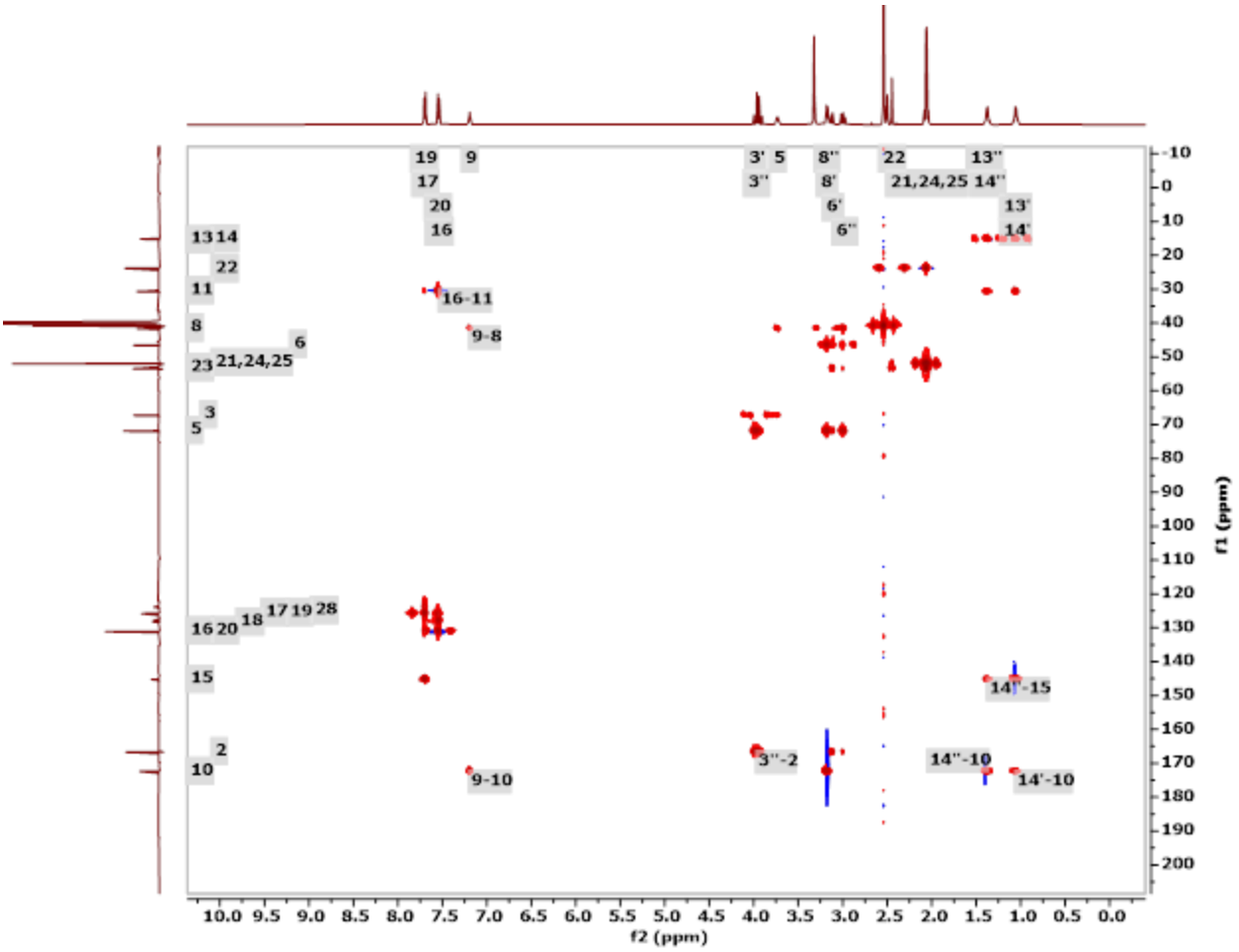
Supplementary Figure 29. 150.9 MHz ^{13}C spectrum of OXM2 in DMSO-d$_6$.



