Supporting Information

Discovery of a Novel Benzodiazepine Series of Cbl-b Inhibitors for the Enhancement of Antitumor Immunity

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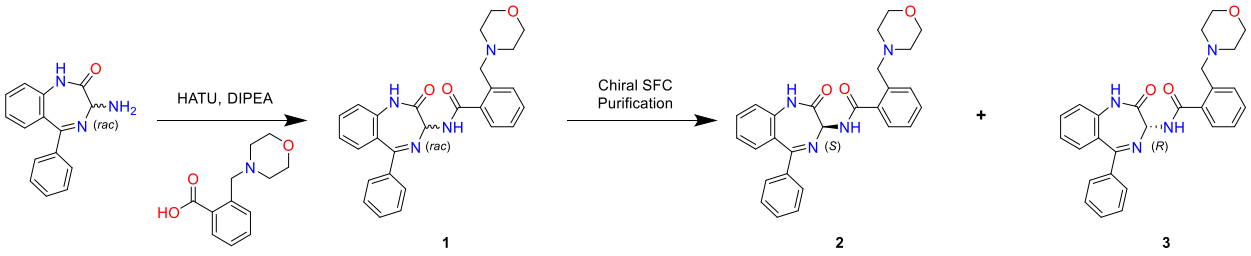
I. General Information:

- Reagents were purchased from commercial sources and were used as received unless otherwise stated.
- All starting materials are either from commercial sources or synthesized according to referenced procedures. **Ex23** was made according to literature procedure.[1]
- All reactions were performed using borosilicate glass vials of the reported sizes. Heating sources were used as specified, using either an oil bath or a Biotage® Initiator+.
- Chromatographic purification of products was accomplished by C_{18} or silica gel chromatography using Biotage® Selekt SEL-2SV.
- ^{1}H spectra were recorded on a BRUKER AscendTM 400/500/600 MHz spectrometer and were internally referenced to residual protio-solvent signals ($CDCl_3$ referenced at 7.27 ppm, CD_2Cl_2 referenced at 5.32 ppm, CD_3OD referenced at 3.31 ppm, and DMSO-d_6 referenced at 2.49 ppm). Data for ^{1}H NMR were reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, t = triplet, q = quartet, m = multiplet, b = broad), integration, and coupling constant (Hz).
- The HRMS were obtained using a hybrid quadrupole time-of-flight mass spectrometer in ESI+ and ESI- mode.
- Stereochemistry of **2** and **25** are determined by crystal structure analysis through binding to Cbl-b to be (*S*)-configuration on the azepine core (see section VII), with the (*S*)-isomer at least 10x more potent in the TR-FRET assay than the respective (*R*)-isomer in both cases. Therefore, all other chirally purified compounds (**21-24**) have stereochemistry assigned based on comparative TR-FRET activity, where the (*S*)-isomer on the azepine core is assumed to be the more potent isomer.
- Chiral separation data has an output style change after compound **22** due to upgrading to a newer SFC instrument.
- Safety Statement: No unexpected or unusually high safety hazards were encountered. All laboratory practices were done under strict adherence to SHE department policy. (SHE = Safety, Health and Environment)

II. Synthetic Procedures for Key Compounds:

Synthesis of Compounds **1**-**3**:



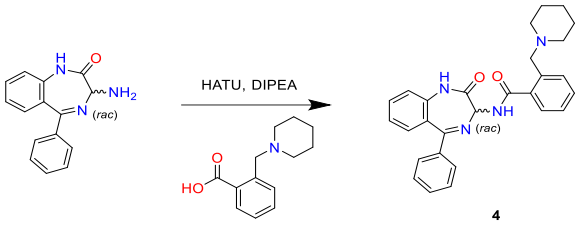
In a 30 mL scintillation vial equipped with a stir bar, *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (100 mg, 0.40 mmol, 1.0 equiv), 2-(morpholinomethyl)benzoic acid (97 mg, 0.44 mmol, 1.1 equiv), and HATU (227 mg, 0.60 mmol, 1.5 equiv) were added. The mixture was dissolved in DMF (4 mL), followed by the addition of DIPEA (174 µl, 0.99 mmol, 2.5 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **1** (158 mg, 87%) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) 2.51 (br s, 4H), 3.46 - 3.61 (m, 4H), 3.67 (d, *J* = 12.1 Hz, 1H), 3.90 (d, *J* = 11.9 Hz, 1H), 5.46 (d, *J* = 7.6 Hz, 1H), 7.23 - 7.29 (m, 1H), 7.31 - 7.39 (m, 3H), 7.42 - 7.55 (m, 7H), 7.62 - 7.70 (m, 1H), 7.70 - 7.75 (m, 1H), 10.89 (s, 1H), 11.60 (d, *J* = 7.6 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{27}H_{27}N_4O_3^+$: 455.2083. Found: 455.2086.

Compound **1** was further purified by chiral SFC to obtain compound **2** (67 mg, >98:2 er) and compound **3** (68 mg, 97:3 er) as white solids. (see SFC Report in Spectral Data section III). Absolute stereochemistry of **2** was determined by crystal structure analysis (see section VII). Absolute stereochemistry of **3** was inferred via crystal structure analysis of **2**.

Compound **2**: ^{1}H NMR (500 MHz, DMSO-d_6) 2.50 - 2.57 (m, 4H), 3.49 - 3.62 (m, 4H), 3.67 (d, *J* = 12.1 Hz, 1H), 3.90 (d, *J* = 12.1 Hz, 1H), 5.44 (d, *J* = 7.5 Hz, 1H), 7.23 - 7.28 (m, 1H), 7.30 - 7.38 (m, 3H), 7.41 - 7.49 (m, 4H), 7.49 - 7.54 (m, 3H), 7.65 (t, *J* = 7.7 Hz, 1H), 7.72 (dd, *J* = 7.5, 1.1 Hz, 1H), 10.88 (br s, 1H), 11.57 (br d, *J* = 7.3 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{27}H_{27}N_4O_3^+$: 455.2083. Found: 455.2083.

Compound **3**: ^{1}H NMR (500 MHz, DMSO-d_6) 2.50 - 2.57 (m, 4H), 3.50 - 3.60 (m, 4H), 3.67 (br d, *J* = 12.1 Hz, 1H), 3.90 (br d, *J* = 11.9 Hz, 1H), 5.45 (d, *J* = 7.5 Hz, 1H), 7.23 - 7.28 (m, 1H), 7.31 - 7.39 (m, 3H), 7.41 - 7.49 (m, 4H), 7.51 (br d, *J* = 6.9 Hz, 3H), 7.66 (br t, *J* = 7.6 Hz, 1H), 7.72 (br d, *J* = 7.2 Hz, 1H), 10.88 (br s, 1H), 11.59 (br d, *J* = 7.5 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{27}H_{27}N_4O_3^+$: 455.2083. Found: 455.2089.

Synthesis of Compound **4**:



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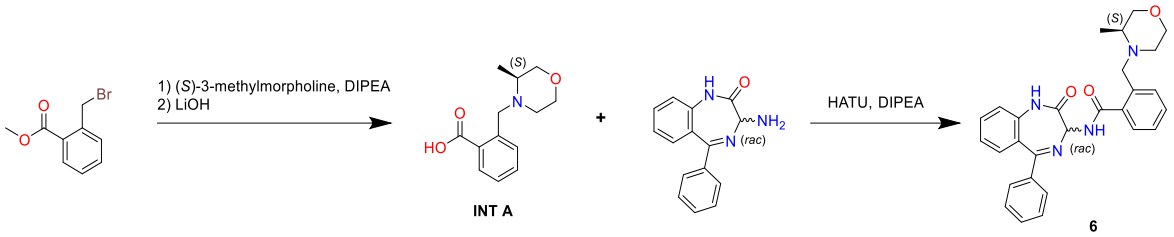
In a 30 mL scintillation vial equipped with a stir bar, *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (50 mg, 0.20 mmol, 1.0 equiv), 2-(piperidin-1-ylmethyl)benzoic acid (48 mg, 0.22 mmol, 1.1 equiv), and HATU (114 mg, 0.30 mmol, 1.5 equiv) were added. The mixture was dissolved in DMF (2 mL), followed by the addition of DIPEA (87 µl, 0.50 mmol, 2.5 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **4** (25 mg, 28%) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) 1.25 - 1.39 (m, 2H), 1.40 - 1.47 (m, 2H), 1.48 - 1.58 (m, 2H), 2.40 - 2.49 (m, 4H), 3.55 - 3.66 (m, 1H), 3.78 - 3.87 (m, 1H), 5.40 - 5.49 (m, 1H), 7.24 - 7.29 (m, 1H), 7.31 - 7.38 (m, 3H), 7.41 - 7.47 (m, 4H), 7.48 - 7.55 (m, 3H), 7.63 - 7.68 (m, 1H), 7.71 - 7.77 (m, 1H), 10.81 - 10.87 (m, 1H), 11.97 - 12.10 (m, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{28}H_{29}N_4O_2^+$: 453.2291. Found: 453.2290.

Synthesis of Compound **5**: Compound **5** was synthesized according to literature procedure.[2]

Synthesis of Compound **6**:

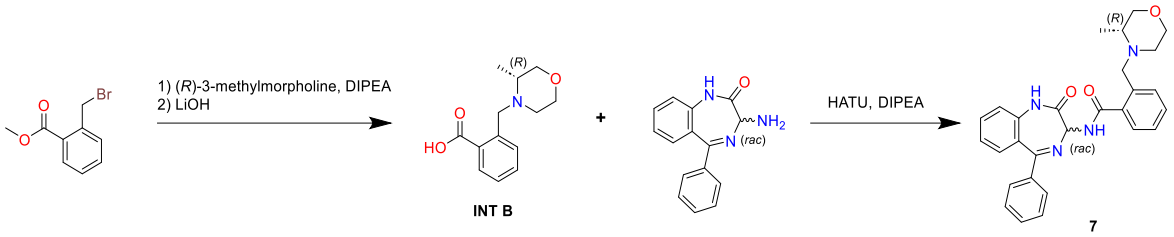


In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (150 mg, 0.65 mmol, 1.0 equiv) and (*S*)-3-methylmorpholine (79 mg, 0.79 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (5 mL) and DIPEA (343 µl, 1.96 mmol, 3.0 equiv) was added. The reaction was stirred at rt for 1 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and concentrated to afford methyl (*S*)-2-((3-methylmorpholino)methyl)benzoate (0.153 g, 94%) as a viscous oil. LC-MS (ES+): 250.1 m/z [M+H], tR = 0.91 min

In a 2-5 mL microwave vial equipped with a stir bar, methyl (*S*)-2-((3-methylmorpholino)methyl)benzoate (153 mg, 0.61 mmol, 1.0 equiv) and LiOH (73.5 mg, 3.07 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (0.8 mL total) was added to the vial before it was sealed with a microwave cap. The reaction was heated at 100 ^oC with stirring for 1 h in a microwave. Once the reaction was cooled to rt, the microwave cap was removed, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~3 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT A** (0.128 g, 89%) as a viscous oil. LC-MS (ES+): 236.1 m/z [M+H], *tR* = 0.39 min

In a 30 mL scintillation vial equipped with a stir bar, **INT A** (37.0 mg, 0.16 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (47.4 mg, 0.19 mmol, 1.2 equiv), and HATU (71.8 mg, 0.19 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (4 mL), followed by the addition of DIPEA (110 µl, 0.63 mmol, 4.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **6** (42.2 mg, 57%) as a white solid (1:1 mixture of diastereomers). ^1H NMR (400 MHz, DMSO-d_6) 1.05 - 1.15 (m, 3H), 2.24 - 2.34 (m, 1H), 2.57 - 2.69 (m, 1H), 3.25 - 3.30 (m, 1H), 3.32 - 3.37 (m, 1H), 3.45 - 3.68 (m, 4H), 4.12 - 4.42 (m, 1H), 5.36 - 5.57 (m, 1H), 7.24 - 7.30 (m, 1H), 7.31 - 7.39 (m, 2H), 7.39 - 7.49 (m, 5H), 7.49 - 7.56 (m, 3H), 7.64 - 7.70 (m, 1H), 7.73 (d, *J* = 7.3 Hz, 1H), 10.84 - 10.90 (m, 1H), 11.56 - 11.66 (m, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{28}H_{29}N_4O_3^+$: 469.2240. Found: 469.2245.

Synthesis of Compound **7**:

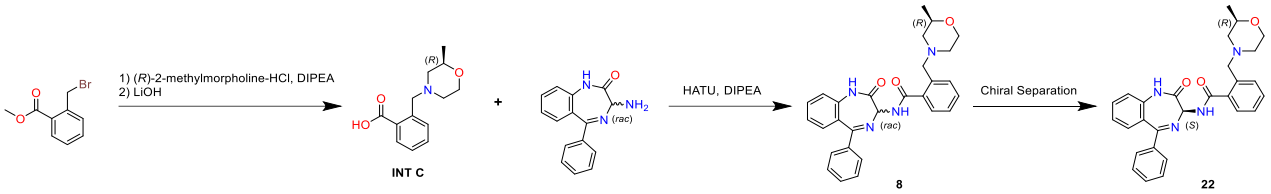


In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (150 mg, 0.65 mmol, 1.0 equiv) and (*R*)-3-methylmorpholine (79 mg, 0.79 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (5 mL) and DIPEA (343 µl, 1.96 mmol, 3.0 equiv) was added. The reaction was stirred at rt for 1 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and concentrated to afford methyl (*R*)-2-((3-methylmorpholino)methyl)benzoate (0.128 g, 78%) as a viscous oil. LC-MS (ES+): 250.1 m/z [M+H], tR = 0.91 min

In a 2-5 mL microwave vial equipped with a stir bar, methyl (*R*)-2-((3-methylmorpholino)methyl)benzoate (128 mg, 0.51 mmol, 1.0 equiv) and LiOH (61.5 mg, 2.57 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (0.8 mL total) was added to the vial before it was sealed with a microwave cap. The reaction was heated at 100 ^oC with stirring for 1 h in a microwave. Once the reaction was cooled to rt, the microwave cap was removed, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~3 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT B** (0.115 g, 95%) as a viscous oil. LC-MS (ES+): 236.1 m/z [M+H], *tR* = 0.38 min

In a 30 mL scintillation vial equipped with a stir bar, **INT B** (47.0 mg, 0.20 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (60.2 mg, 0.24 mmol, 1.2 equiv), and HATU (91.0 mg, 0.24 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (4 mL), followed by the addition of DIPEA (140 µl, 0.80 mmol, 4.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **7** (39.1 mg, 42%) as a white solid (1:1 mixture of diastereomers). ^1H NMR (500 MHz, DMSO-d_6) 1.06 - 1.15 (m, 3H), 2.22 - 2.36 (m, 1H), 2.61 - 2.72 (m, 1H), 3.24 - 3.30 (m, 1H), 3.32 - 3.37 (m, 1H), 3.46 - 3.68 (m, 4H), 4.18 - 4.41 (m, 1H), 5.39 - 5.55 (m, 1H), 7.24 - 7.30 (m, 1H), 7.32 - 7.39 (m, 2H), 7.40 - 7.50 (m, 5H), 7.51 - 7.56 (m, 3H), 7.64 - 7.69 (m, 1H), 7.71 - 7.76 (m, 1H), 10.84 - 10.92 (m, 1H), 11.56 - 11.65 (m, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{28}H_{29}N_4O_3^+$: 469.2240. Found: 469.2244.

Synthesis of Compound **8** and **22**:



In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (150 mg, 0.65 mmol, 1.0 equiv) and (*R*)-2-methylmorpholine, HCl (108 mg, 0.79 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (4 mL) and DIPEA (457 µl, 2.62 mmol, 4.0 equiv) was added. The reaction was stirred at rt for 2 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford methyl (*R*)-2-((2-methylmorpholino)methyl)benzoate (0.133 g, 81%) as a viscous oil. LC-MS (ES+): 250.1 m/z [M+H], tR = 0.42 min

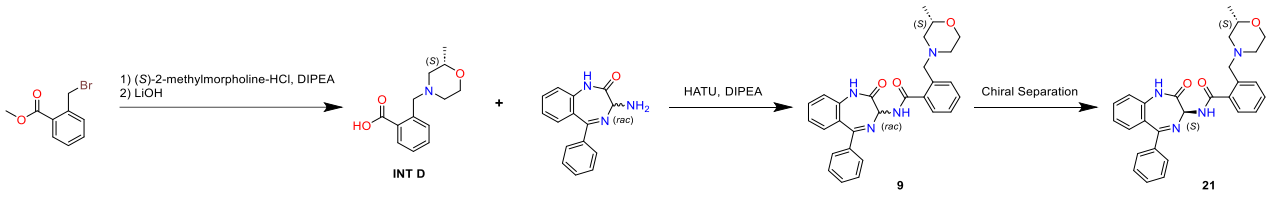
In a 2-5 mL microwave vial equipped with a stir bar, methyl (*R*)-2-((2-methylmorpholino)methyl)benzoate (133 mg, 0.53 mmol, 1.0 equiv) and LiOH (63.9 mg, 2.67 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (0.7 mL total) was added to the vial before it was sealed with a microwave cap. The reaction was heated at 100 ^oC with stirring for 1 h in a microwave. Once the reaction was cooled to rt, the microwave cap was removed, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~3 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT C** (0.093 g, 74%) as a viscous oil. LC-MS (ES+): 236.1 m/z [M+H], *tR* = 0.40 min

In a 30 mL scintillation vial equipped with a stir bar, **INT C** (51.0 mg, 0.22 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (65.4 mg, 0.26 mmol, 1.2 equiv), and HATU (99.0 mg, 0.26 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (5 mL), followed by the addition of DIPEA (151 µl, 0.87 mmol, 4.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **8** (0.087 g, 86%) as a white solid (1:1 mixture of diastereomers). ^1H NMR (500 MHz, DMSO-d_6) 0.85 - 0.89 (m, 3H), 1.88 - 1.94 (m, 1H), 2.13 - 2.28 (m, 1H), 2.65 - 2.98 (m, 2H), 3.42 - 3.57 (m, 2H), 3.57 - 3.64 (m, 1H), 3.71 (br dd, *J* = 19.4, 12.2 Hz, 1H), 3.80 - 3.90 (m, 1H), 5.47 (dd, *J* = 11.0, 7.8 Hz, 1H), 7.24 - 7.30 (m, 1H), 7.31 - 7.40 (m, 3H), 7.43 - 7.57 (m, 7H), 7.67 (br t, *J* = 7.6 Hz, 1H), 7.74 (br t, *J* = 6.1 Hz, 1H), 10.90 (s, 1H), 11.46 - 11.73 (m, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{28}H_{29}N_4O_3^+$: 469.2240. Found: 469.2245.

Compound **8** was further purified by chiral SFC to obtain compound **22** (17.1 mg, 17%, >98:2 er) and the less active isomer (17.0 mg, 17%, >98:2 er) as white solids. (see SFC Report in Spectral Data section III).

Compound **22**: ^{1}H NMR (500 MHz, DMSO-d$_6$) 0.85 (br d, *J* = 5.6 Hz, 3H), 1.90 (br t, *J* = 10.4 Hz, 1H), 2.23 (br t, *J* = 11.4 Hz, 1H), 2.69 - 2.84 (m, 2H), 3.49 (br t, *J* = 11.1 Hz, 1H), 3.55 - 3.62 (m, 1H), 3.69 (br d, *J* = 12.8 Hz, 2H), 3.86 (br d, *J* = 11.7 Hz, 1H), 5.44 (br d, *J* = 7.3 Hz, 1H), 7.22 - 7.30 (m, 1H), 7.31 - 7.40 (m, 3H), 7.43 - 7.57 (m, 7H), 7.66 (br t, *J* = 7.4 Hz, 1H), 7.73 (br d, *J* = 7.2 Hz, 1H), 10.81 (br s, 1H), 11.51 (br d, *J* = 7.0 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{28}H_{29}N_4O_3^+$: 469.2240. Found: 469.2244.

Synthesis of Compound **9** and **21**:



In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (226 mg, 0.99 mmol, 1.0 equiv) and (*S*)-2-methylmorpholine, HCl (163 mg, 1.18 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (5 mL) and DIPEA (689 µl, 3.95 mmol, 4.0 equiv) was added. The reaction was stirred at rt for 30 min. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford methyl (*S*)-2-((2-methylmorpholino)methyl)benzoate (0.229 g, 93%) as a viscous oil. LC-MS (ES+): 250.1 m/z [M+H], tR = 0.41 min

In a 30 mL scintillation vial equipped with a stir bar, methyl (*S*)-2-((2-methylmorpholino)methyl)benzoate (229 mg, 0.92 mmol, 1.0 equiv) and LiOH (110 mg, 4.59 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (2.0 mL total) was added to the vial. The reaction was heated at 100 ^{o}C with stirring for 1 h in an oil bath. The reaction was then cooled to rt, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~3 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT D** (0.158 g, 73%) as a waxy solid. LC-MS (ES+): 236.1 m/z [M+H], *tR* = 0.43 min

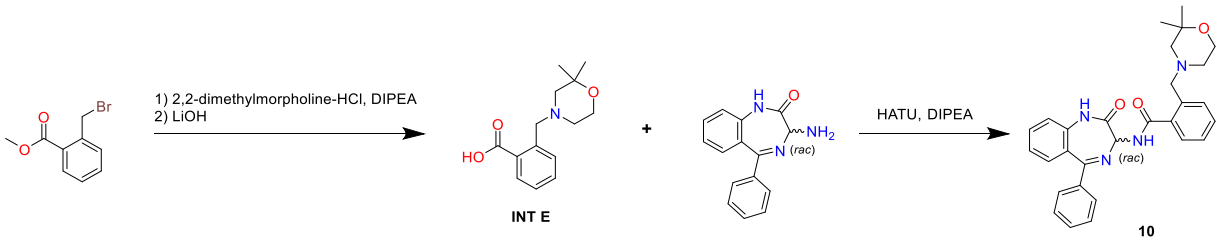
In a 30 mL scintillation vial equipped with a stir bar, **INT D** (72.0 mg, 0.31 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (92.0 mg, 0.37 mmol, 1.2 equiv), and HATU (140 mg, 0.37 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (5 mL), followed by the addition of DIPEA (214 µl, 1.22 mmol, 4.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer).

The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **9** (0.128 g, 89%) as a white solid (1:1 mixture of diastereomers). ^{1}H NMR (500 MHz, DMSO-d_6) 0.85 - 0.89 (m, 3H), 1.88 - 1.94 (m, 1H), 2.15 - 2.26 (m, 1H), 2.66 - 2.96 (m, 2H), 3.46 - 3.76 (m, 4H), 3.81 - 3.87 (m, 1H), 5.45 - 5.49 (m, 1H), 7.25 - 7.30 (m, 1H), 7.32 - 7.41 (m, 3H), 7.45 - 7.56 (m, 7H), 7.67 (br t, *J* = 7.6 Hz, 1H), 7.74 (br t, *J* = 5.8 Hz, 1H), 10.90 (br s, 1H), 11.48 - 11.72 (m, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{28}H_{29}N_4O_3^+$: 469.2240. Found: 469.2245.

Compound **9** was further purified by chiral SFC to obtain compound **21** (40.0 mg, 28%, >98:2 er) and the less active isomer (34.1 mg, 24%, >98:2 er) as white solids. (see SFC Report in Spectral Data section III).

Compound **21**: ^{1}H NMR (500 MHz, DMSO-d_6) 0.85 (d, *J* = 6.4 Hz, 3H), 1.90 (t, *J* = 10.5 Hz, 1H), 2.24 (td, *J* = 11.3, 3.0 Hz, 1H), 2.68 - 2.85 (m, 2H), 3.49 (td, *J* = 11.4, 2.2 Hz, 1H), 3.54 - 3.62 (m, 1H), 3.64 - 3.73 (m, 2H), 3.86 (d, *J* = 12.1 Hz, 1H), 5.45 (d, *J* = 7.6 Hz, 1H), 7.24 - 7.30 (m, 1H), 7.31 - 7.39 (m, 3H), 7.41 - 7.56 (m, 7H), 7.67 (ddd, *J* = 8.3, 7.1, 1.5 Hz, 1H), 7.73 (dd, *J* = 7.5, 1.5 Hz, 1H), 10.91 (s, 1H), 11.52 (d, *J* = 7.5 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{28}H_{29}N_4O_3^+$: 469.2240. Found: 469.2242.

Synthesis of Compound **10**:



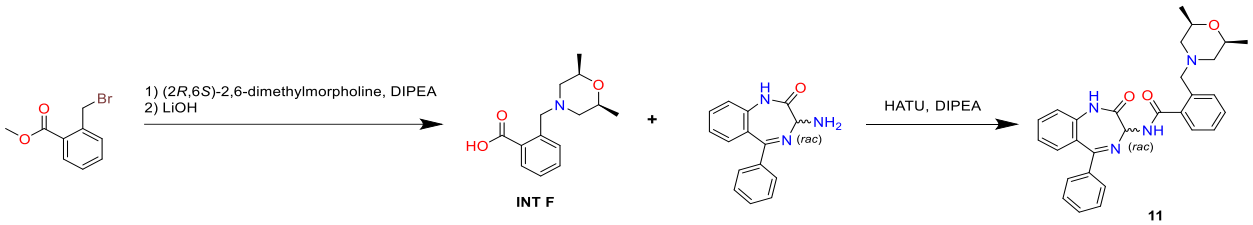
In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (345 mg, 1.51 mmol, 1.0 equiv) and 2,2-dimethylmorpholine, HCl (274 mg, 1.81 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (4 mL) and DIPEA (789 µl, 4.52 mmol, 3.0 equiv) was added. The reaction was stirred at rt for 2 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and concentrated to afford methyl 2-((2,2-dimethylmorpholino)methyl)benzoate (0.340 g, 86%) as a viscous oil. LC-MS (ES+): 264.2 m/z [M+H], tR = 1.01 min

In a 20 mL microwave vial equipped with a stir bar, methyl 2-((2,2-dimethylmorpholino)methyl)benzoate (340 mg, 1.29 mmol, 1.0 equiv) and LiOH (155 mg, 6.46 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (1.6 mL total) was added to the vial before it was sealed with a microwave cap. The reaction was heated at 100 ºC with stirring for 1 h in a microwave. Once the reaction was cooled to rt, the microwave cap was removed, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~7

with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT E** (0.289 g, 90%) as a viscous oil. LC-MS (ES+): 250.2 m/z [M+H], *tR* = 0.45 min

In a 30 mL scintillation vial equipped with a stir bar, **INT E** (50.0 mg, 0.20 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (60.5 mg, 0.24 mmol, 1.2 equiv), and HATU (92.0 mg, 0.24 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (3 mL), followed by the addition of DIPEA (140 µl, 0.80 mmol, 4.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **10** (0.058 g, 60%) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) 1.05 (s, 3H), 1.19 (s, 3H), 2.14 (br d, *J* = 10.1 Hz, 1H), 2.27 - 2.45 (m, 2H), 2.59 - 2.71 (m, 1H), 3.43 - 3.50 (m, 1H), 3.53 (br d, *J* = 11.0 Hz, 1H), 3.59 - 3.70 (m, 1H), 4.01 (br s, 1H), 5.50 (d, *J* = 7.8 Hz, 1H), 7.24 - 7.30 (m, 1H), 7.31 - 7.39 (m, 3H), 7.43 - 7.54 (m, 7H), 7.65 - 7.70 (m, 1H), 7.73 (dd, *J* = 7.6, 1.4 Hz, 1H), 10.90 (s, 1H), 11.44 (br d, *J* = 5.8 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{29}H_{31}N_4O_3^+$: 483.2396. Found: 483.2391.

Synthesis of Compound **11**:



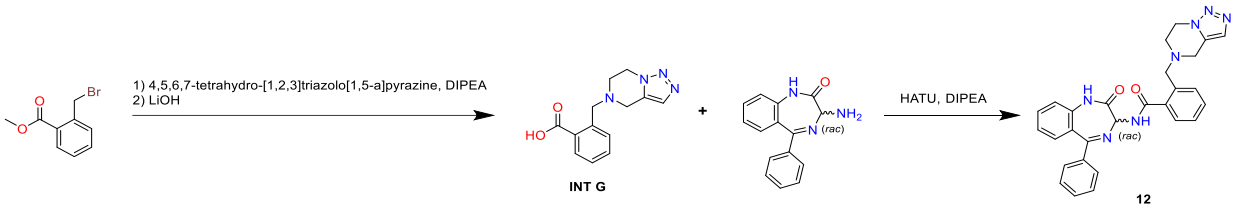
In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (345 mg, 1.51 mmol, 1.0 equiv) and (2*R*,6*S*)-2,6-dimethylmorpholine (208 mg, 1.81 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (4 mL) and DIPEA (789 µl, 4.52 mmol, 3.0 equiv) was added. The reaction was stirred at rt for 2 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and concentrated to afford methyl 2-(((2*R*,6*S*)-2,6-dimethylmorpholino)methyl)benzoate (0.209 g, 53%) as a viscous oil. LC-MS (ES+): 264.2 m/z [M+H], tR = 1.16 min

In a 20 mL microwave vial equipped with a stir bar, methyl 2-(((2*R*,6*S*)-2,6-dimethylmorpholino)methyl)benzoate (209 mg, 0.79 mmol, 1.0 equiv) and LiOH (95 mg, 3.97 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (1.6 mL total) was added to the vial before it was sealed with a microwave cap. The reaction was heated at 100 ^{o}C with stirring

for 1 h in a microwave. Once the reaction was cooled to rt, the microwave cap was removed, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~7 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT F** (0.188 g, 95%) as a viscous oil. LC-MS (ES+): 250.1 m/z [M+H], *tR* = 0.40 min

In a 30 mL scintillation vial equipped with a stir bar, *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (100 mg, 0.40 mmol, 1.0 equiv), **INT F** (119 mg, 0.48 mmol, 1.2 equiv), and HATU (378 mg, 0.99 mmol, 2.5 equiv) were added. The mixture was dissolved in DMF (5 mL), followed by the addition of DIPEA (209 µl, 1.19 mmol, 3.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **11** (0.113 g, 59%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 0.85 (d, *J* = 6.2 Hz, 6H), 1.77 - 1.88 (m, 2H), 2.68 (br d, *J* = 12.0 Hz, 1H), 2.93 (br d, *J* = 10.9 Hz, 1H), 3.46 - 3.53 (m, 1H), 3.55 - 3.64 (m, 1H), 3.75 (s, 2H), 5.47 (d, *J* = 7.7 Hz, 1H), 7.24 - 7.29 (m, 1H), 7.30 - 7.38 (m, 3H), 7.42 - 7.53 (m, 7H), 7.63 - 7.69 (m, 1H), 7.74 (dd, *J* = 7.5, 1.3 Hz, 1H), 10.88 (s, 1H), 11.57 (d, *J* = 7.8 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{29}H_{31}N_4O_3^+$: 483.2396. Found: 483.2396.

Synthesis of Compound **12**:



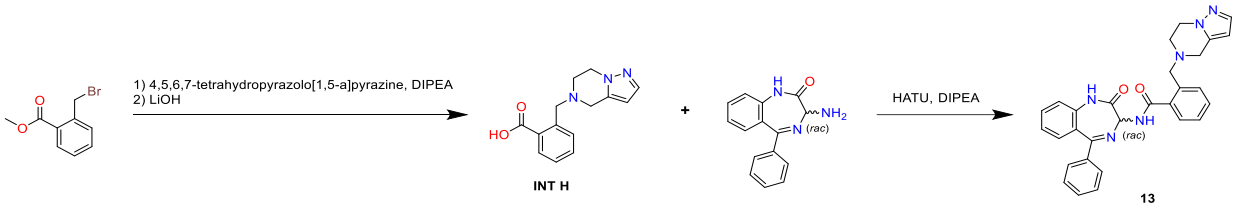
In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (150 mg, 0.65 mmol, 1.0 equiv) and 4,5,6,7-tetrahydro-[1,2,3]triazolo[1,5-a]pyrazine (81.0 mg, 0.65 mmol, 1.0 equiv) were added. The mixture was dissolved in DMF (4 mL) and DIPEA (343 µl, 1.96 mmol, 3.0 equiv) was added. The reaction was stirred at rt for 2 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford methyl 2-((6,7-dihydro-[1,2,3]triazolo[1,5-a]pyrazin-5(4*H*)-yl)methyl)benzoate (0.130 g, 73%) as a viscous oil. LC-MS (ES+): 273.1 m/z [M+H], tR = 0.57 min

In a 20 mL microwave vial equipped with a stir bar, methyl 2-((6,7-dihydro-[1,2,3]triazolo[1,5-a]pyrazin-5(4H)-yl)methyl)benzoate (130 mg, 0.48 mmol, 1.0 equiv) and LiOH (57.2 mg, 2.39 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (1.6 mL total) was added to the

vial before it was sealed with a microwave cap. The reaction was heated at 100 °C with stirring for 1 h in a microwave. Once the reaction was cooled to rt, the microwave cap was removed, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~7 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT G** (0.101 g, 60%) as a viscous oil. LC-MS (ES+): 259.1 m/z [M+H], *tR* = 0.37 min

In a 30 mL scintillation vial equipped with a stir bar, **INT G** (30.0 mg, 0.12 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (35.0 mg, 0.14 mmol, 1.2 equiv), and HATU (53.0 mg, 0.14 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (4 mL), followed by the addition of DIPEA (84 µl, 0.48 mmol, 4.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **12** (0.044 g, 77%) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) 3.03 - 3.19 (m, 2H), 3.75 - 3.80 (m, 1H), 3.83 - 3.93 (m, 1H), 4.00 (br d, *J* = 12.4 Hz, 1H), 4.15 (br d, *J* = 12.4 Hz, 1H), 4.32 - 4.44 (m, 2H), 5.39 (d, *J* = 7.3 Hz, 1H), 7.24 - 7.29 (m, 1H), 7.33 (t, *J* = 7.6 Hz, 2H), 7.38 - 7.41 (m, 2H), 7.42 - 7.51 (m, 5H), 7.51 - 7.58 (m, 2H), 7.66 (br t, *J* = 7.6 Hz, 1H), 7.71 (br d, *J* = 7.3 Hz, 1H), 10.78 (br d, *J* = 7.5 Hz, 1H), 10.90 (s, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{28}H_{26}N_7O_2^+$: 492.2148. Found: 492.2138.

Synthesis of Compound **13**:



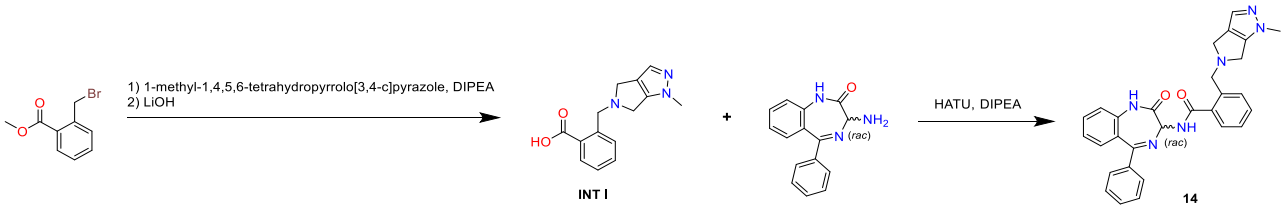
In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (150 mg, 0.65 mmol, 1.0 equiv) and 4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazine (81.0 mg, 0.65 mmol, 1.0 equiv) were added. The mixture was dissolved in DMF (4 mL) and DIPEA (343 µl, 1.96 mmol, 3.0 equiv) was added. The reaction was stirred at rt for 2 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford methyl 2-((6,7-dihydropyrazolo[1,5-a]pyrazin-5(4*H*)-yl)methyl)benzoate (0.134 g, 75%) as a viscous oil. LC-MS (ES+): 272.1 m/z [M+H], tR = 0.49 min

In a 20 mL microwave vial equipped with a stir bar, methyl 2-((6,7-dihydro-[1,2,3]triazolo[1,5-a]pyrazin-5(4*H*)-yl)methyl)benzoate (134 mg, 0.49 mmol, 1.0 equiv) and LiOH (59.1 mg, 2.47

mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (1.6 mL total) was added to the vial before it was sealed with a microwave cap. The reaction was heated at 100 ^oC with stirring for 1 h in a microwave. Once the reaction was cooled to rt, the microwave cap was removed, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~7 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT H** (0.110 g, 65%) as a viscous oil. LC-MS (ES+): 258.2 m/z [M+H], *tR* = 0.40 min

In a 30 mL scintillation vial equipped with a stir bar, **INT H** (20.0 mg, 0.078 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (23.4 mg, 0.093 mmol, 1.2 equiv), and HATU (35.5 mg, 0.093 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (3 mL), followed by the addition of DIPEA (55 µl, 0.31 mmol, 4.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **13** (31.9 mg, 84%) as a white solid. ^1H NMR (500 MHz, DMSO-d_6) 3.04 - 3.14 (m, 2H), 3.66 - 3.73 (m, 1H), 3.74 - 3.81 (m, 1H), 3.97 (br d, *J* = 12.4 Hz, 1H), 4.00 - 4.08 (m, 1H), 4.10 - 4.18 (m, 2H), 5.41 (d, *J* = 7.5 Hz, 1H), 5.92 (s, 1H), 7.24 - 7.30 (m, 1H), 7.30 - 7.37 (m, 3H), 7.40 - 7.51 (m, 6H), 7.51 - 7.57 (m, 2H), 7.66 (br t, *J* = 7.7 Hz, 1H), 7.72 (br d, *J* = 7.8 Hz, 1H), 10.84 - 10.94 (m, 2H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{29}H_{27}N_6O_2^+$: 491.2195. Found: 491.2196.