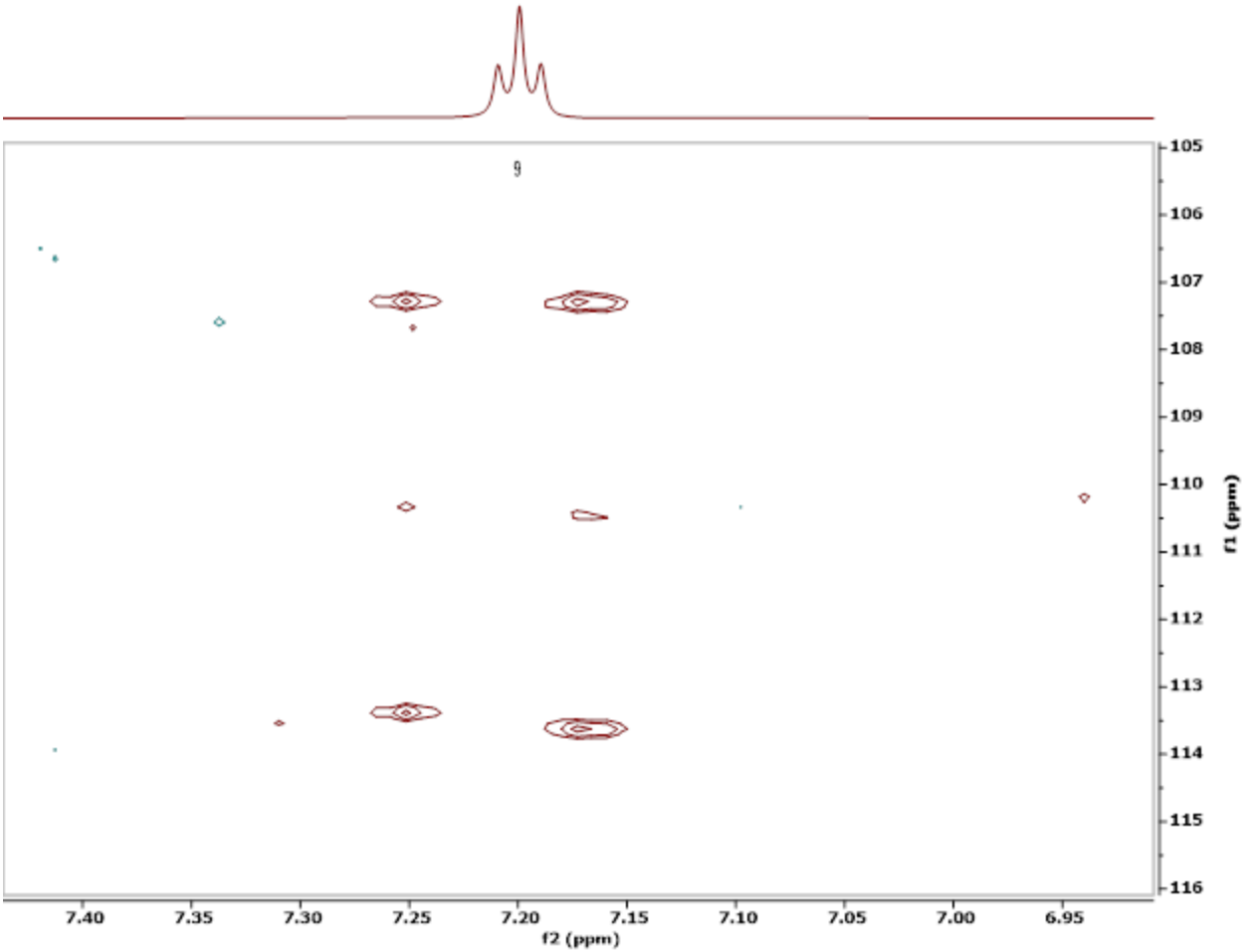
Supplementary Figure 30. 600.1 MHz COSY spectrum of OXM2 in DMSO-d$_6$.