Synthesis of Compound **14**:

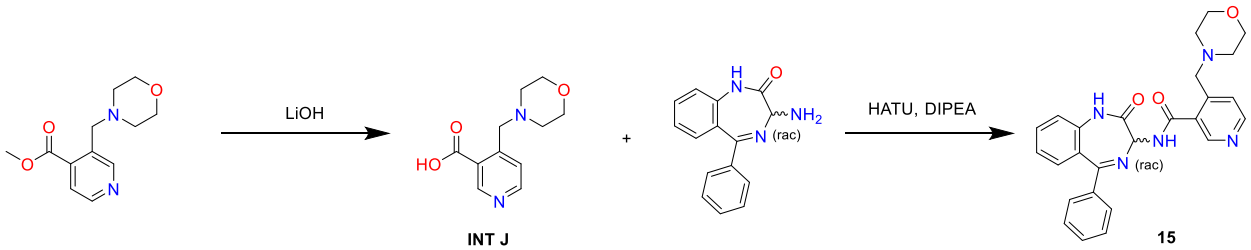


In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)benzoate (186 mg, 0.81 mmol, 1.0 equiv) and 1-methyl-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazole (100 mg, 0.81 mmol, 1.0 equiv) were added. The mixture was dissolved in DMF (4 mL) and DIPEA (425 µl, 2.44 mmol, 3.0 equiv) was added. The reaction was stirred at rt for 1 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford methyl 2-((1-methyl-4,6-dihydropyrrolo[3,4-c]pyrazol-5(1*H*)-yl)methyl)benzoate (0.112 g, 51%) as a waxy solid. LC-MS (ES+): 272.1 m/z [M+H], tR = 0.41 min

In a 30 mL scintillation vial equipped with a stir bar, methyl 2-((1-methyl-4,6-dihydropyrrolo[3,4-c]pyrazol-5(1*H*)-yl)methyl)benzoate (112 mg, 0.41 mmol, 1.0 equiv) and LiOH (49.4 mg, 2.06 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (2.0 mL total) was added to the vial. The reaction was heated at 60 ^oC with stirring for 3 h in an oil bath. Once the reaction was cooled to rt, and the THF was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~7 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT I** (0.092 g, 87%) as a waxy solid. LC-MS (ES+): 258.1 m/z [M+H], *tR* = 0.42 min

In a 30 mL scintillation vial equipped with a stir bar, **INT I** (92.0 mg, 0.36 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (99.0 mg, 0.39 mmol, 1.1 equiv), and HATU (150 mg, 0.39 mmol, 1.1 equiv) were added. The mixture was dissolved in DMF (5 mL), followed by the addition of DIPEA (312 µl, 1.79 mmol, 5.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% ammonium hydroxide buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **14** (39.5 mg, 21%) as an off-white solid. ^1H NMR (600 MHz, DMSO-d_6) 3.57 (s, 3H), 3.80 - 3.93 (m, 4H), 4.14 (br d, *J* = 12.0 Hz, 1H), 4.28 (br d, *J* = 12.0 Hz, 1H), 5.37 (d, *J* = 6.6 Hz, 1H), 7.11 (s, 1H), 7.25 - 7.29 (m, 1H), 7.34 (d, *J* = 8.3 Hz, 2H), 7.42 - 7.44 (m, 4H), 7.45 - 7.49 (m, 1H), 7.51 - 7.56 (m, 3H), 7.64 - 7.68 (m, 1H), 7.76 (d, *J* = 7.5 Hz, 1H), 10.87 (s, 1H), 11.52 (br d, *J* = 6.6 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{29}H_{27}N_6O_2^+$: 491.2195. Found: 491.2200.

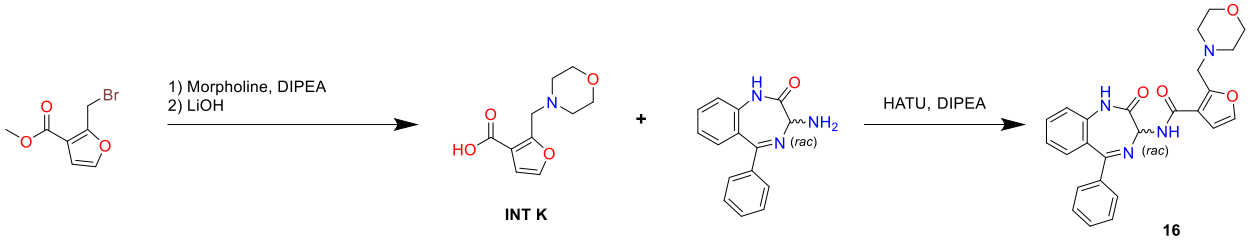
Synthesis of Compound **15**:



In a 20 mL scintillation vial equipped with a stir bar, methyl 4-(morpholinomethyl)nicotinate (110 mg, 0.47 mmol, 1.0 equiv) and LiOH (56.0 mg, 2.33 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (4.6 mL total) was added to the vial. The reaction was stirred at 70 °C for 15 h. The reaction was then allowed to cool to rt and was acidified with 1N HCl to pH ~4-5. The resulting precipitate was filtered and washed with cold water. The solid was then dried under vacuum to provide **INT J** (93.1 mg, 90%) as a white solid. The material was then used directly in the next reaction without further purification. LC-MS (ES+): 223.2 m/z [M+H], *tR* = 0.18 min

In a 2 dram vial equipped with a stir bar, *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (90.0 mg, 0.36 mmol, 1.0 equiv) and **INT J** (80.0 mg, 0.36 mmol, 1.0 equiv) were dissolved in DMF (3.4 ml). DIPEA (189 µl, 1.08 mmol, 3.0 equiv) was then added, followed by HATU (151 mg, 0.40 mmol, 1.1 equiv). The reaction was stirred at rt for 1 h. The reaction was then directly purified without workup by C18 column chromatography, eluting with a 5-100% MeCN in water gradient (0.1% formic acid buffer). The combined organic layers were dried over sodium sulfate and concentrated to afford compound **15** (116 mg, 71%) as a white solid. ^{1}H NMR (500 MHz, CD_3OD) 2.61 (br s, 4H), 3.61 - 3.68 (m, 2H), 3.70 - 3.81 (m, 3H), 4.01 (d, *J* = 12.4 Hz, 1H), 5.63 (s, 1H), 7.25 - 7.31 (m, 1H), 7.35 - 7.40 (m, 2H), 7.42 - 7.48 (m, 3H), 7.49 - 7.54 (m, 1H), 7.54 - 7.59 (m, 2H), 7.63 - 7.68 (m, 1H), 8.62 (d, *J* = 5.0 Hz, 1H), 8.94 (s, 1H) (NH protons not present). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{26}H_{26}N_5O_3^+$: 456.2036. Found: 456.2037.

Synthesis of Compound **16**:



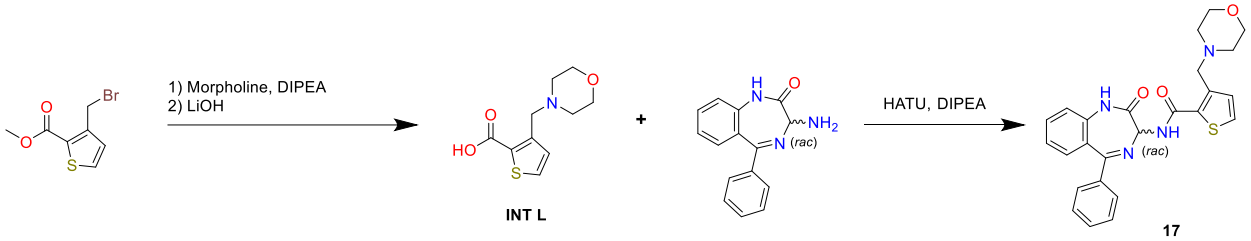
In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(bromomethyl)furan-3-carboxylate (400 mg, 1.83 mmol, 1.0 equiv) was added. The mixture was dissolved in DMF (5.0 mL), followed by the addition of DIPEA (0.638 mL, 3.65 mmol, 2.0 equiv) and morpholine (0.318 mL, 3.65 mmol, 2.0 equiv). The reaction was stirred at rt for 1 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and concentrated to afford methyl 2-(morpholinomethyl)furan-3-carboxylate (354 mg, 86 %) as a viscous oil. LC-MS (ES+) 226.4 m/z [M+H], *tR* = 0.59 min

In a 30 mL scintillation vial equipped with a stir bar, methyl 2-(morpholinomethyl)furan-3-carboxylate (354 mg, 1.57 mmol) and LiOH (188 mg, 7.86 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (4.0 mL total) was added to the vial. The reaction was stirred at 100 °C for 1 h in an oil bath. The reaction was then cooled to room temperature and was placed under a stream of nitrogen to remove the THF. The resulting mixture was then acidified to pH ~6-7 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT K** (296 mg, 89 %) as a viscous oil. LC-MS (ES+): 212.3 m/z [M+H], *tR* = 0.33 min

In a 30 mL scintillation vial equipped with a stir bar, **INT K** (100 mg, 0.400 mmol, 1.0 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (96.0 mg, 0.45 mmol, 1.14 equiv), and HATU (182 mg, 0.48 mmol, 1.2 equiv) were added. The mixture was dissolved in

DMF (3.7 mL), followed by the addition of DIPEA (209 µl, 1.19 mmol, 3.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **16** (19.7 mg, 11%) as a white solid. ^1H NMR (500 MHz, $CDCl_3$) 2.44 - 2.79 (m, 4H), 3.70 - 3.79 (m, 5H), 3.91 (d, *J* = 12.8 Hz, 1H), 5.81 (d, *J* = 7.9 Hz, 1H), 6.96 (d, *J* = 4.9 Hz, 1H), 7.17 (d, *J* = 7.9 Hz, 1H), 7.21 (t, *J* = 7.6 Hz, 1H), 7.36 - 7.43 (m, 4H), 7.45 - 7.50 (m, 1H), 7.52 - 7.60 (m, 3H), 7.94 (s, 1H), 12.06 (br d, *J* = 7.9 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{25}H_{25}N_4O_4^+$: 445.1876. Found: 445.1878.

Synthesis of Compound **17**:



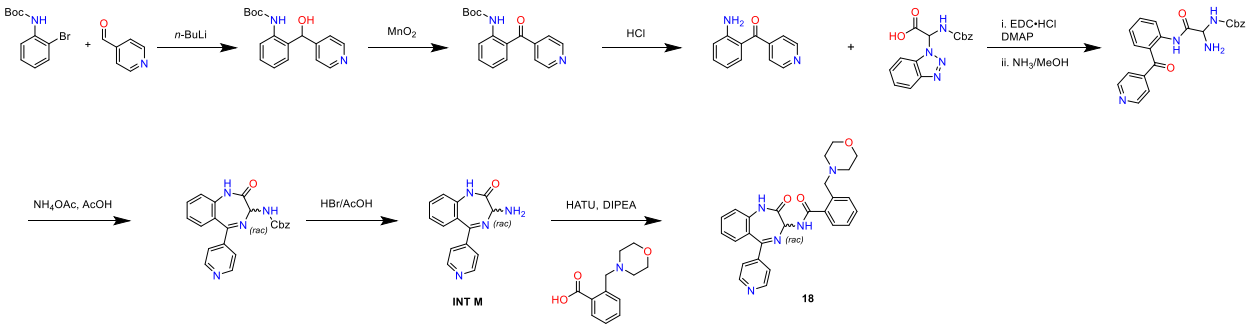
In a 30 mL scintillation vial equipped with a stir bar, methyl 3-(bromomethyl)thiophene-2-carboxylate (400 mg, 1.70 mmol, 1.0 equiv) was added. The mixture was dissolved in DMF (5.0 mL), followed by the addition of DIPEA (0.594 mL, 3.40 mmol, 2.0 equiv) and morpholine (0.318 mL, 3.65 mmol, 2.0 equiv). The reaction was stirred at rt for 1 h. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and concentrated to afford methyl 3-(morpholinomethyl)thiophene-2-carboxylate (368 mg, 90 %) as a viscous oil. LC-MS (ES+) 242.1 m/z [M+H], *tR* = 0.72 min

In a 30 mL scintillation vial equipped with a stir bar, methyl 3-(morpholinomethyl)thiophene-2-carboxylate (368 mg, 1.53 mmol, 1.0 equiv) and LiOH (183 mg, 7.63 mmol, 5.0 equiv) were added. Then a 1:1 mixture of THF/H_2O (4.0 mL total) was added to the vial. The reaction was stirred at 100 °C for 1 h in an oil bath. The reaction was then cooled to room temperature and was placed under a stream of nitrogen to remove the THF. The resulting mixture was then acidified to pH ~6-7 with a 2N solution of HCl. The homogeneous solution was then directly purified by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT L** (338 mg, 98 %) as a viscous oil. LC-MS (ES+): 228.1 m/z [M+H], *tR* = 0.34 min

In a 30 mL scintillation vial equipped with a stir bar, **INT L** (96 mg, 0.42 mmol, 1.1 equiv), *rac*-3-amino-5-phenyl-1,3-dihydro-2*H*-benzo[e][1,4]diazepin-2-one (100 mg, 0.40 mmol, 1.00 equiv), and HATU (182 mg, 0.48 mmol, 1.2 equiv) were added. The mixture was dissolved in DMF (3.7 mL), followed by the addition of DIPEA (209 µl, 1.19 mmol, 3.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18

column chromatography eluting with a 10-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined, diluted with a saturated aqueous $NaHCO_3$ solution, and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **17** (71.3 mg, 39%) as a white solid. ^1H NMR (500 MHz, $CDCl_3$) 2.49 - 2.76 (m, 4H), 3.70 - 3.85 (m, 5H), 3.95 (br d, *J* = 13.9 Hz, 1H), 5.78 (br d, *J* = 8.1 Hz, 1H), 6.86 (d, *J* = 1.5 Hz, 1H), 7.17 (br d, *J* = 8.2 Hz, 2H), 7.33 (s, 1H), 7.35 - 7.42 (m, 3H), 7.43 - 7.49 (m, 1H), 7.51 - 7.60 (m, 3H), 8.28 (br s, 1H), 11.06 (br d, *J* = 7.5 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{25}H_{25}N_4O_3S^+$: 461.1647. Found: 461.1651.

Synthesis of Compound **18**:



In a 250 mL RB flask equipped with a stir bar, *tert*-butyl (2-bromophenyl)carbamate (2.00 g, 7.35 mmol, 1.0 equiv) was added. The flask was then purged with nitrogen before the addition of 50 mL of THF. The reaction was then cooled to -78 °C before the dropwise addition of 2.5 M nBuLi in hexanes (6.47 mL, 16.2 mmol, 2.2 equiv). The reaction was stirred for 30 min at -78 °C before the addition of isonicotinaldehyde (0.762 mL, 8.08 mmol, 1.1 equiv) in 9 mL of THF. The reaction was then warmed to 0 °C and stirred at that temperature for 1 h. The mixture was then neutralized with a saturated aqueous NH_4Cl solution and was concentrated to remove THF. The solution was then extracted with DCM, and the combined organic layers were dried over sodium sulfate and concentrated. The residue was dissolved in minimal DMSO and purified by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford *tert*-butyl (2-(hydroxy(pyridin-4-yl)methyl)phenyl)carbamate (0.681 g, 31%) as a white solid. LC-MS (ES+): 301.2 m/z [M+H], *tR* = 0.61 min

In a 30 mL scintillation vial equipped with a stir bar, *tert*-butyl (2-(hydroxy(pyridin-4-yl)methyl)phenyl)carbamate (660 mg, 2.20 mmol, 1.0 equiv) and manganese dioxide (1.80 g, 17.6 mmol, 8.0 equiv) were added. The vial was then purged with nitrogen before the addition of DCM (15 mL). The reaction was then stirred at rt for 5 h. The reaction was filtered over celite and concentrated to afford *tert*-butyl (2-isonicotinoylphenyl)carbamate (0.625 g, 95%) as a light yellow solid. LC-MS (ES+): 299.0 m/z [M+H], *tR* = 1.08 min

In a 30 mL scintillation vial equipped with a stir bar, *tert*-butyl (2-isonicotinoylphenyl)carbamate (625 mg, 2.16 mmol, 1.0 equiv) was added. The solid was dissolved in 4 mL of dioxane before the addition of a 4N solution of HCl (10.8 mL, 43.2 mmol, 20 equiv) in dioxane. The reaction was stirred at rt for 4 h. The reaction was then concentrated, diluted with a 3:1 $CHCl_3$/IPA

solution, and washed with a saturated aqueous $NaHCO_3$ solution. The organic layer was separated and was then dried over sodium sulfate and concentrated to afford (2-aminophenyl)(pyridin-4-yl)methanone (0.427 g, quant.) as a yellow solid. LC-MS (ES+): 199.1 m/z [M+H], *tR* = 0.58 min

In a 250 mL RB flask equipped with a stir bar, 2-(1*H*-benzo[d][1,2,3]triazol-1-yl)-2-(((benzyloxy)carbonyl)amino)acetic acid[3] (800 mg, 2.45 mmol, 1.5 equiv) and (2-aminophenyl)(pyridin-4-yl)methanone (324 mg, 1.63 mmol, 1.0 equiv) were added, and the flask was placed under nitrogen before the addition of DCM (32 mL). The solution was cooled to 0 °C before the addition of EDC-HCl (627 mg, 3.27 mmol, 2.0 equiv) and DMAP (39.9 mg, 0.33 mmol, 0.2 equiv). The reaction was then warmed to rt and stirred for 1 h. At this point, ammonia (11.7 mL, 81.9 mmol, 50 equiv) as added as a 7M solution in MeOH, and the reaction was stirred at rt for 15 h. The reaction was then concentrated, and was diluted with a 3:1 $CHCl_3$/IPA solution. The organic solution was then washed with an aqueous 1M NaOH solution. The organic layer was then dried over sodium sulfate and concentrated to provide benzyl (1-amino-2-((2-isonicotinoylphenyl)amino)-2-oxoethyl)carbamate as a crude residue, which was used directly in the next step without further purification. LC-MS (ES+): 405.3 m/z [M+H], *tR* = 0.67 min

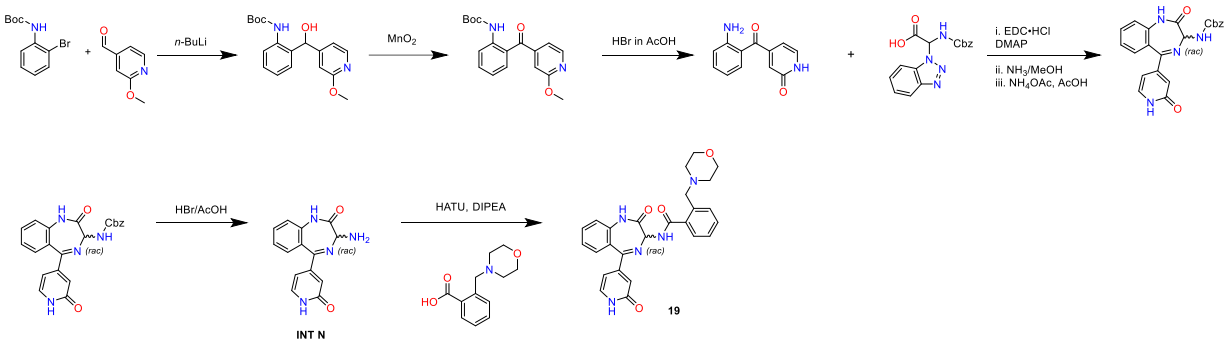
In a 250 mL RB flask equipped with a stir bar containing crude benzyl (1-amino-2-((2-isonicotinoylphenyl)amino)-2-oxoethyl)carbamate, ammonium acetate (0.754 g, 9.78 mmol, 6.0 equiv) was added. Then 23 mL of AcOH was added, and the reaction was stirred at rt for 6 h. The reaction was then slowly added to a saturated aqueous $NaHCO_3$ solution to quench the AcOH. The solution was then washed 5x with a 3:1 $CHCl_3$/IPA solution. The combined organic layers were dried over sodium sulfate and concentrated. The residue was dissolved in minimal DMSO and purified by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford benzyl (2-oxo-5-(pyridin-4-yl)-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-yl)carbamate (0.373 g, 59%) as an off-white solid. LC-MS (ES+): 387.0 m/z [M+H], *tR* = 0.75 min

In a 20 mL microwave vial equipped with a stir bar was added benzyl (2-oxo-5-(pyridin-4-yl)-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-yl)carbamate (200 mg, 0.52 mmol), and HBr (3.0 mL, 33 wt. % in acetic acid). The vial was sealed and was then stirred at 70 °C for 40 min in an oil bath. The reaction was then cooled 0 °C before dropwise addition of the reaction into a saturated aqueous Na_2CO_3 solution to neutralize the acid. The solution was then extracted with a 3:1 $CHCl_3$/IPA solution. The combined organic layers were dried over sodium sulfate and concentrated to afford **INT M** (0.168 g, 129%) as a crude dark green solid, which was used directly in the next step without further purification. LC-MS (ES+): 253.1 m/z [M+H], *tR* = 0.30 min

In a 30 mL scintillation vial equipped with a stir bar, **INT M** (50.0 mg, 0.20 mmol, 1.0 equiv) and 2-(morpholinomethyl)benzoic acid (48.2 mg, 0.22 mmol, 1.1 equiv) were added, along with HATU (83.0 mg, 0.22 mmol) and DMF (5 mL). Then DIPEA (0.138 ml, 0.79 mmol, 4.0 equiv) was added and the reaction was stirred for 45 min at rt. The reaction was directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water

gradient using (0.1% NH_4OH buffer). The desired fractions were combined, diluted with a saturated aqueous $NaHCO_3$ solution, and extracted with a 3:1 $CHCl_3$:IPA solution. The combined organic layers were dried over sodium sulfate and concentrated to afford compound **18** (7.6 mg, 8%) as a white solid. ^1H NMR (500 MHz, DMSO-d_6) 2.51 - 2.59 (m, 4H), 3.49 - 3.62 (m, 4H), 3.70 (br d, *J* = 12.1 Hz, 1H), 3.89 (br d, *J* = 12.1 Hz, 1H), 5.52 (d, *J* = 7.6 Hz, 1H), 7.26 - 7.33 (m, 1H), 7.34 - 7.41 (m, 3H), 7.43 - 7.55 (m, 4H), 7.65 - 7.79 (m, 2H), 8.70 (br d, *J* = 5.3 Hz, 2H), 10.95 (br s, 1H), 11.68 (br d, *J* = 7.6 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{26}H_{26}N_5O_3^+$: 456.2036. Found: 456.2036.