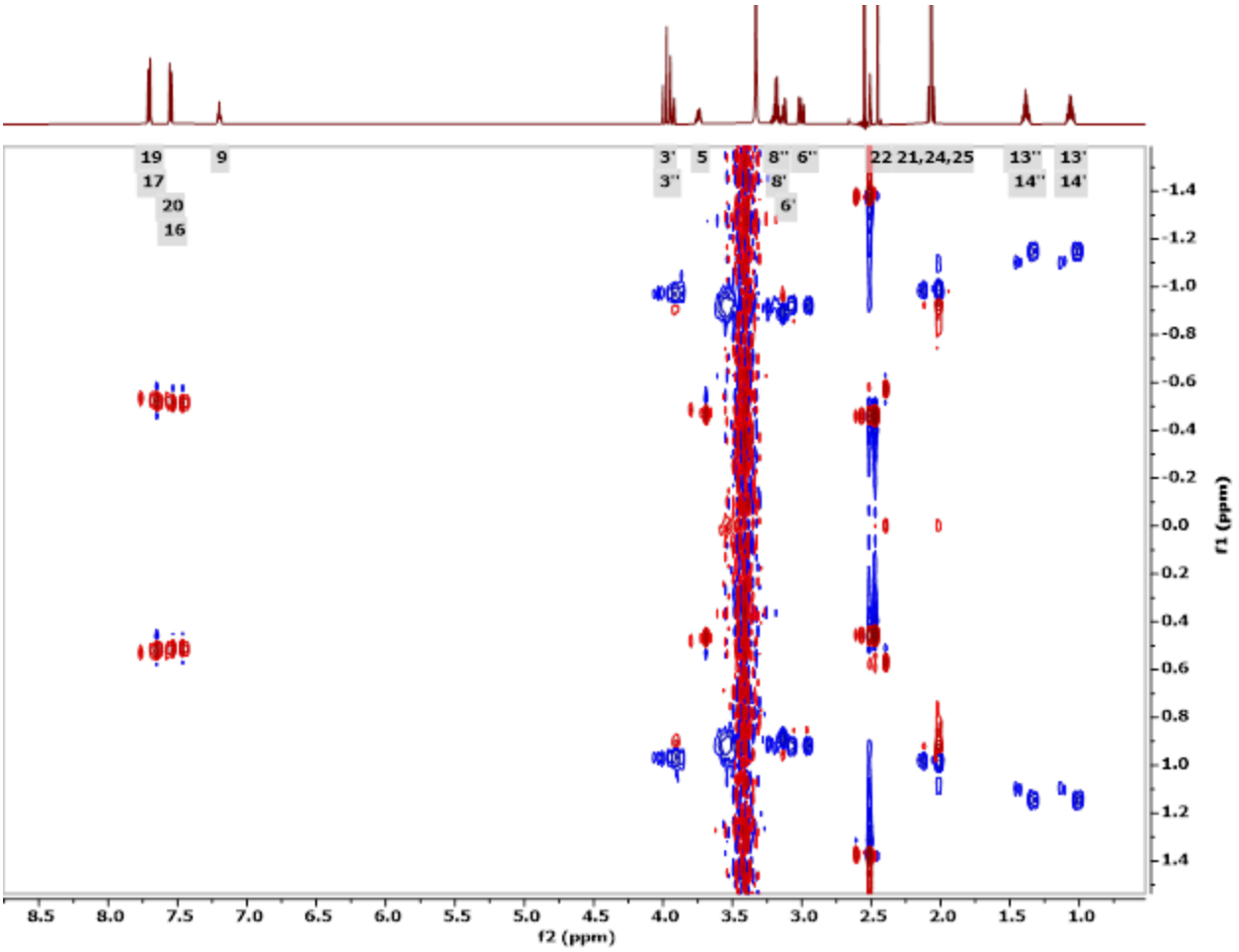
Supplementary Figure 31. 600.1 MHz HC-HSQC spectrum of OXM2 in DMSO-d_6.