Synthesis of Compound **19**:



In a 250 mL RB flask equipped with a stir bar, *tert*-butyl (2-bromophenyl)carbamate (2.00 g, 7.35 mmol, 1.0 equiv) was added. The flask was then purged with nitrogen before addition of THF (50 mL). The reaction was then cooled to -78 °C before dropwise addition of 2.5 M nBuLi in hexanes (6.47 mL, 16.17 mmol, 2.2 equiv). The reaction was stirred for 30 min at -78 °C before the addition of 2-methoxyisonicotinaldehyde (1.11 g, 8.08 mmol, 1.1 equiv) in THF (9.0 mL). The reaction was then warmed to 0 °C and stirred for 1 h. The mixture was neutralized at 0 °C with a saturated aqueous NH_4Cl solution, and was then concentrated to remove THF. The solution was then extracted with DCM, and the combined organic layers were dried over sodium sulfate and concentrated. The residue was dissolved in minimal DMSO and purified by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined concentrated to afford *tert*-butyl (2-(hydroxy(2-methoxypyridin-4-yl)methyl)phenyl)carbamate (0.690 g, 28%) as a white solid. LC-MS (ES+): 331.2 m/z [M+H], *tR* = 1.02 min

In a 30 mL scintillation vial equipped with a stir bar, *tert*-butyl (2-(hydroxy(2-methoxypyridin-4-yl)methyl)phenyl)carbamate (0.690 g, 2.09 mmol, 1.0 equiv) and manganese dioxide (1.709 g, 16.71 mmol, 8.0 equiv) were added. The vial was then purged with nitrogen before addition of DCM (15 mL). The reaction was then stirred at rt for 5 h. The reaction was filtered over celite and concentrated to afford *tert*-butyl (2-(2-methoxyisonicotinoyl)phenyl)carbamate as a light yellow solid. The crude material was used directly in the next step without purification assuming 100% yield. LC-MS (ES+): 329.1 m/z [M+H], *tR* = 1.33 min

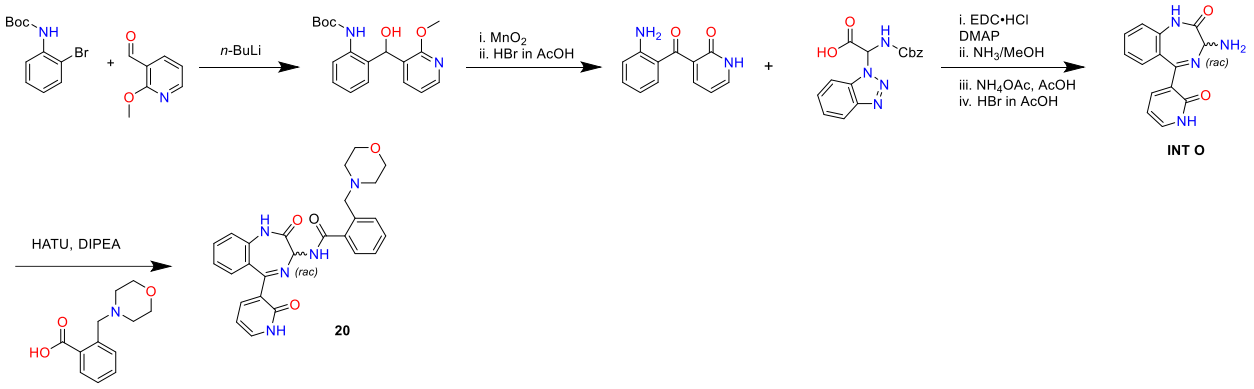
In a 20 mL microwave vial equipped with a stir bar, *tert*-butyl (2-(2-methoxyisonicotinoyl)phenyl)carbamate was added. To the vial was then added HBr in AcOH (2.0 mL, 33 wt. % in acetic acid). The vial was sealed and then heated to 70 °C for 30 min. The vial was cooled to room temperature and was then concentrated to afford crude 4-(2-aminobenzoyl)pyridin-2(1*H*)-one (0.246 g, 55%) was carried forwards to the next reaction without further purification. LC-MS (ES+): 215.3 m/z [M+H], *tR* = 0.58 min

In a 2 dram vial equipped with stir bar 4-(2-aminobenzoyl)pyridin-2(1*H*)-one (0.246 g, 1.15 mmol, 1.0 equiv) and 2-(1*H*-benzo[d][1,2,3]triazol-1-yl)-2-(((benzyloxy)carbonyl)amino)acetic acid[3] (0.450 g, 1.38 mmol, 1.2 equiv) were added. To the vial was then added DCM (18 mL), followed by the addition of DMAP (0.028 g, 0.23 mmol, 0.2 equiv) and EDC-HCl (0.440 g, 2.30 mmol). The reaction was stirred at rt for 1 h. At this point, ammonia (8.0 mL) was then added as a 7M solution in MeOH, and the reaction was left to stir at rt overnight. The excess solvent was then removed *in vaccuo* and redissolved in a 3:1 $CHCl_3$/IPA solvent system. The solution was washed with a 1N NaOH solution, and the organic layer was then dried over sodium sulfate and concentrated to afford a crude oil. The oil was then dissolved in acetic acid (18 mL) and ammonium acetate (0.531 g, 6.89 mmol, 6.0 equiv) was added. The reaction was stirred at rt for 1 h. The reaction was then concentrated to afford *rac*-benzyl-(2-oxo-5-(2-oxo-1,2-dihydropyridin-4-yl)-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-yl)carbamate which was used directly in the next reaction without purification. LC-MS (ES+): 403.3 m/z [M+H], *tR* = 0.73 min

In a 20 mL microwave vial equipped with a stir bar was added the crude *rac*-benzyl-(2-oxo-5-(2-oxo-1,2-dihydropyridin-4-yl)-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-yl)carbamate and HBr (0.572 mL, 33 wt% in acetic acid). The vial was sealed and the reaction was stirred at 70 °C for 40 min in an oil bath. The reaction was then cooled 0 °C before pipetting the solution slowly into a saturated aqueous Na_2CO_3 solution to neutralize the acid. All excess solvent was then removed *in vaccuo*. The solids were then dissolved in H_2O and purified directly by C18 column chromatography eluting with a 0-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and concentrated to afford **INT N** (18.9 mg, 6% over four steps) as a white solid. LC-MS (ES+): 269.2 m/z [M+H], *tR* = 0.37 min

In a 30 mL scintillation vial equipped with a stir bar, **INT N** (18.9 mg, 0.07 mmol, 1.0 equiv) and 2-(morpholinomethyl)benzoic acid (16.0 mg, 0.07 mmol, 1.0 equiv) were added, along with HATU (29.0 mg, 0.08 mmol, 1.1 equiv) and DMF (0.668 mL). Then DIPEA (0.037 mL, 0.21 mmol, 3.0 equiv) was added and the reaction was stirred at rt for 45 min. The reaction was then diluted with additional DMF (2 mL), and was directly purified without workup using C18 column chromatography, eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford compound **19** (11.0 mg, 33%) as a white solid. ^{1}H NMR (500 MHz, CD_3OD) 2.63 (br s, 4H), 3.65 - 3.71 (m, 2H), 3.71 - 3.78 (m, 3H), 4.02 (d, *J* = 12.2 Hz, 1H), 5.68 (s, 1H), 6.55 (s, 1H), 6.68 (dd, *J* = 6.7, 1.8 Hz, 1H), 7.31 - 7.43 (m, 3H), 7.45 - 7.57 (m, 4H), 7.66 - 7.74 (m, 1H), 7.81 - 7.89 (m, 1H) (NH protons not present). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{26}H_{26}N_5O_4^+$: 472.1985. Found: 472.1981.

Synthesis of Compound **20**:



In a 250 mL RB flask equipped with a stir bar, *tert*-butyl (2-bromophenyl)carbamate (2.00 g, 7.35 mmol, 1.0 equiv) was added. The flask was then purged with nitrogen before addition of THF (50 mL). The reaction was then cooled to -78 °C before dropwise addition of 2.5 M nBuLi in hexanes (6.47 ml, 16.2 mmol, 2.2 equiv). The reaction was stirred for 30 min at -78 °C before the addition of 2-methoxynicotinaldehyde (1.11 g, 8.08 mmol, 1.1 equiv) in THF (9.0 mL). The reaction was then warmed to 0 °C and stirred at that temperature for 1 h. The mixture was then neutralized with a saturated aqueous NH_4Cl solution, and then concentrated to remove THF. The solution was then extracted 3x with DCM, and the combined organic layers were dried over sodium sulfate and concentrated. The residue was dissolved in minimal DMSO and purified by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford *tert*-butyl (2-(hydroxy(2-methoxypyridin-3-yl)methyl)phenyl)carbamate (0.478 g, 20%) as a white solid. LC-MS (ES+): 353.1 m/z [M+Na], *tR* = 1.11 min

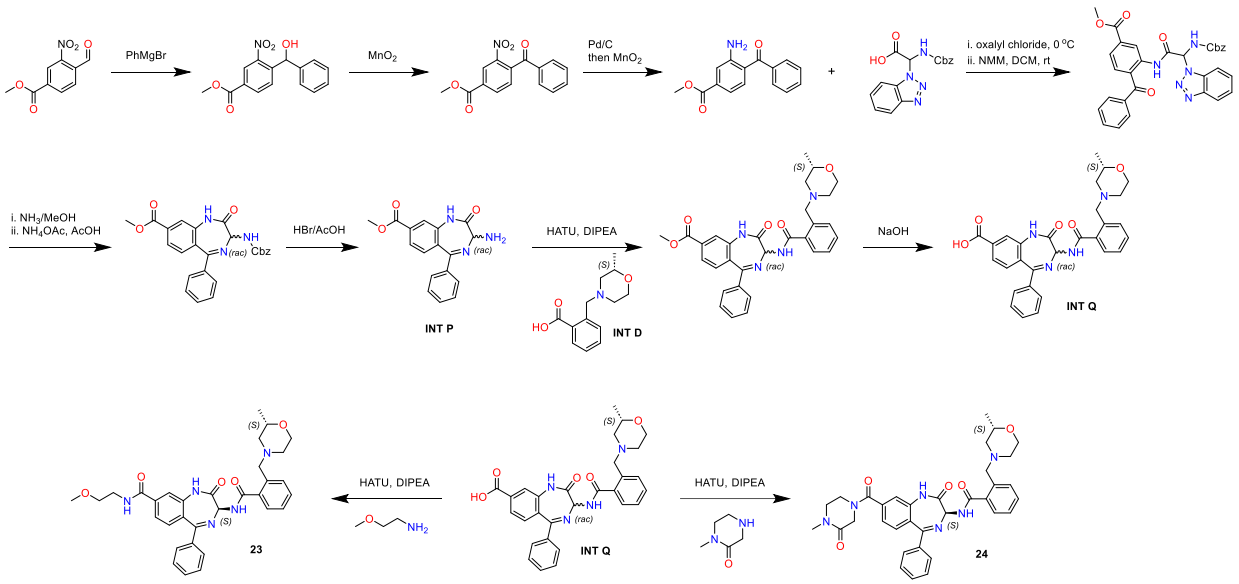
In a 30 mL scintillation vial equipped with a stir bar, *tert*-butyl (2-(hydroxy(2-methoxypyridin-3-yl)methyl)phenyl)carbamate (0.478 g, 1.45 mmol, 1.0 equiv) and manganese dioxide (1.18 g, 11.6 mmol, 8.0 equiv) were added. The vial was then purged with nitrogen before addition of DCM (15 mL). The reaction was then stirred at rt for 5 h. The reaction was filtered over celite and concentrated to afford *tert*-butyl (2-(2-methoxynicotinoyl)phenyl)carbamate as a light yellow solid. The crude material was then transferred to a 20 mL microwave vial equipped with a stir bar, followed by the addition of HBr (8.33 mL, 33 wt. % in acetic acid). The vial was sealed and stirred at 70 °C for 1 h. The reaction was then cooled to 0 °C and was slowly added to a saturated aqueous solution of Na_2CO_3 to neutralize the acid. The resulting solution was then purified directly via C18 column chromatography eluting with a 0-100% MeCN in water gradient (0.1% NH_4OH buffer). The desired fractions were combined and concentrated to afford 3-(2-aminobenzoyl)pyridin-2(1*H*)-one (0.126 g, 41%) as a viscous oil. LC-MS (ES+): 237.1 m/z [M+Na], *tR* = 0.55 min

In a 2 dram vial equipped with stir bar, 3-(2-aminobenzoyl)pyridin-2(1*H*)-one (0.106 g, 0.46 mmol, 1.2 equiv) and 2-(1*H*-benzo[d][1,2,3]triazol-1-yl)-2-(((benzyloxy)carbonyl)amino)acetic acid[3] (0.126 g, 0.39 mmol, 1.0 equiv) were added. To the vial was added DCM (0.29 mL), followed by the addition of DMAP (9.4 mg, 0.08 mmol, 0.2 equiv) and EDC-HCl (0.148 mg,

0.77 mmol, 2.0 equiv). The reaction was then stirred at rt for 1 h. At this point, ammonia (2.75 mL) was added as a 7M solution in MeOH, and the reaction was stirred at rt overnight. The excess solvent was then removed *in vaccuo*, redissolved in a 3:1 $CHCl_3$/IPA solution, and was then washed with a 1N NaOH solution. The organic layer was then dried over sodium sulfate and concentrated. The crude oil was then dissolved in acetic acid (18 mL), and ammonium acetate (0.179 g, 2.32 mmol, 6.0 equiv) was added. The reaction was stirred at rt for 1 h. The reaction was then concentrated and the crude residue was transferred to a 20 mL microwave vial equipped with a stir bar. To the vial was then added HBr (3.18 mL, 33 wt. % in acetic acid). The vial was sealed and was stirred at 70 °C for 30 min. The reaction was then cooled to 0 °C and slowly added to a saturated aqueous Na_2CO_3 solution to neutralize the acid. The resulting solution was then directly purified by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford **INT O** (23.3 mg, 22%) as a white solid. LC-MS (ES+): 269.2 m/z [M+H], *tR* = 0.30 min

In a 2 dram vial equipped with a stir bar, **INT O** (23.3 mg, 0.08 mmol, 1.0 equiv) and 2-(morpholinomethyl)benzoic acid (0.018 g, 0.08 mmol, 1.0 equiv) were added. To the vial was added DMF (0.78 mL), followed by the addition of DIPEA (0.043 mL, 0.25 mmol, 3.0 equiv) and HATU (34.0 mg, 0.09 mmol, 1.1 equiv). The reaction was stirred at rt for 30 min. The reaction was then directly purified without workup by C18 column chromatography eluting with a 0-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and concentrated to afford compound **20** (17.0 mg, 44.0% yield) as a white solid. ^{1}H NMR (500 MHz, CD_3OD) 2.55 - 2.79 (m, 4H), 3.66 - 3.75 (m, 4H), 3.84 (br d, *J* = 12.2 Hz, 1H), 4.03 (br d, *J* = 12.2 Hz, 1H), 5.59 (s, 1H), 6.53 (t, *J* = 6.6 Hz, 1H), 7.23 (t, *J* = 7.6 Hz, 1H), 7.31 (d, *J* = 8.1 Hz, 1H), 7.37 - 7.44 (m, 2H), 7.47 - 7.55 (m, 2H), 7.57 - 7.62 (m, 2H), 7.89 (d, *J* = 7.3 Hz, 1H), 7.94 (br d, *J* = 6.9 Hz, 1H) (NH protons not present). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{26}H_{26}N_5O_4^+$: 472.1985. Found: 472.1988.

Synthesis of Compound **23** and **24**:



In a 1 L RB flask, methyl 4-formyl-3-nitrobenzoate (84.0 g, 402 mmol, 1.0 equiv) was added, along with a stir bar. The flask was purged with nitrogen, followed by the addition of THF (800 mL). The reaction was then cooled to -40 °C with stirring before the dropwise addition of phenylmagnesium bromide (402 mL, 402 mmol, 1.0 equiv) as a 1M solution in THF. The reaction was then stirred at -40 °C for 1 h. The reaction was then allowed to warm to rt and was quenched with an aqueous saturated NH_4Cl solution. The solution was then extracted with ethyl acetate, and the combined organic layers were washed with brine. The combined organic layers were then dried over sodium sulfate and were concentrated. The residue was then dissolved in minimal DCM and purified by silica gel chromatography eluting with a 0-20% ethyl acetate in pentane gradient. The purified fractions were combined and concentrated to afford methyl 4-(hydroxy(phenyl)methyl)-3-nitrobenzoate (54.0 g, 47%) as a yellow gum. LC-MS (ES+): 288.1 m/z [M+H], *tR* = 0.88 min

In a 1 L RB flask, methyl 4-(hydroxy(phenyl)methyl)-3-nitrobenzoate (34.0 g, 118 mmol, 1.0 equiv) was added, along with a stir bar. The flask was purged with nitrogen, followed by the addition of ethyl acetate (800 mL). The reaction was stirred at rt before the addition of manganese dioxide (51.4 g, 592 mmol, 5.0 equiv). The reaction was then heated at 80 °C to stir for 16 h. The reaction was then allowed to cool to rt and was filtered over celite. The filtrate was concentrated to afford methyl 4-benzoyl-3-nitrobenzoate (32.1 g, 95%) as a yellow solid. LC-MS (ES+): 286.1 m/z [M+H], *tR* = 0.72 min

In a 500 mL RB flask, methyl 4-benzoyl-3-nitrobenzoate (32.0 g, 112 mmol, 1.0 equiv) was added, along with a stir bar. The flask was purged with nitrogen, followed by the addition of methanol (800 mL). The reaction was stirred at rt before the addition of 3.0 g of Pd/C (10 wt. %), at which point the nitrogen inlet was swapped for an H_2 balloon. The reaction was then stirred at rt for 3 h. The reaction mixture was then filtered over celite and the filtrate was concentrated in a

1 L RB flask. To the flask was then added a stir bar, and the flask was purged with nitrogen before dissolving the residue in ethyl acetate (600 mL). The reaction was stirred at rt before the addition of manganese dioxide (29.3 g, 337 mmol, 3.0 equiv). The reaction was then heated at 80 ^{o}C to stir for 2 h. The reaction was then allowed to cool to rt and was filtered over celite. The filtrate was concentrated to afford the crude residue. The residue was dissolved in minimal DCM to be purified by silica gel chromatography eluting with a 0-10% ethyl acetate in petroleum ether gradient. The purified fractions were combined and concentrated to afford methyl 3-amino-4-benzoylbenzoate (18.0 g, 63%) as a yellow solid. LC-MS (ES+): 256.1 m/z [M+H], *tR* = 0.90 min

In a 500 mL RB flask equipped with a stir bar, 2-(1*H*-benzo[d][1,2,3]triazol-1-yl)-2-(((benzyloxy)carbonyl)amino)acetic acid[3] (12.8 g, 39.2 mmol, 1.0 equiv) was added. The flask was purged with nitrogen before the addition of THF (150 mL). The reaction was cooled to 0 ^{o}C with stirring before the dropwise addition of a solution of oxalyl chloride (5.48 g, 43.2 mmol, 1.1 equiv) in DCM (75 mL). The reaction mixture was then stirred at 0 ^{o}C for 5 min before the addition of DMF (3.04 mL, 39.2 mmol, 1.0 equiv). The reaction mixture was stirred at 0 ^{o}C for 2 h. At this point, a solution of methyl 3-amino-4-benzoylbenzoate (11.01 g, 43.2 mmol, 1.1 equiv) and 4-methylmorpholine (7.94 g, 78.5 mmol, 2.0 equiv) in THF (75 mL) was added dropwise to the reaction mixture over a period of 15 min. The resulting reaction mixture was then allowed to warm to rt and was stirred 2 h. At this point, the reaction mixture was filtered over celite, and the filtrate was concentrated. The crude product was taken onto the next step without purification.

The crude product from the previous step was added to a 250 mL RB flask equipped with a stir bar. The flask was purged with nitrogen before the addition of ammonia (80.0 mL, 560 mmol, 14.3 equiv) as a 7M solution in MeOH. The reaction was stirred at rt for 18 h. The reaction mixture was then concentrated, and the residue was partitioned between ethyl acetate and a 1M aqueous solution of NaOH. The organic layer was separated, dried over sodium sulfate, and concentrated. The crude oil was then transferred to a 250 mL RB flask equipped with a stir bar and was dissolved in acetic acid (150 mL). To this mixture was added ammonium acetate (18.1 g, 235 mmol, 6.0 equiv), and the reaction was stirred at rt for 18 h. The reaction mixture was then concentrated down to ~10-15 mL of mixture before diluting with ethyl ether (300 mL), causing a precipitate to crash out of solution. The mixture was then filtered, and the precipitate was washed with ethyl ether. The solid was then dissolved in minimal DCM was purified by silica gel chromatography eluting with a 0-100% ethyl acetate in petroleum ether gradient. The purified fractions were combined and concentrated to afford *rac*-methyl-3-(((benzyloxy)carbonyl)amino)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-8-carboxylate (10.1 g, 58%) as a yellow solid. LC-MS (ES+): 444.2 m/z [M+H], tR = 0.92 min

In a 250 mL RB flask equipped with a stir bar was added *rac*-methyl-3-(((benzyloxy)carbonyl)amino)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-8-carboxylate (6.50 g, 14.7 mmol, 1.0 equiv). This was followed by the addition of HBr (90 mL, 33 wt. % in acetic acid). The reaction mixture was heated to 70 ^{o}C and stirred for 30 min. The reaction mixture was allowed to cool to rt and was poured into ethyl ether (250 mL). The precipitate was collected by filtration and washed with ethyl ether. The solid was then suspended in an aqueous saturated $NaHCO_3$ solution adjust the pH to ~8-9, and was filtered once more, washing with a 1:1 DCM/petroleum ether solution. The solid was then dried under vacuum to afford **INT P** (3.4 g, 75%) as a light yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) 2.59 (s, 2H), 3.91 (s, 3H), 4.28 (s, 1H), 7.37 - 7.58 (m, 6H), 7.73 (dd, *J* = 8.1, 1.7 Hz, 1H), 7.86 (d, *J* = 1.7 Hz, 1H), 10.86 (s, 1H). LC-MS (ES+): 310.1 m/z [M+H], tR = 0.49 min

In a 30 mL scintillation vial equipped with a stir bar, **INT D** (277 mg, 1.18 mmol, 1.0 equiv), **INT P** (400 mg, 1.29 mmol, 1.1 equiv), and HATU (492 mg, 1.29 mmol, 1.1 equiv) were added. The mixture was dissolved in DMF (4 mL), followed by the addition of DIPEA (1.03 mL, 5.90 mmol, 5.0 equiv). The reaction was stirred overnight at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford methyl (*rac*)-3-(2-(((*S*)-2-methylmorpholino)methyl)benzamido)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-8-carboxylate (377 mg, 61%) as a pale yellow solid. LC-MS (ES+): 527.2 m/z [M+H], tR = 0.79 min

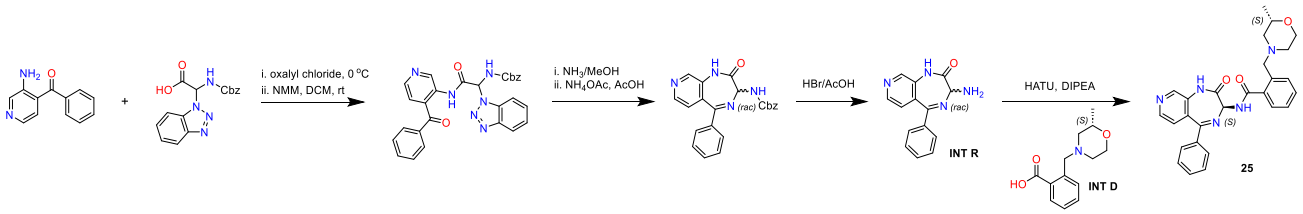
In a 30 mL scintillation vial equipped with a stir bar, methyl (*rac*)-3-(2-(((*S*)-2-methylmorpholino)methyl)benzamido)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-8-carboxylate (350 mg, 0.66 mmol, 1.0 equiv) and a 1M aqueous solution of NaOH (1.66 mL, 1.66 mmol, 2.5 equiv) were added. The reaction mixture was then diluted with methanol (3 mL) and was heated at 60 ^{o}C with stirring for 1 h in an oil bath. The reaction was then cooled to rt, and the methanol was removed under a stream of nitrogen. The resulting mixture was acidified to pH ~5 with a 2N solution of HCl, causing the product to precipitate out of solution. The solid was filtered, washed with water, and dried under vacuum to afford **INT Q** (256 mg, 75%) as a yellow solid. LC-MS (ES+): 513.3 m/z [M+H], tR = 0.66 min

In a 30 mL scintillation vial equipped with a stir bar, **INT Q** (132 mg, 0.26 mmol, 1.0 equiv), 2-methoxyethan-1-amine (29.0 mg, 0.39 mmol, 1.5 equiv), and HATU (108 mg, 0.28 mmol, 1.1 equiv) were added. The mixture was dissolved in DMF (5 mL), followed by the addition of DIPEA (180 µl, 1.03 mmol, 4.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% ammonium hydroxide buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford the product (83.0 mg, 57%) as an off-white solid (1:1 mixture of diastereomers). The product was further purified by chiral SFC to obtain compound **23** (26.5 mg, 17%, >98:2 er) and the less active isomer (27.2 mg, 18%, >98:2 er) as white solids. (see SFC

Report in Spectral Data section III). ^{1}H NMR (500 MHz, DMSO-d_6) 0.85 (br d, *J* = 6.3 Hz, 3H), 1.91 (br t, *J* = 10.5 Hz, 1H), 2.20 - 2.30 (m, 1H), 2.75 (br dd, *J* = 19.7, 11.1 Hz, 2H), 3.28 (s, 3H), 3.44 - 3.53 (m, 5H), 3.56 - 3.63 (m, 1H), 3.69 (br d, *J* = 11.7 Hz, 2H), 3.87 (br d, *J* = 12.1 Hz, 1H), 5.49 (d, *J* = 7.5 Hz, 1H), 7.37 (br d, *J* = 7.2 Hz, 1H), 7.41 - 7.50 (m, 5H), 7.51 - 7.56 (m, 3H), 7.67 (br d, *J* = 7.9 Hz, 1H), 7.74 (br d, *J* = 7.2 Hz, 1H), 7.79 (s, 1H), 8.73 - 8.82 (m, 1H), 11.02 (br s, 1H), 11.55 (br d, *J* = 7.5 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{32}H_{36}N_5O_5^+$: 570.2716. Found: 570.2722.

In a 30 mL scintillation vial equipped with a stir bar, **INT Q** (80.0 mg, 0.16 mmol, 1.0 equiv), 1-methylpiperazin-2-one (21.4 mg, 0.19 mmol, 1.2 equiv), and HATU (77.0 mg, 0.20 mmol, 1.3 equiv) were added. The mixture was dissolved in DMF (1 mL), followed by the addition of DIPEA (140 µl, 0.80 mmol, 5.0 equiv). The reaction was stirred for 2 h at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% ammonium hydroxide buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 $CHCl_3$:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford the product as a 1:1 mixture of diastereomers. The product was further purified by chiral SFC to obtain compound **24** (16.0 mg, 17%, >98:2 er) and the less active isomer (13.1 mg, 14%, >98:2 er) as white solids. (see SFC Report in Spectral Data section III). ^{1}H NMR (500 MHz, CD_2Cl_2) 0.89 (d, *J* = 6.4 Hz, 3H), 1.92 (t, *J* = 10.6 Hz, 1H), 2.27 (td, *J* = 11.2, 3.4 Hz, 1H), 2.81 - 2.93 (m, 2H), 2.98 (s, 3H), 3.33 - 3.52 (m, 2H), 3.60 - 3.77 (m, 5H), 3.86 - 4.05 (m, 2H), 4.12 - 4.36 (m, 2H), 5.80 (br d, *J* = 6.9 Hz, 1H), 7.22 - 7.30 (m, 3H), 7.40 - 7.51 (m, 6H), 7.56 - 7.59 (m, 2H), 7.86 - 7.92 (m, 1H), 8.14 (s, 1H), 11.83 (br s, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{34}H_{37}N_6O_5^+$: 609.2825. Found: 609.2825.

Synthesis of Compound **25**:



In a 250 mL RB flask equipped with a stir bar, 2-(1*H*-benzo[d][1,2,3]triazol-1-yl)-2-(((benzyloxy)carbonyl)amino)acetic acid[3] (3.32 g, 10.2 mmol, 1.2 equiv) was added. The flask was purged with nitrogen before the addition of THF (54 mL) and DCM (18 mL). The reaction was cooled to 0 °C with stirring before the dropwise addition of a 2M solution of oxalyl chloride (5.3 mL, 10.6 mmol, 1.25 equiv) in DCM. The reaction mixture was then stirred at 0 °C for 5 min before the addition of DMF (50 µL, 0.65 mmol, 0.08 equiv). The reaction mixture was stirred at 0 °C for 1 h. At this point, a solution of (3-aminopyridin-4-yl)phenylmethanone[4] (1.68 g, 8.48 mmol, 1.0 equiv) and 4-methylmorpholine (2.14 g, 21.2 mmol, 2.5 equiv) in THF (40 mL) was added dropwise to the reaction mixture over a period of 15 min. The resulting reaction mixture was then allowed to warm to rt and was stirred overnight. At this point, the reaction

mixture was filtered over celite, and the filtrate was concentrated. The crude product was taken onto the next step without purification.

The crude product from the previous step was added to a 250 mL RB flask equipped with a stir bar. The flask was purged with nitrogen before the addition of ammonia (50 mL) as a 7M solution in MeOH. The reaction was stirred at 35 ^{o}C in an oil bath for 3 h. The reaction mixture was then concentrated, and the residue was dissolved in acetic acid (50 mL). To this mixture was added ammonium acetate (6.53 g, 84.8 mmol, 10 equiv), and the reaction was stirred at rt for 3 h. The reaction mixture was then concentrated down to ~5 mL of mixture before diluting with water (50 mL). The mixture was cooled to 0 ^{o}C and was neutralized with a 2M aqueous NaOH solution to a pH of ~8-9. This caused a precipitate to crash out of solution. The mixture was then filtered, and the precipitate was washed with water. The solid was then dried under vacuum to afford *rac*-benzyl 2-oxo-5-phenyl-2,3-dihydro-1*H*-pyrido[3,4-e][1,4]diazepin-3-ylcarbamate (3.10 g, 95%) as an off-white solid. LC-MS (ES+): 387.1 m/z [M+H], tR = 0.94 min

In a 30 mL scintillation vial equipped with a stir bar was added *rac*-benzyl 2-oxo-5-phenyl-2,3-dihydro-1*H*-pyrido[3,4-e][1,4]diazepin-3-ylcarbamate (230 mg, 0.60 mmol, 1.0 equiv). This was followed by the addition of HBr (6.0 mL, 33 wt. % in acetic acid). The reaction mixture was heated to 70 ^{o}C and stirred for 30 min. The reaction mixture was allowed to cool to rt and was poured into ethyl ether (20 mL). The precipitate was collected by filtration and washed with ethyl ether. The solid was then dried under vacuum to afford **INT R** (210 mg, 85%) as a brown solid. LC-MS (ES+): 253.2 m/z [M+H], tR = 0.41 min

In a 30 mL scintillation vial equipped with a stir bar, **INT R** (37.0 mg, 0.15 mmol, 1.0 equiv), **INT D** (41.4 mg, 0.18 mmol, 1.2 equiv), and HATU (61.3 mg, 0.16 mmol, 1.1 equiv) were added. The mixture was dissolved in DMF (5 mL), followed by the addition of DIPEA (128 µl, 0.73 mmol, 5.0 equiv). The reaction was stirred for 30 min at rt. The reaction was then directly purified without workup by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% formic acid buffer). The desired fractions were combined and diluted with a saturated aqueous $NaHCO_3$ solution and extracted with a 3:1 CHCl3:IPA solvent system. The combined organic layers were dried over sodium sulfate and concentrated to afford the product as a 1:1 mixture of diastereomers. The product was further purified by chiral SFC to obtain compound **25** (6.1 mg, 9%, >98:2 er) and the less active isomer (6.2 mg, 9%, >98:2 er) as off-white solids. (see SFC Report in Spectral Data section III). ^{1}H NMR (500 MHz, DMSO-d_6) 0.86 (d, *J* = 6.3 Hz, 3H), 1.91 (t, *J* = 10.5 Hz, 1H), 2.24 (td, *J* = 11.3, 3.0 Hz, 1H), 2.69 - 2.81 (m, 2H), 3.48 (td, *J* = 11.3, 1.9 Hz, 1H), 3.53 - 3.61 (m, 1H), 3.65 - 3.73 (m, 2H), 3.85 (d, *J* = 12.1 Hz, 1H), 5.49 (d, *J* = 7.6 Hz, 1H), 7.30 (d, *J* = 5.2 Hz, 1H), 7.35 - 7.41 (m, 1H), 7.44 - 7.52 (m, 4H), 7.53 - 7.59 (m, 3H), 7.72 - 7.76 (m, 1H), 8.43 (d, *J* = 5.2 Hz, 1H), 8.68 (s, 1H), 11.14 (br s, 1H), 11.55 (br d, *J* = 7.6 Hz, 1H). HRMS (ESI) m/z $(M+H)^+$ calculated for $C_{27}H_{28}N_5O_3^+$: 470.2192. Found: 470.2199.

Abbreviation Table for Synthesis Terms:

HATU- Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium (IUPAC: 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate)
DMF- Dimethylformamide
DIPEA- Diisopropylethylamine
MeCN- Acetonitrile
NH_4OH- Ammonium hydroxide
$NaHCO_3$- Sodium bicarbonate
$CHCl_3$- Chloroform
IPA- Isopropyl alcohol
DMSO- Dimethylsulfoxide
SFC- Supercritical fluid chromatography
LiOH- Lithium hydroxide
THF- Tetrahydrofuran
HCl- Hydrochloric acid
LC-MS- Liquid chromatography mass spectrometry
ES- Electrospray
tR- Retention time
HRMS- High resolution mass spectrometry
ESI- Electrospray ionization
$CDCl_3$- Deuterated chloroform
CD_2Cl_2- Deuterated dichloromethane
CD_3OD- Deuterated methanol
NMR- Nuclear magnetic resonance
nBuLi- n-Butyllithium
NH_4Cl- Ammonium chloride
DCM- Dichloromethane
RB flask- Round bottom flask
DMAP- 4-Dimethylaminopyridine
EDC-HCl- 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride
MeOH- Methanol
NaOH- Sodium hydroxide
AcOH- Acetic acid
HBr- Hydrobromic acid
Na_2CO_3- Sodium carbonate
Pd/C- Palladium on carbon
H_2- Hydrogen

III. Spectral Data

Chiral SFC Data:

Compound 2:

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Points Count	7201	***Run Time***	6.00 min		
Sample Name	EN12183-46-1A_ID_MeOH40%6min_01082021-1				
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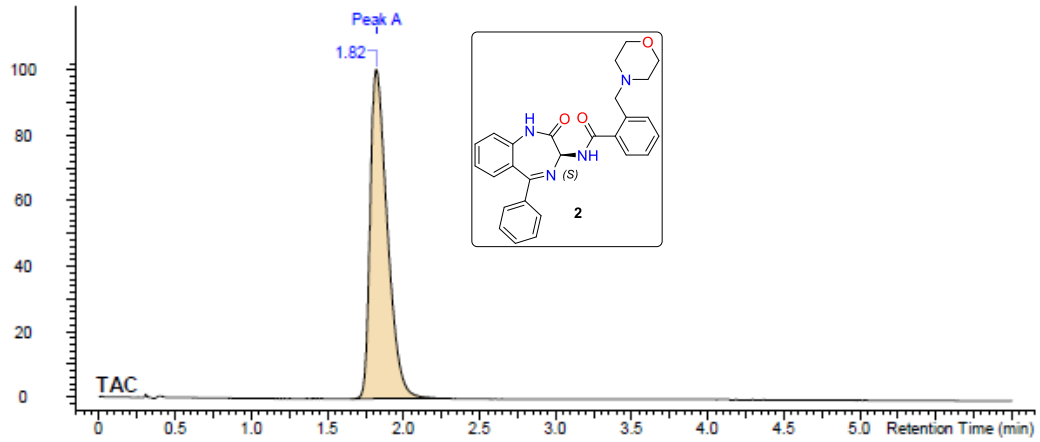
Type	HPLC	***X Axis***	Wavelength (nanometers)	***Y Axis***	Arbitrary