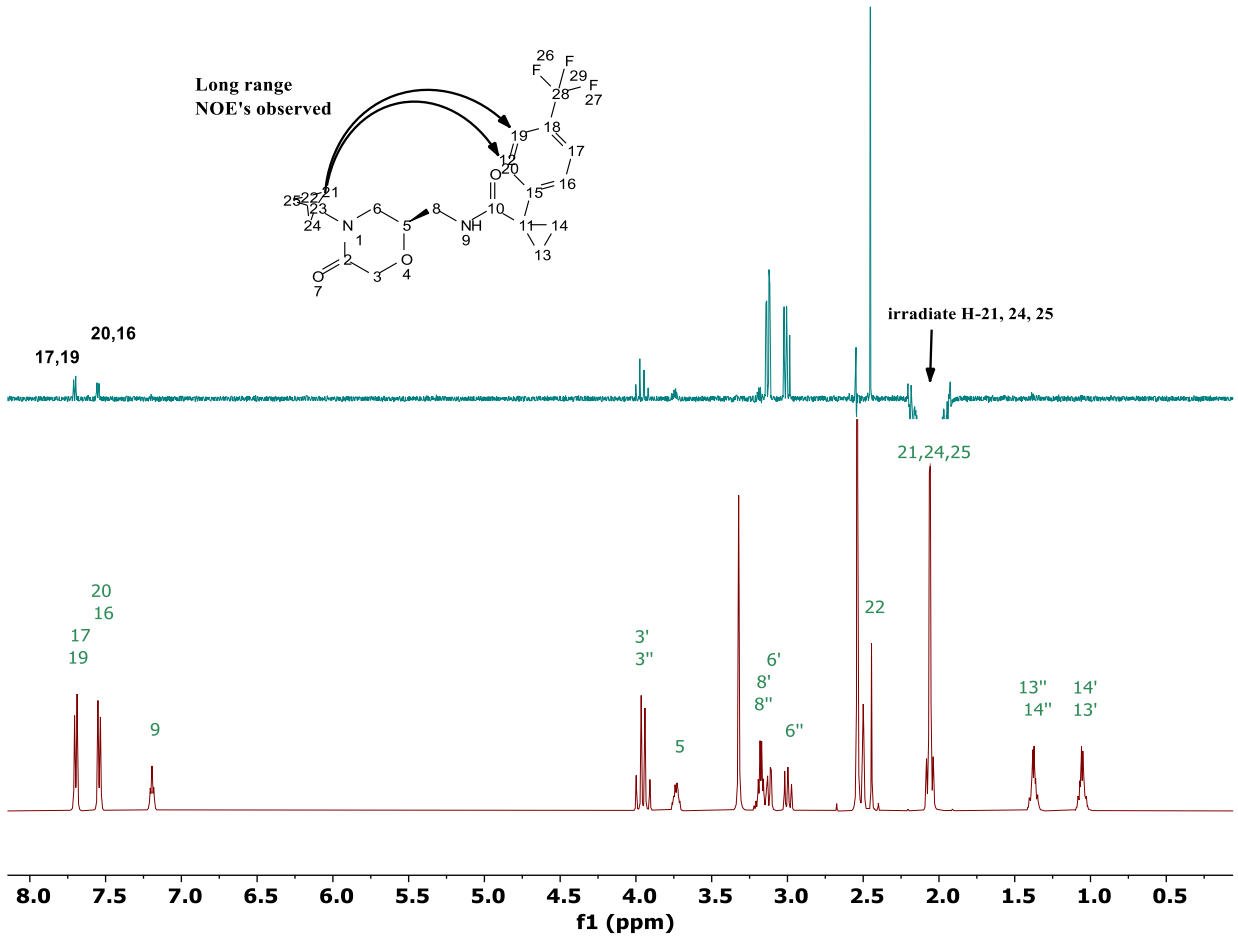
Supplementary Figure 32. 600.1 MHz HC-HMBC spectrum of OXM2 in DMSO-d6.



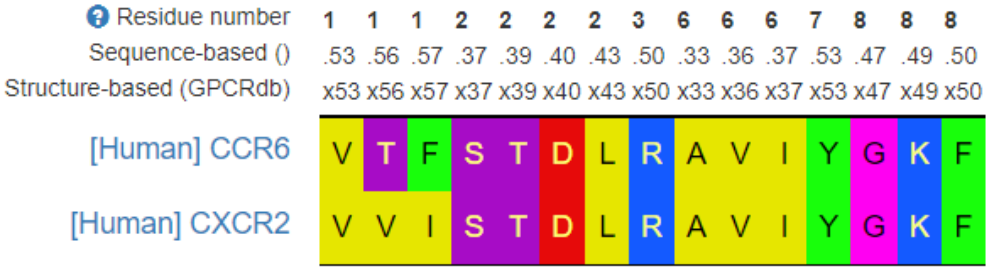
Supplementary Figure 33. 600.1 MHz JSBHN-HSQC spectrum of OXM2 in poly-HEMA gel in DMSO-d_6.



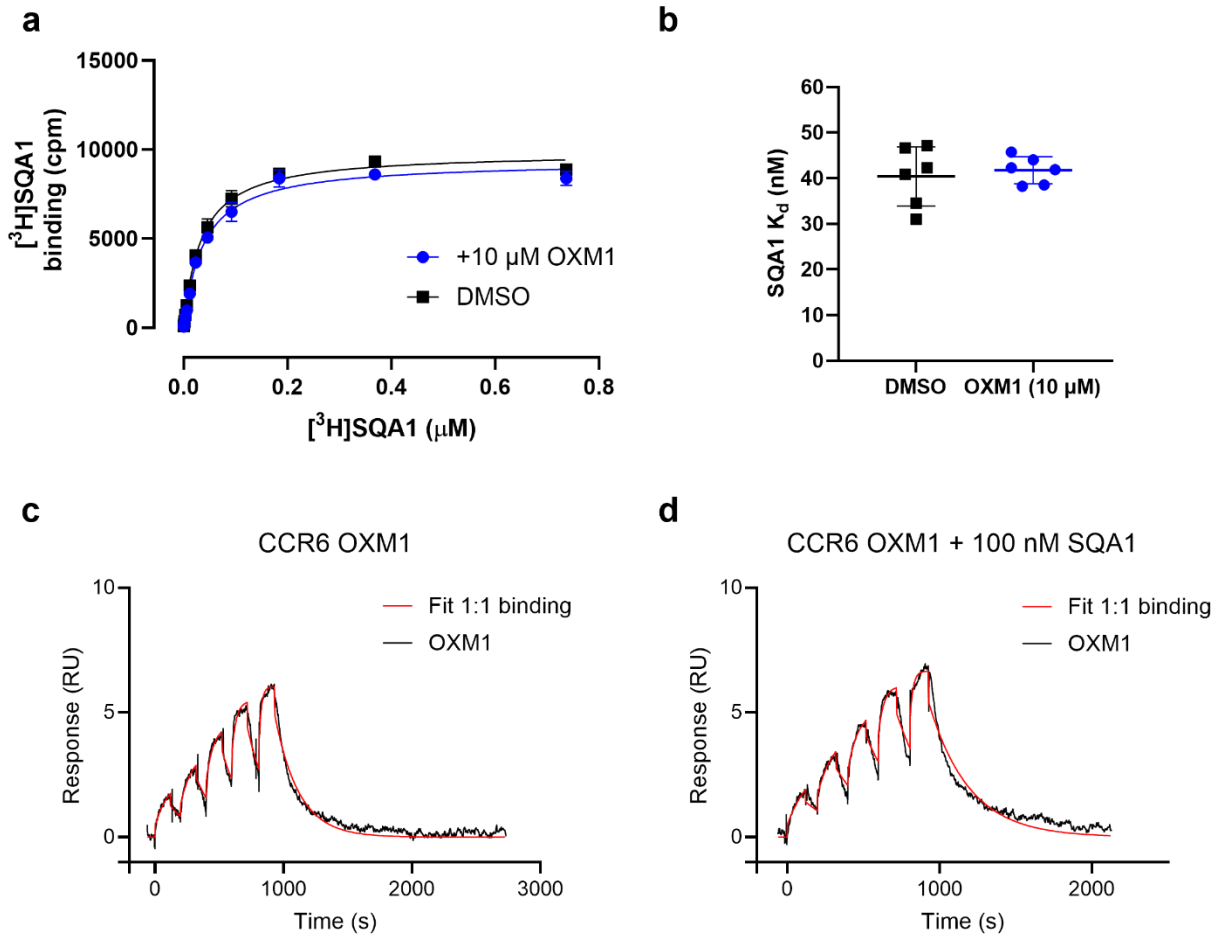
Supplementary Figure 34. 600.1 MHz JSBHC-HSQC spectrum of OXM2 in poly-HEMA gel in DMSO-d_6.



Supplementary Figure 35. Nuclear Overhauser Enhancement signals (NOEs) observed for OXM2 in DMSO-d_6. The observation of long-range NOEs from H-21/24/25 to H-17/H-19 and to H20/H-16 suggests that OXM2 adopts a U-shaped conformation in solution.



Supplementary Figure 36. Structure-based sequence alignment of human CXCR2 and human CCR6 at the SQA analogue SQA1 binding site. The sequence alignment was performed using servers from www.GPCRdb.org[55].



Supplementary Figure 37. No crosstalk between OXM1 and SQA1 on binding to CCR6. a, Representative saturation binding curves of [^{3}H]-SQA1 to WT CCR6 in the absence (black squares) and presence (blue circles) of 10 μM of OXM1. Data are presented in mean ± s.d. from four technical replicates or two biological replicates within the representative experiment. **b**, Kd of SQA1 from the saturation binding experiment in the absence (black squares) and presence (blue circles) of 10 μM of OXM1. Kd values from each biological replicate are shown. Average Kd shown as mean ± s.d. are from n = 3 independent experiments. **c-d**, Representative SPR sensorgrams of OXM1 binding to purified CCR6_Nβ10.2 protein in the absence **c**, or preincubated with 100 nM of SQA1, **d**. Experimental data (black curves) and fitting using a model of 1:1 binding (red curves) are shown. Average k_a, k_d, and K_D in mean ± s.d. from n = 3 independent experiments are 5.2E+04 ± 9.0E+03 1/Ms, 6.1E-03 ± 5.4E-04 1/s, and 121.107 ± 25 nM, respecitvely, for SQA1-free condition, and 6.4E+04 ± 1.5E+04 1/Ms, 4.9E-03 ± 1.6E-03 1/s, and 78.6 ± 20.7 nM, respectively, for SQA1-preincubated condition (**Supplementary Table 4**). Data graphs in **a-d** were prepared using GraphPad Prism v9.5.1. Source data are provided as a Source Data file.

Supplementary Tables

Supplementary Table 1. Cryo-EM data collection, refinement, and validation statistics.