No.	Peak Name	tR (min)	Peak Area (Y units*min)	Area (%)
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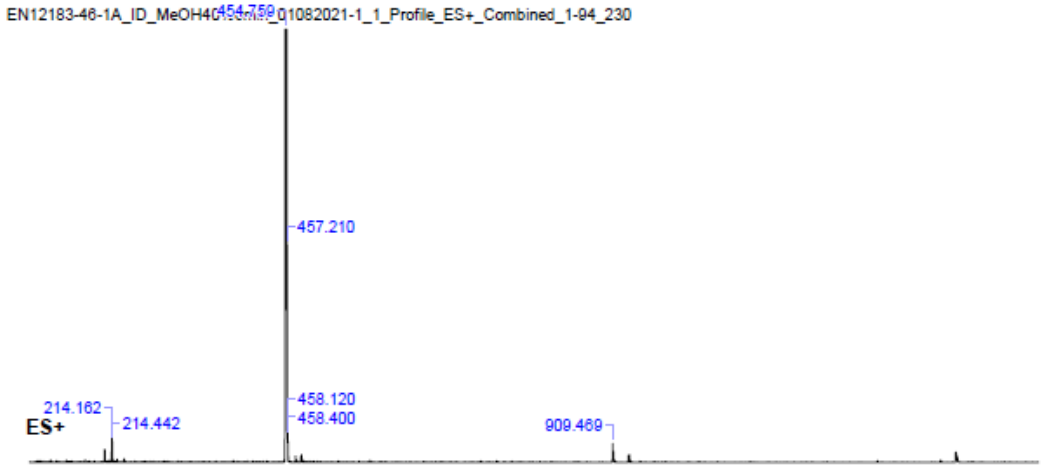

Compound 3:

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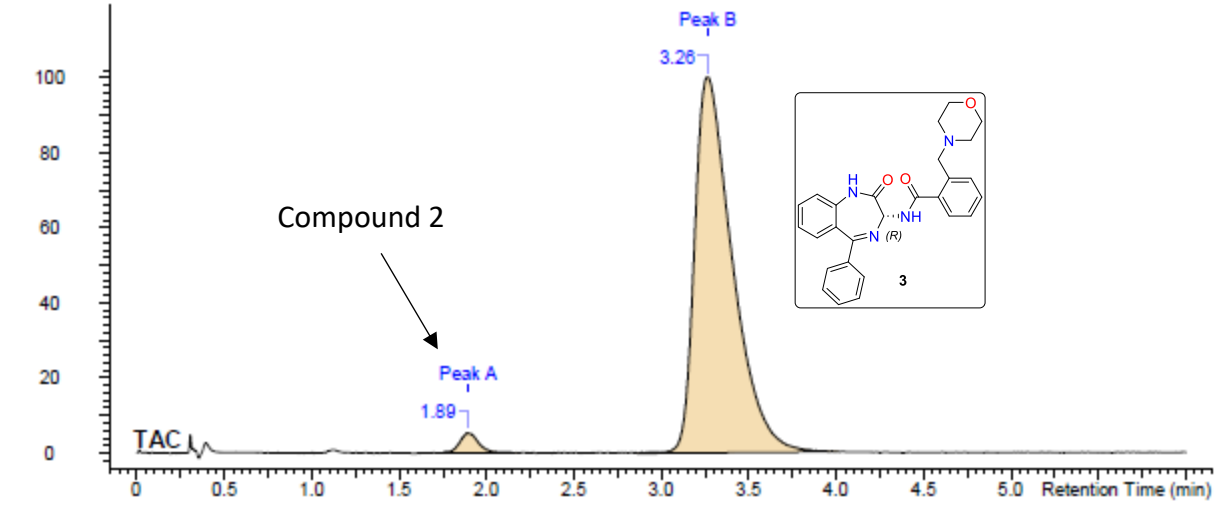
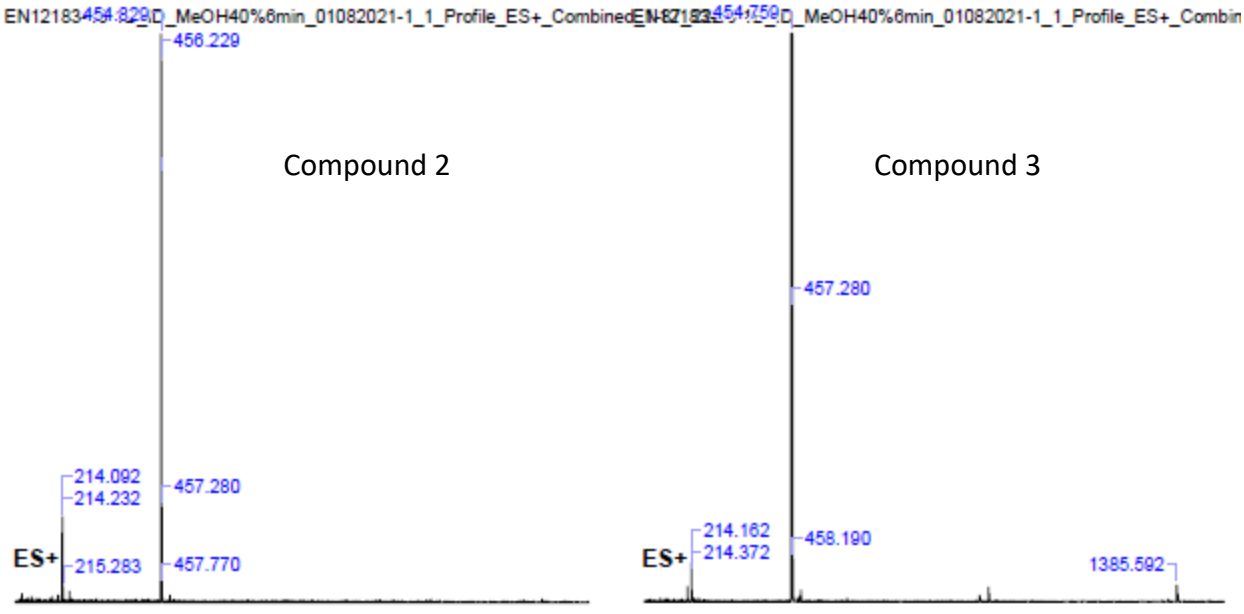
Points Count	7201	***Run Time***	6.00 min		
Sample Name	EN12183-46-1B ID MeOH40%6min 01082021-1			***Short File Name***	EN12183-46-1B.spectrus
Spectral Region	UV	***Spectrum Range***	189.9669 - 359.9669	***Technique***	UV-Visible
Temperature	40°C	***Type***	HPLC	***X Axis***	Wavelength (nanometers)
Y Axis	Arbitrary				



No.	Peak Name	tR (min)	Peak Area (Y units*min)	Area (%)
1	Peak A	1.890	152123.156	2.606
2	Peak B	3.262	5685733.500	97.394



Compound 21:

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Points Count	7201	*Run Time*	6.00 min		
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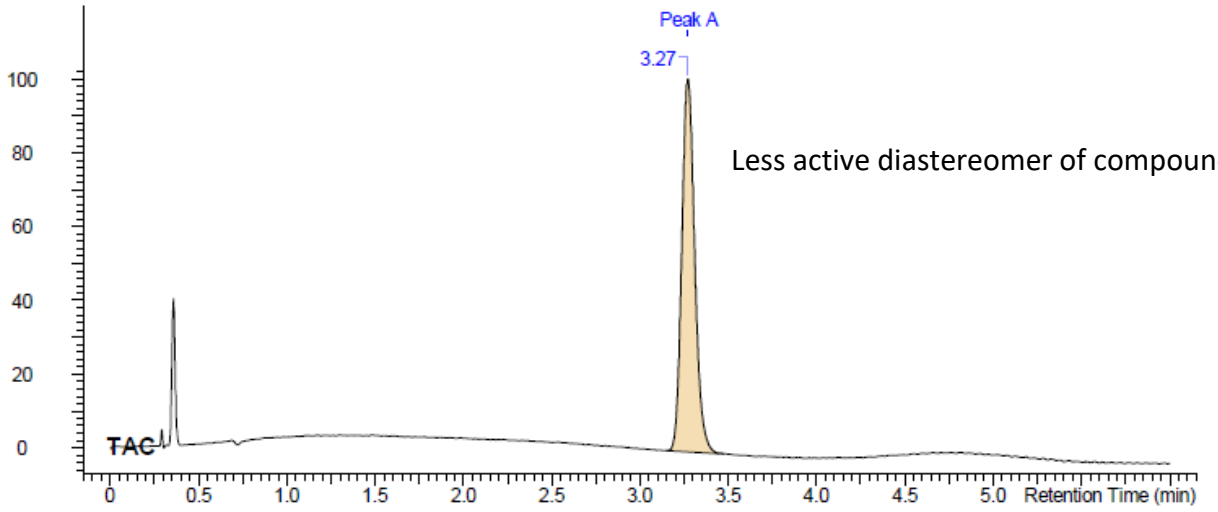
Y Axis	Arbitrary				



Less active diastereomer of compound 21

No.	Peak Name	tR (min)	Peak Area (Y units*min)	Area (%)
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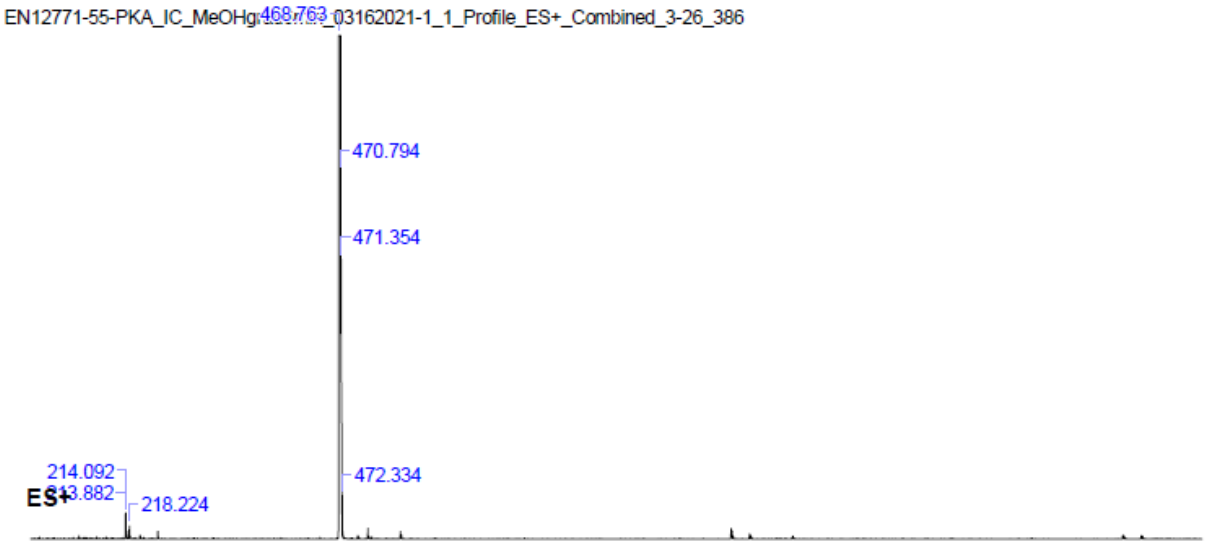

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Points Count	7201	*Run Time*	6.00 min		
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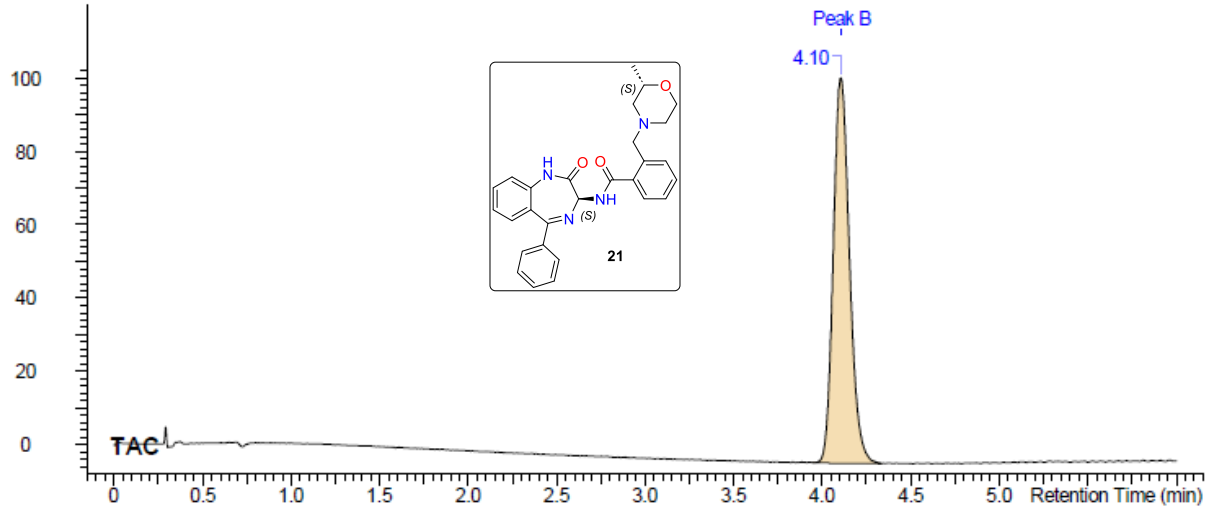
Temperature	40°C	*Type*	HPLC	*X Axis*	Wavelength (nanometers)
Y Axis	Arbitrary				



No.	Peak Name	tR (min)	Peak Area (Y units*min)	Area (%)
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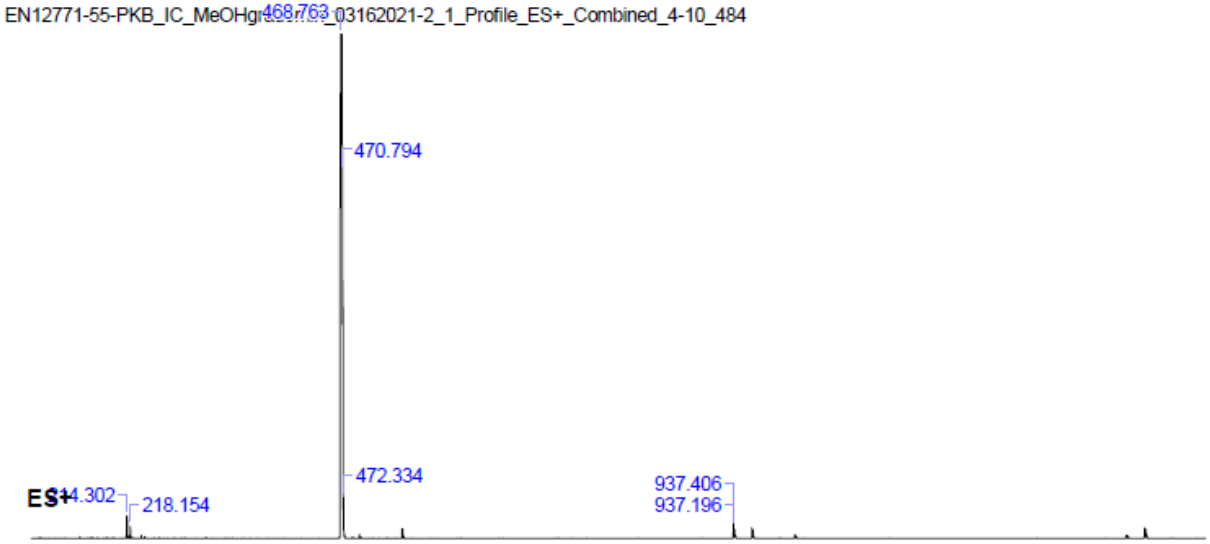
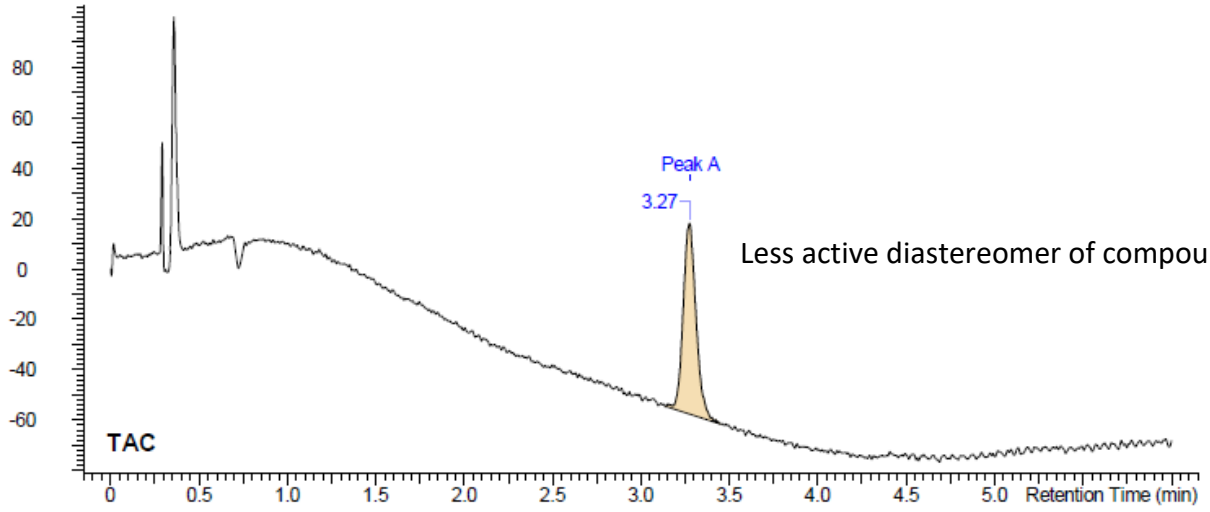


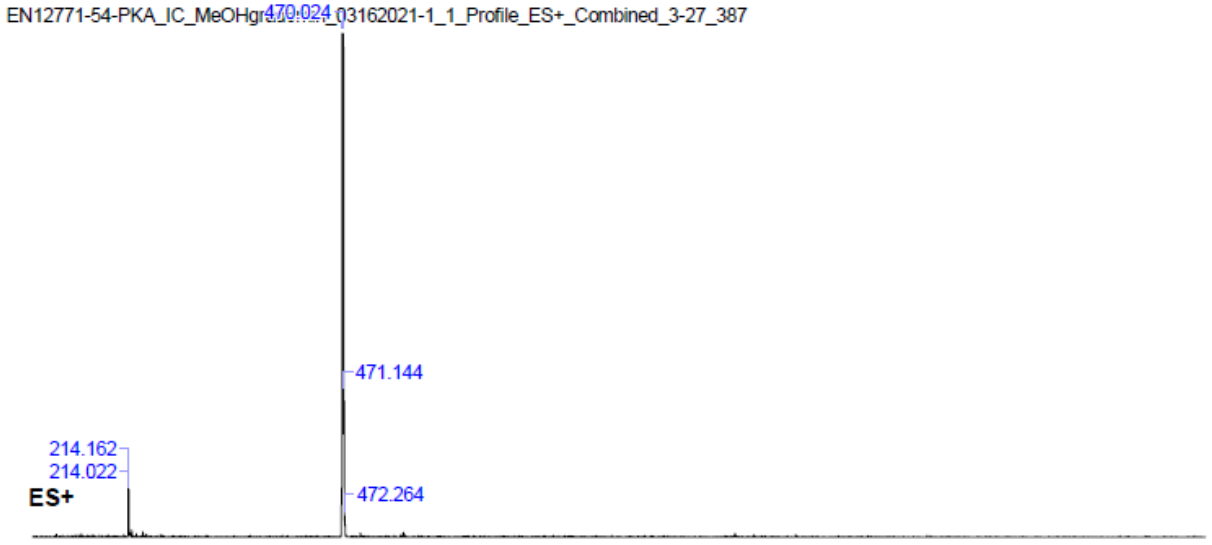
Compound 22:

Data Spacing	1.0000	*Detector*	UV	*Detector Lag*	0
File Name	C:\Users\ktjt918\OneDrive - AZCollaboration\Desktop\Raw data\EN12771-54-PKA_IC_MeOHgrad6min_03162021-1.raw				
Injection Volume	2 uL	*Instrument Name*	ACQ-SQD#LBA950		
Internal Name	EN12771-54-PKA_IC_MeOHgrad6min_03162021-1_2_DAD			*Mobile Phase*	MeOH
Points Count	7201	*Run Time*	6.00 min		
Sample Name	EN12771-54-PKA_IC_MeOHgrad6min_03162021-1				
Short File Name	EN12771-54-PKA_IC_MeOHgrad6min_03162021-1.raw			*Spectral Region*	UV
Spectrum Range	189.9669 - 359.9669	*Technique*	UV-Visible	*Temperature*	40°C
Type	HPLC	*X Axis*	Wavelength (nanometers)	*Y Axis*	Arbitrary

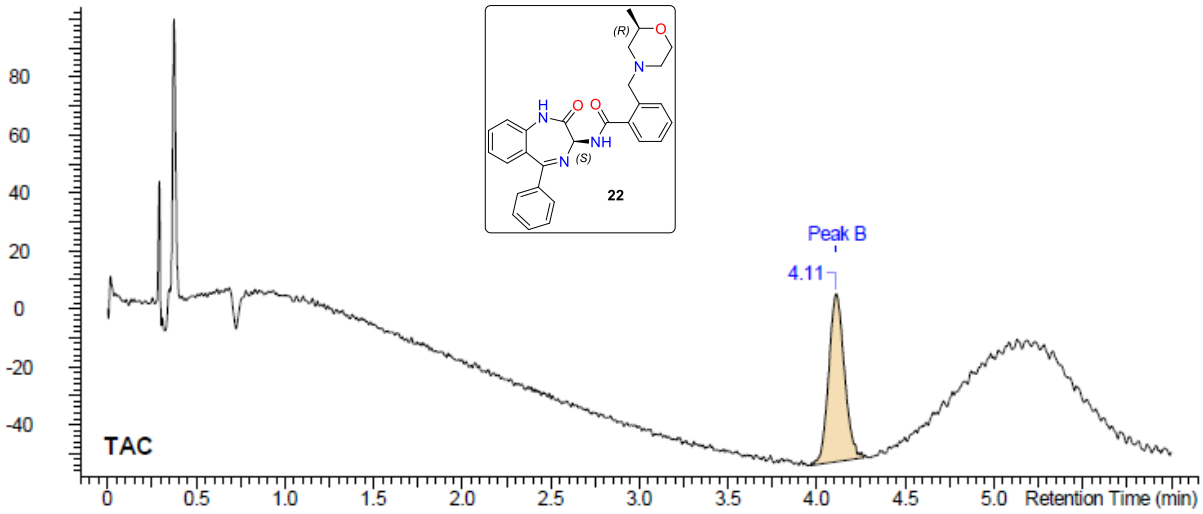


Less active diastereomer of compound 22

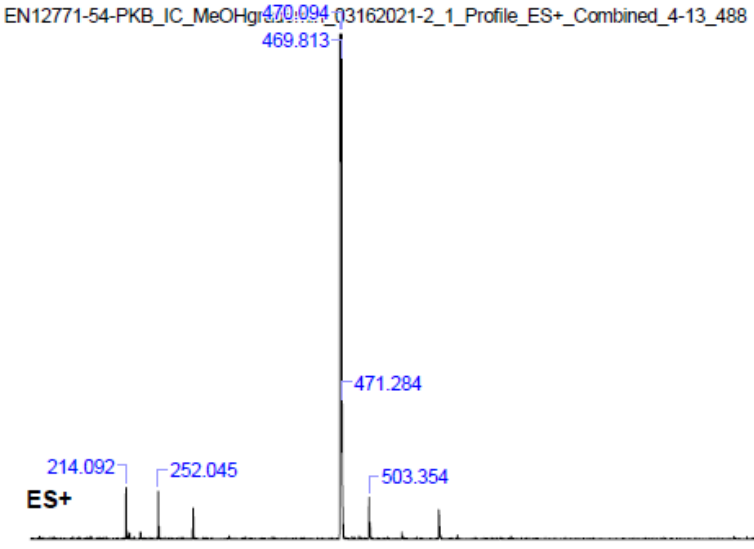
No.	Peak Name	tR (min)	Peak Area (Y units*min)	Area (%)
1	Peak A	3.275	194175.641	100.000



Data Spacing	1.0000	*Detector*	UV	*Detector Lag*	0
File Name	C:\Users\ktjt918\OneDrive - AZCollaboration\ACD\processed data examples\EN12771-54-B.spectrus				
Injection Volume	2 uL	*Instrument Name*	ACQ-SQD#LBA950		
Internal Name	EN12771-54-PKB_IC_MeOHgrad6min_03162021-2_2_DAD			*Mobile Phase*	MeOH
Points Count	7201	*Run Time*	6.00 min		
Sample Name	EN12771-54-PKB_IC_MeOHgrad6min_03162021-2			*Short File Name*	EN12771-54-B.spectrus
Spectral Region	UV	*Spectrum Range*	189.9669 - 359.9669	*Technique*	UV-Visible
Temperature	40°C	*Type*	HPLC	*X Axis*	Wavelength (nanometers)
Y Axis	Arbitrary				



No.	Peak Name	tR (min)	Peak Area (Y units*min)	Area (%)
1	Peak B	4.110	171322.094	100.000

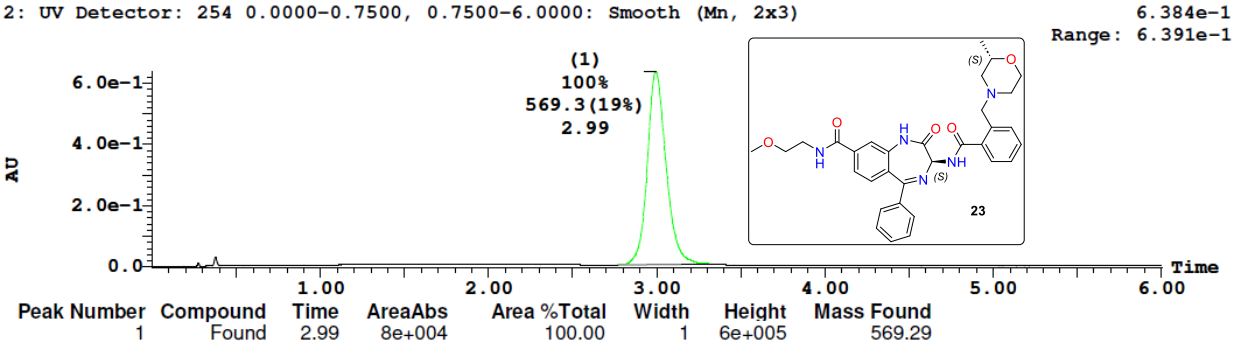


Compound 23:

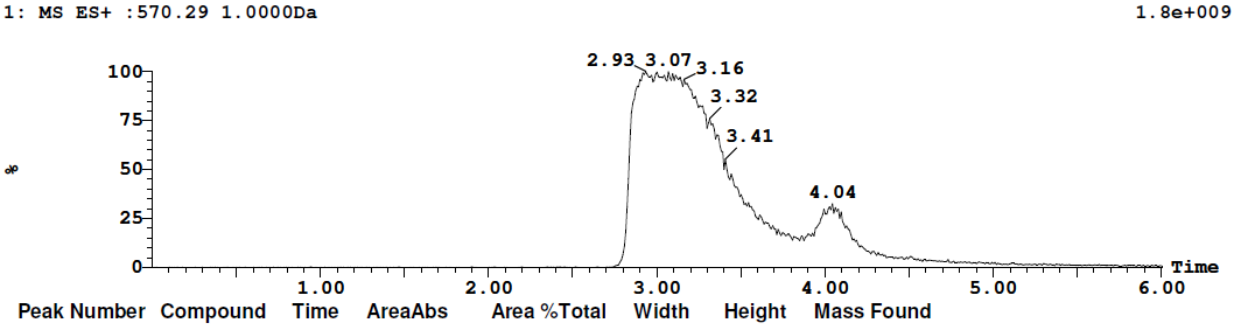
AstraZeneca RD Boston Page 1

Date:30-Jun-2021 File:EN12871-99_IA_MeOHgrad6min_6302021-pkA Inlet Method:C2_B2_1060_6min
Time:08:00:17 Sample:EN12871-99 Column:IA

Sample Report:

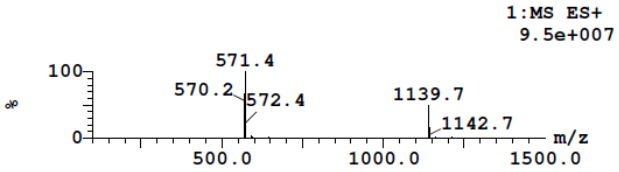


Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1	Found	2.99	8e+004	100.00	1	6e+005	569.29



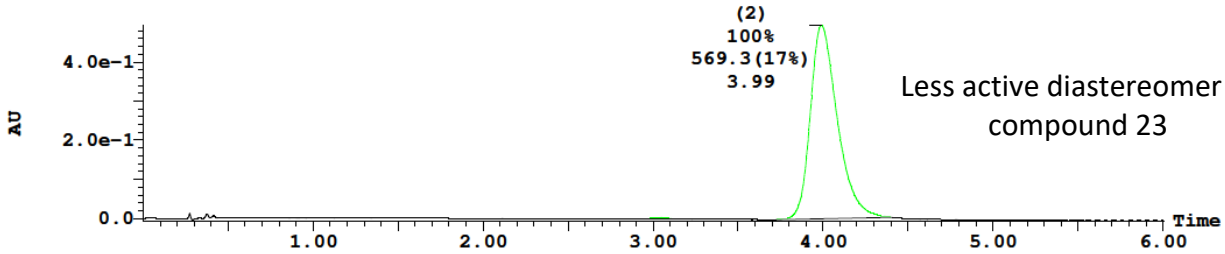
Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found

Peak ID	Compound	Time	Mass Found
1	Found	2.99	570.29



Sample Report (continued):

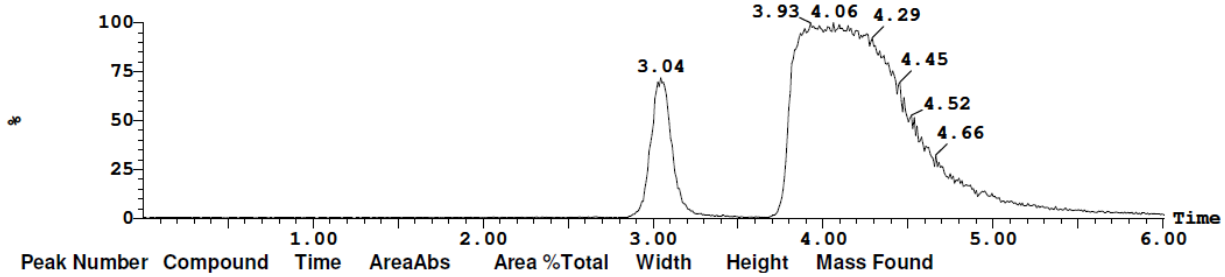
2: UV Detector: 254 0.0000-0.7500, 0.7500-6.0000: Smooth (Mn, 2x3) 4.94e-1
Range: 4.999e-1



Less active diastereomer of compound 23

Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1	Found	3.04	5e+002	0.50	0	4e+003	569.29
2	Found	3.99	9e+004	99.50	1	5e+005	569.29

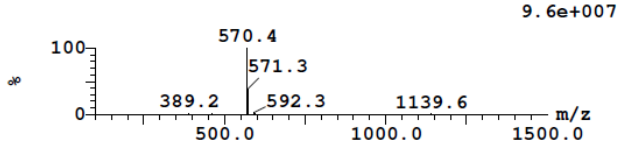
1: MS ES+ :570.29 1.0000Da 1.9e+009



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found

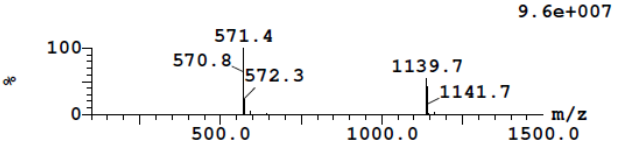
Peak ID	Compound	Time	Mass Found
1	Found	3.04	570.29

1:MS ES+
9.6e+007



Peak ID	Compound	Time	Mass Found
2	Found	3.99	570.29

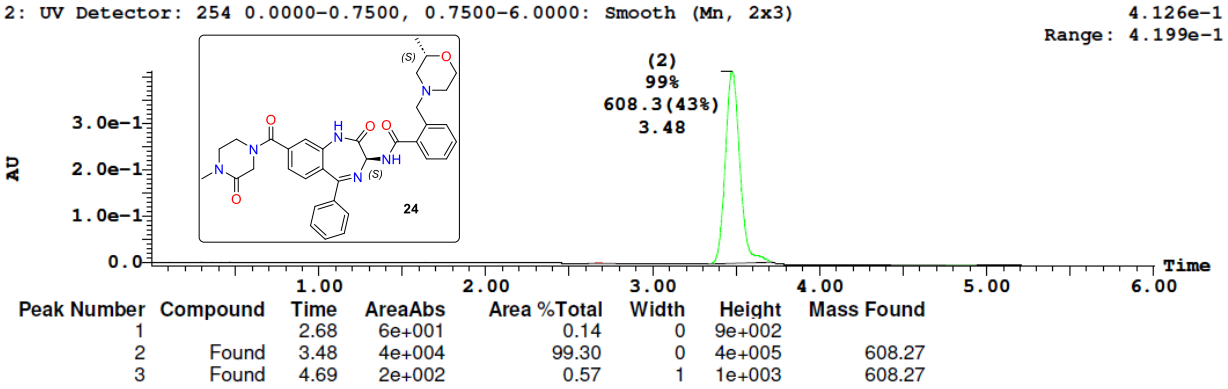
1:MS ES+
9.6e+007



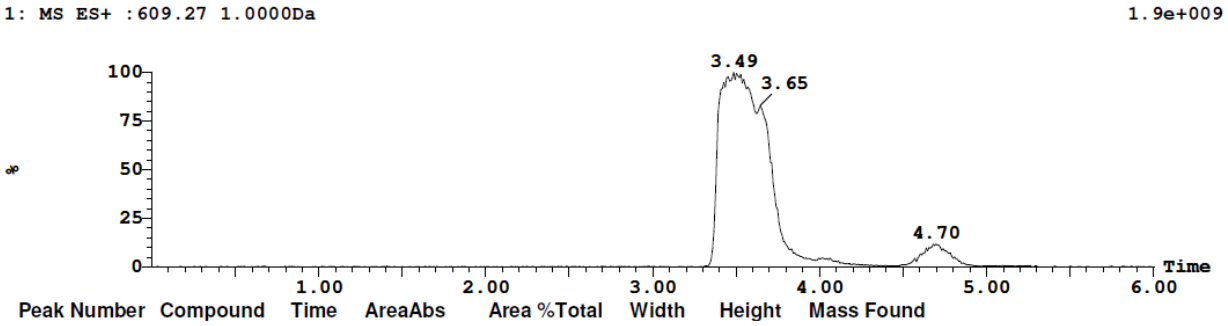
Compound 24:

AstraZeneca RD Boston Page 1

Date:01-Nov-2021 File:EN13196-09_11012021-8 Inlet Method:C2_B2_1060_6min
Time:09:49:10 Description:EN13196-09-pkA Mobile Phase:Methanol w/ 0.2% NH4OH
Vial:1:43 Column Name:IH 4.6x150mm, 5um Injection Volume:2.0000

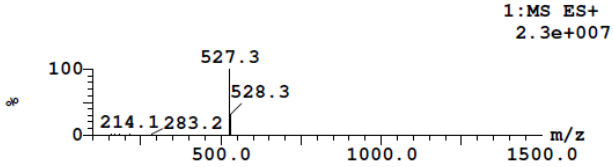


Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1		2.68	6e+001	0.14	0	9e+002	
2	Found	3.48	4e+004	99.30	0	4e+005	608.27
3	Found	4.69	2e+002	0.57	1	1e+003	608.27

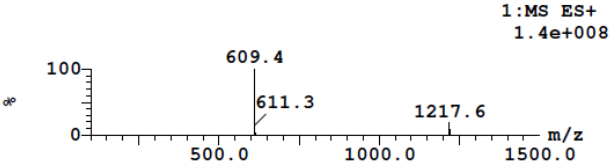


Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found

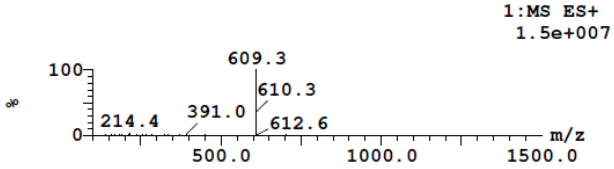
Peak ID	Compound	Time	Mass Found
1		2.68	



Peak ID	Compound	Time	Mass Found
2	Found	3.48	609.27



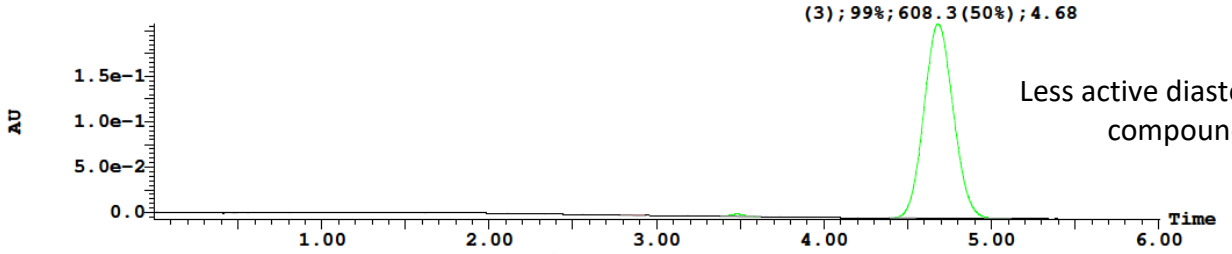
Peak ID	Compound	Time	Mass Found
3	Found	4.69	609.27



AstraZeneca RD Boston

Date:01-Nov-2021	File:EN13196-09_11012021-9	Inlet Method:C2_B2_1060_6min
Time:09:56:11	Description:EN13196-09-pkB	Mobile Phase:Methanol w/ 0.2% NH4OH
Vial:1:44	Column Name:IH 4.6x150mm, 5um	Injection Volume:2.0000