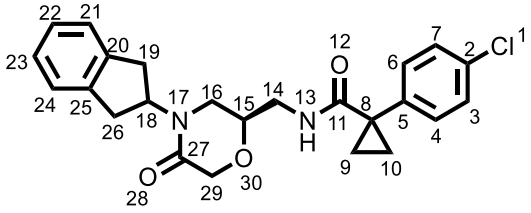
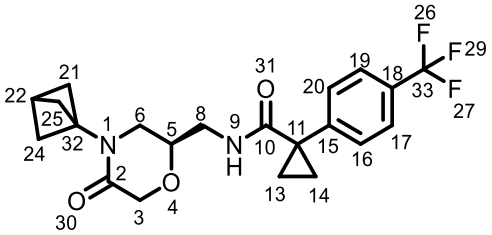
	CCR6/SQA1/OXM1 (EMD-46534) (PDB ID 9D3G)	CCR6/SQA1/OXM2 (EMD-46533) (PDB ID 9D3E)
Data collection and processing		
Magnification	215,000	130,000
Voltage (kV)	300	300
Electron exposure (e–/Å^2)	40	45
Defocus range (μm)	-0.8 to -2.4	-0.4 to -1.8
Pixel size (Å)	0.575	0.654
Symmetry imposed	C1	C1
Initial particle images (no.)	5,332,464	10,786,639
Final particle images (no.)	482,484	368,745
Map resolution (Å)	3.3	3.0
FSC threshold	0.143	0.143
Refinement		
Initial model used (PDB code)	Alphafold, 6WW2	Alphafold, 6WW2
Model resolution (Å)	3.3	3.17
FSC threshold	0.5	0.5
Map sharpening *B* factor (Å^2)	-92	-112
Model composition		
Non-hydrogen atoms	7403	7173
Protein residues	946	918
B factors (Å^2)		
Protein	79.55	79.23
Ligand	110.12	109.95
R.m.s. deviations		
Bond lengths (Å)	0.005	0.004
Bond angles (°)	0.945	0.854
Validation		
MolProbity score	1.54	1.24
Clashscore	6.26	4.00
Poor rotamers (%)	0.0	0.0
Ramachandran plot		
Favored (%)	96.78	97.78
Allowed (%)	3.22	2.22
Disallowed (%)	0.0	0.0

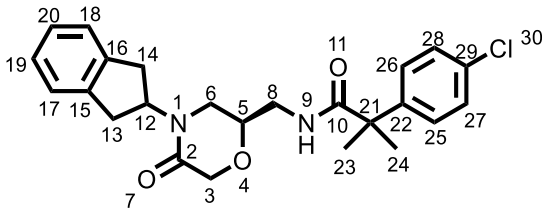
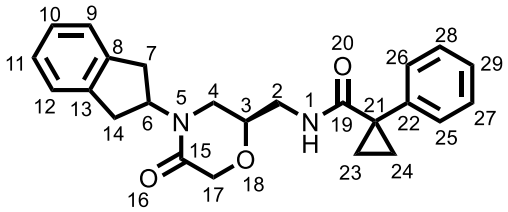
Supplementary Table 2. OXM1 profiling against the chemokine receptor panel from DiscoveRx® in both agonist and antagonist modes. 1 μM of OXM1 was used in the profiling.

GPCR ID	Assay Mode	Conc (μM)	Rep 1 RLU	Rep 2 RLU	Mean RLU	SD	%CV	% Inhibition
CCR1	Antagonist	1	1282680	1296960	1289820	10097	1%	4%
CCR10	Antagonist	1	1303960	1318240	1311100	10097	1%	-4%
CCR2	Antagonist	1	2300480	2274440	2287460	18413	1%	-13%
CCR3	Antagonist	1	814520	780360	797440	24155	3%	-14%
CCR4	Antagonist	1	1175720	1201200	1188460	18017	2%	-2%
CCR5	Antagonist	1	916160	823760	869960	65337	8%	2%
CCR6	Antagonist	1	543200	504000	523600	27719	5%	51%
CCR7	Antagonist	1	3079160	3257240	3168200	125922	4%	2%
CCR8	Antagonist	1	1331400	1292760	1312080	27323	2%	1%
CCR9	Antagonist	1	874160	871640	872900	1782	0%	2%
CMKLR1	Antagonist	1	1523760	1417360	1470560	75236	5%	-2%
CX3CR1	Antagonist	1	143360	147280	145320	2772	2%	8%
CXCR1	Antagonist	1	2775080	3118360	2946720	242736	8%	7%
CXCR2	Antagonist	1	964320	1045520	1004920	57417	6%	0%
CXCR3	Antagonist	1	1285200	1311800	1298500	18809	1%	-4%
CXCR4	Antagonist	1	159880	150640	155260	6534	4%	2%
CXCR5	Antagonist	1	1237600	1119440	1178520	83552	7%	-2%
CXCR6	Antagonist	1	107520	101080	104300	4554	4%	13%
CXCR7	Antagonist	1	980840	1066520	1023680	60585	6%	9%
XCR1	Antagonist	1	18480	19600	19040	792	4%	13%
GPCR ID	**Assay Mode**	**Conc (μM)**	**Rep 1 RLU**	**Rep 2 RLU**	**Mean RLU**	**SD**	**%CV**	**% Activity**
CCR1	Agonist	1	679280	717080	698180	26729	4%	4%
CCR10	Agonist	1	96600	96600	96600	0	0%	0%
CCR2	Agonist	1	71120	95200	83160	17027	20%	1%

CCR3	Agonist	1	249760	244440	247100	3762	2%	1%
CCR4	Agonist	1	174160	148680	161420	18017	11%	1%
CCR5	Agonist	1	106400	111160	108780	3366	3%	0%
CCR6	Agonist	1	67480	66920	67200	396	1%	-1%
CCR7	Agonist	1	788480	758240	773360	21383	3%	-1%
CCR8	Agonist	1	40880	43120	42000	1584	4%	0%
CCR9	Agonist	1	153440	153160	153300	198	0%	0%
CMKLR1	Agonist	1	101360	94640	98000	4752	5%	0%
CX3CR1	Agonist	1	9240	10360	9800	792	8%	0%
CXCR1	Agonist	1	212240	226240	219240	9899	5%	0%
CXCR2	Agonist	1	190400	201600	196000	7920	4%	1%
CXCR3	Agonist	1	509600	470960	490280	27323	6%	2%
CXCR4	Agonist	1	90440	82600	86520	5544	6%	9%
CXCR5	Agonist	1	230720	213920	222320	11879	5%	1%
CXCR6	Agonist	1	24920	26320	25620	990	4%	3%
CXCR7	Agonist	1	92120	107240	99680	10691	11%	0%
XCR1	Agonist	1	6440	5880	6160	396	6%	-2%

Supplementary Table 3. Comparison of RDC data from OXM1, OXM3 and OXM4 in compressed poly-HEMA gels in DMSO-d_6 with calculated $^1D_{CH}$ and $^1D_{NH}$ couplings.

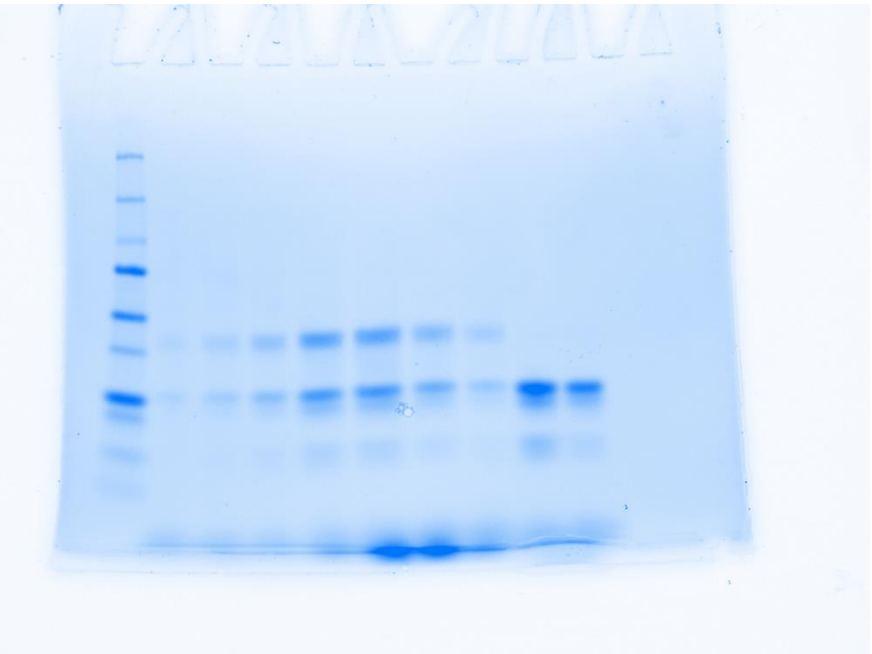
Compound	Experimental NMR Data		RDC-Refined Structure	
	$^1D_{CH}$ (Hz) in DMSO-d_6	$^1D_{NH}$ (Hz) in DMSO-d_6	Calculated $^1D_{CH}$ (Hz)	Calculated ^{1}DNH (Hz)
OXM1				
C22-H49	-0.3	-	-0.3	-
C18-H45	0.6	-	0.6	-
C15-H42	-8.9	-	-8.9	-
C7-H34	-6.1	-	-6.1	-
C29-H54/H55	-3.0	-	-3.0	-
C26-H52/H53	-5.5	-	-5.5	-
C10-H37/H38	6.3	-	6.3	-
N13-H39	-	9.0	-	9.0
OXM2				
C14-H40/H41	5.8	-	6.1	-
C22-H48	-1.8	-	-1.8	-
C6-H34/H33	0.11	-	1.0	-
C8-H35/H36	-2.0	-	-2.2	-
C5-H32	-3.6	-	-3.6	-
C3-H30H/31	-0.02	-	-0.46	-
C20-H45	-7.6	-	-7.6	-
C24-H49/H50	-1.0	-	-1.0	-
N9-H37	-	-4.4	-	-4.4

OXM3				
C5-H33	-8.5	-	-8.8	-
C12-H39	1.0	-	1.6	-
C27-H56	1.7	-	3.3	-
C3-H31/H32	-3.5	-	-3.5	-
C13-H40/H41	0.4	-	0.4	-
C8-H36/H37	-3.3	-	-3.3	-
C23-H48/H49/H50	0.7	-	0.6	-
N9-H38	-	2.8	-	3.2
OXM4				
C6-H36	2.9	-	2.9	-
C3-H33	13.3	-	13.3	-
C27-H53	4.4	-	4.6	-
C17-H45/H46	-2.9	-	-2.4	-
C14-H43/H44	0	-	0.2	-
C23-H47/H48	4.4	-	4.6	-
N1-H30	-	-0.7	-	-0.7

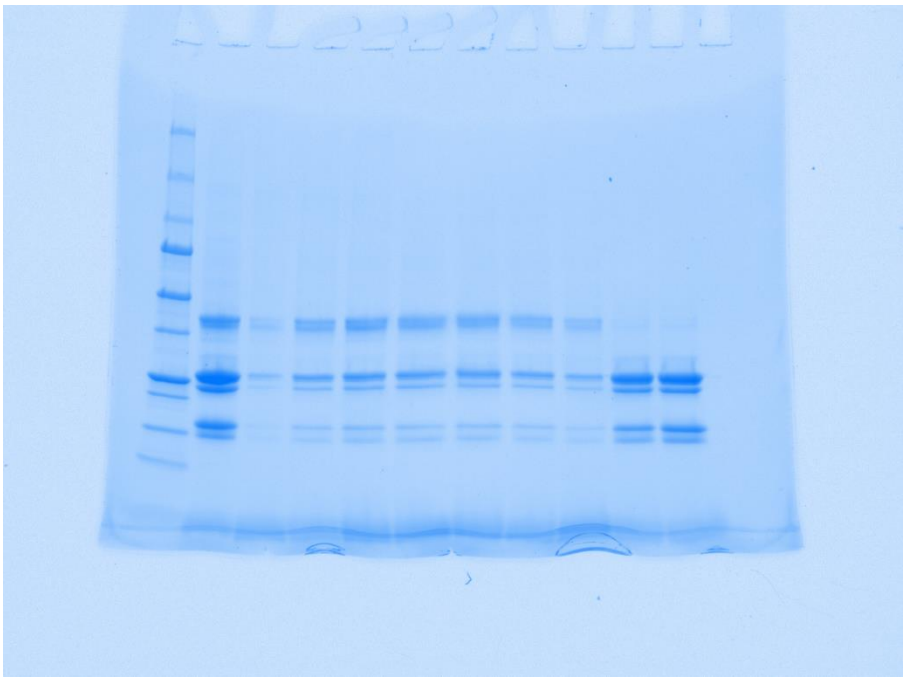
Supplementary Table 4. Summary of crosstalk SPR experimental conditions, maximal surface response (Rmax), binding kinetics (k_a, k_b), and K_D from each individual experiment and average reported in mean ± s.d. from n = 3 independent experiments. SPR experiments to measure OXM1 binding were performed with purified CCR6_Nβ10.2 protein in SQA1-free (-SQA1) and SQA1-preincubated conditions as indicated.

Compound	Condition	Expt.	k_a (1/Ms)	k_d (1/s)	K_D (nM)	Rmax (RU)	Comments
OXM1	- SQA1	1	4.9E+04	5.4E-03	110	5.5	Supplementary Fig. 37c
		2	6.4E+04	6.1E-03	96.0	6.0	
		3	4.3E+04	6.7E-03	156.7	6.3	
		Average	**5.2E+04 (±9.0E+03)**	**6.1E-03 (±5.4E-04)**	**121.107 (±25)**		
OXM1	+ SQA1 (100 nM)	1	7.5E+04	4.2E-03	56.7	4.2	
		2	6.9E+04	6.8E-03	97.9	5.7	
		3	4.7E+04	3.8E-03	81.1	5.9	Supplementary Fig. 37d
		Average	**6.4E+04 (±1.5E+04)**	**4.9E-03 (±1.6E-03)**	**78.6 (±20.7)**		

Uncropped SDS-PAGE Images



Uncropped SDS-PAGE image of Supplementary Figure 3a.



Uncropped SDS-PAGE image of Supplementary Figure 3b.