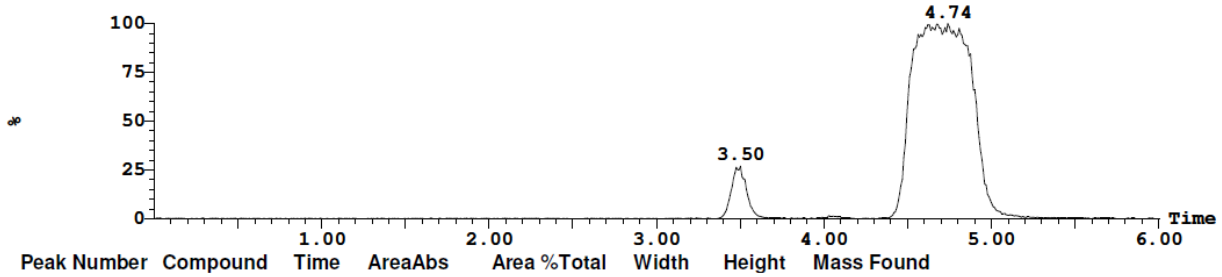
2: UV Detector: 254 0.0000-0.7500, 0.7500-6.0000: Smooth (Mn, 2x3) 2.073e-1
Range: 2.145e-1



Less active diastereomer of compound 24

Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1		2.84	6e+001	0.13	0	8e+002	
2	Found	3.48	3e+002	0.57	0	3e+003	608.27
3	Found	4.68	4e+004	99.30	1	2e+005	608.27

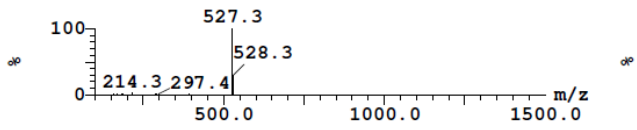
1: MS ES+ :609.27 1.0000Da 1.8e+009



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1		2.84	6e+001	0.13	0	8e+002	
2	Found	3.48	3e+002	0.57	0	3e+003	608.27
3	Found	4.68	4e+004	99.30	1	2e+005	608.27

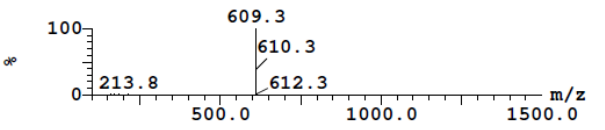
Peak ID	Compound	Time	Mass Found
1		2.84	

1:MS ES+
2.2e+007



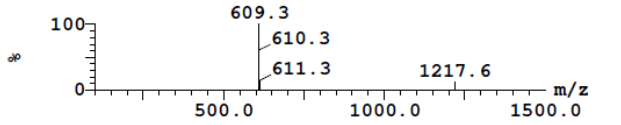
Peak ID	Compound	Time	Mass Found
2	Found	3.48	609.27

1:MS ES+
3.5e+007



Peak ID	Compound	Time	Mass Found
3	Found	4.68	609.27

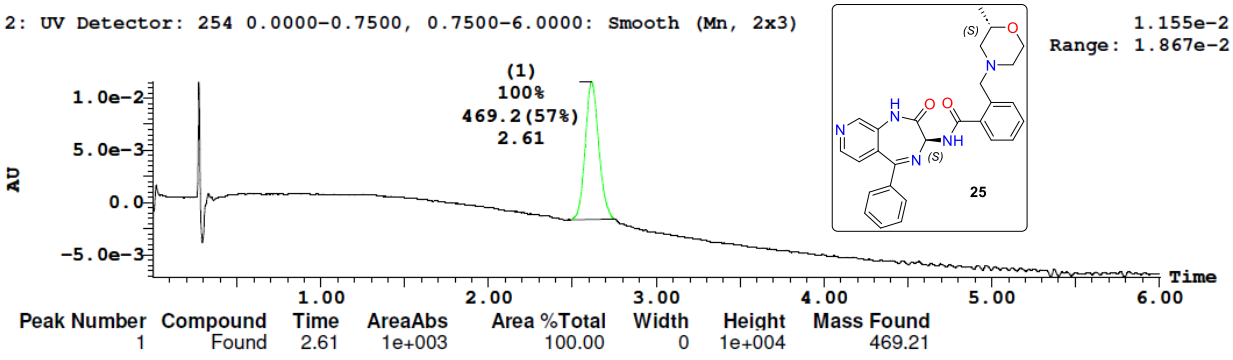
1:MS ES+
1.4e+008



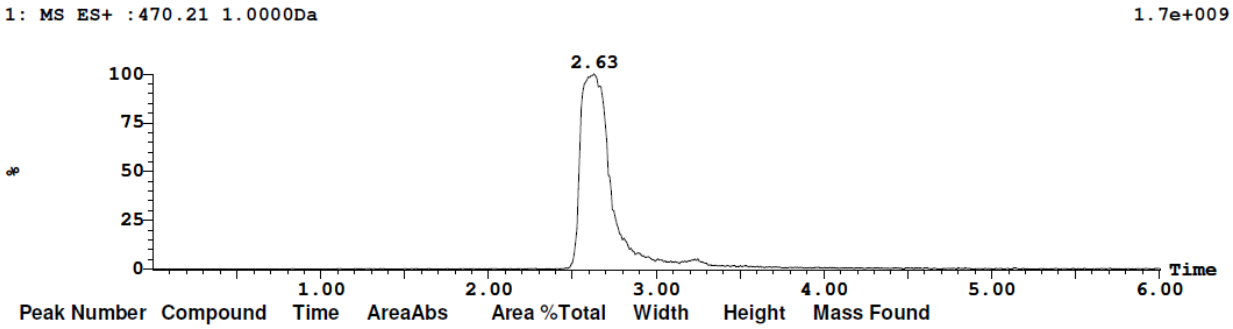
Compound 25:

AstraZeneca RD Boston Page 1

Date:09-Jul-2021 File:EN12995-15_07092021-1 Inlet Method:C2_B2_1060_6min
Time:08:02:27 Description:EN12995-15-pkA Mobile Phase:Methanol w/ 0.2% NH4OH
Vial:1:41 Column Name:IA 4.6x100mm, 5um Injection Volume:2.0000

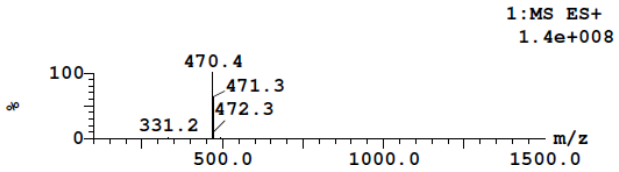


Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1	Found	2.61	1e+003	100.00	0	1e+004	469.21



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found

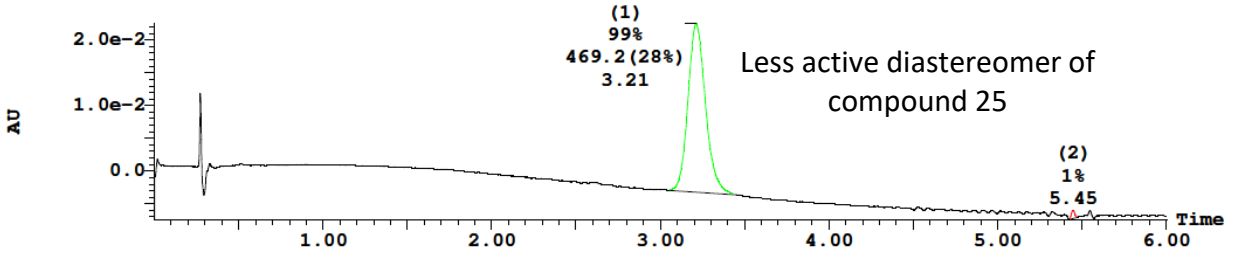
Peak ID	Compound	Time	Mass Found
1	Found	2.61	470.21



AstraZeneca RD Boston

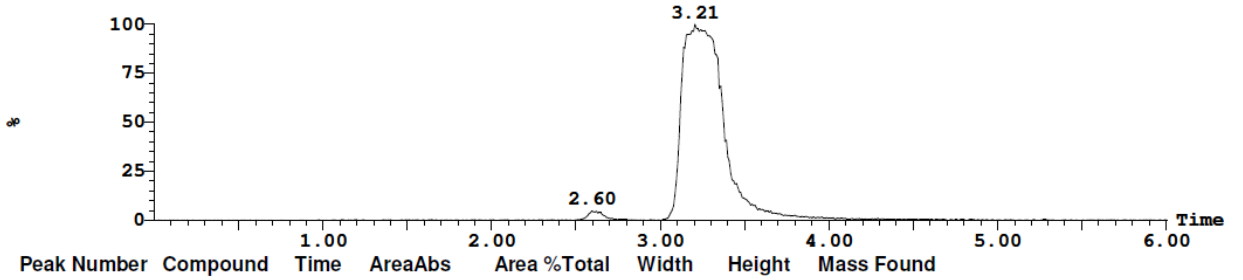
Date:09-Jul-2021 File:EN12995-15_07092021-2 Inlet Method:C2_B2_1060_6min
Time:08:09:34 Description:EN12995-15-pkB Mobile Phase:Methanol w/ 0.2% NH4OH
Vial:1:42 Column Name:IA 4.6x100mm, 5um Injection Volume:2.0000

2: UV Detector: 254 0.0000–0.7500, 0.7500–6.0000: Smooth (Mn, 2x3) 2.253e-2
Range: 2.994e-2



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1	Found	3.21	3e+003	99.07	0	3e+004	469.21
2		5.45	3e+001	0.93	0	1e+003	

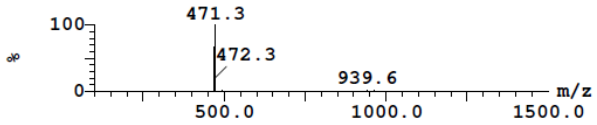
1: MS ES+ :470.21 1.0000Da 1.8e+009



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found

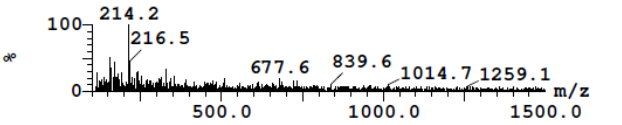
Peak ID	Compound	Time	Mass Found
1	Found	3.21	470.21

1:MS ES+ 1.1e+008

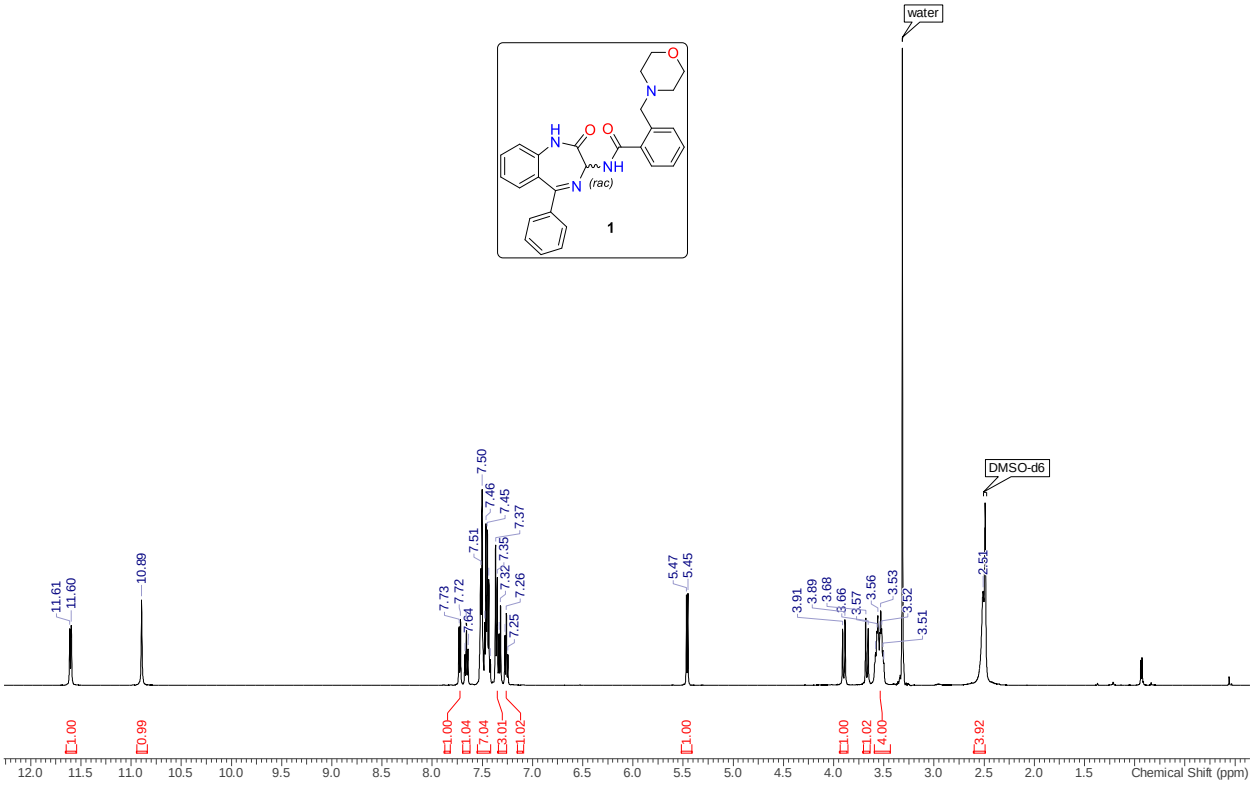


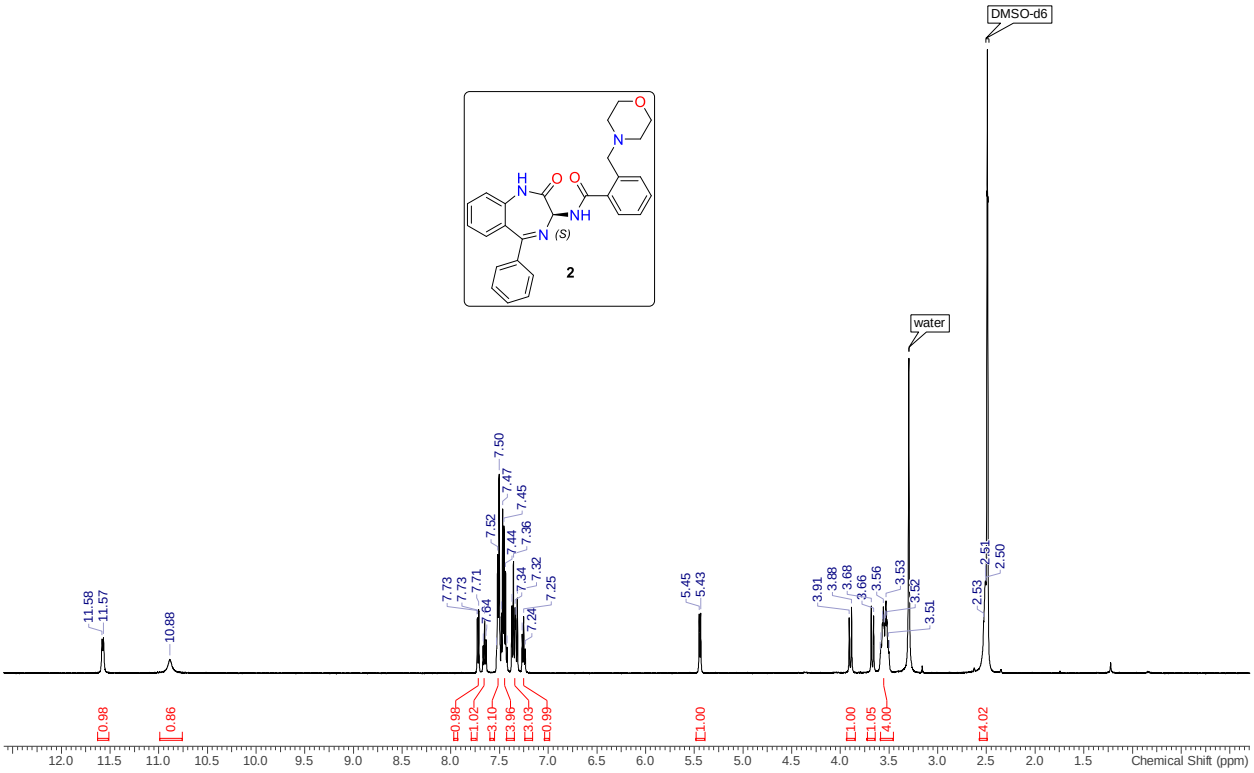
Peak ID	Compound	Time	Mass Found
2		5.45	

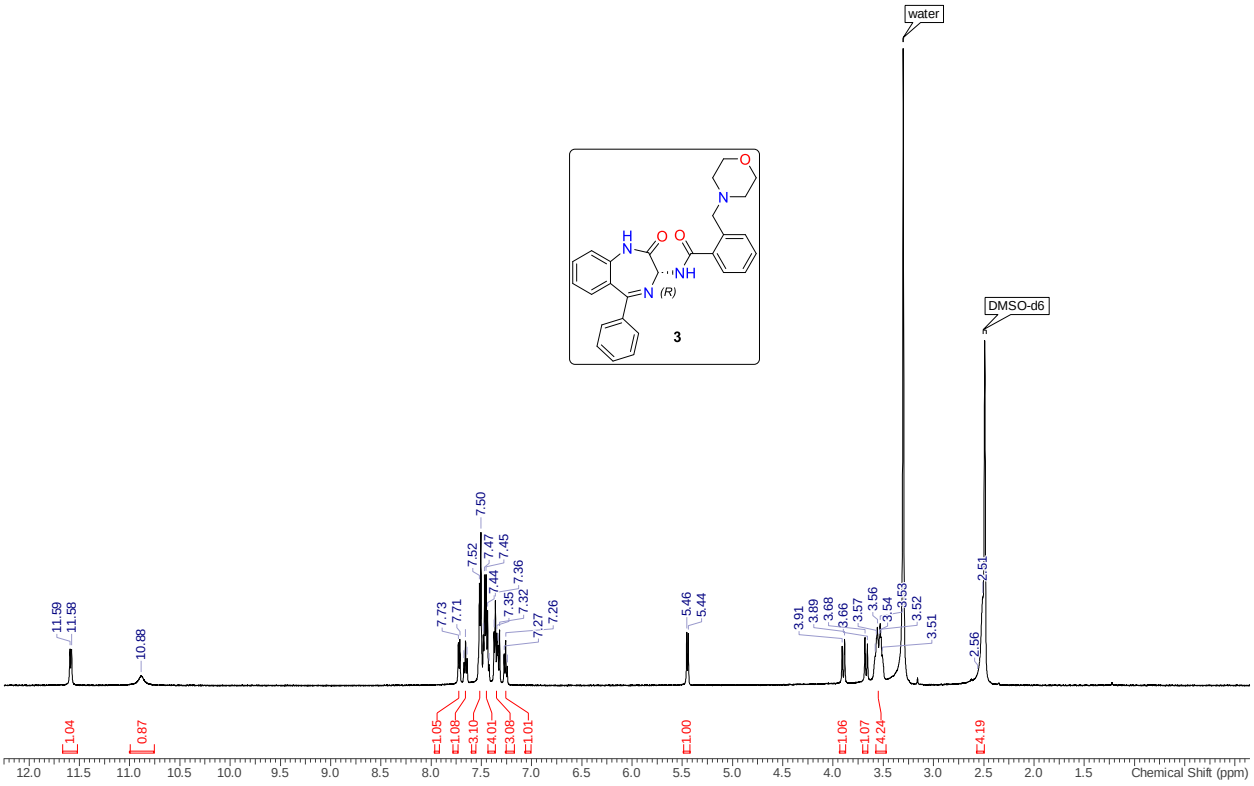
1:MS ES+ 4.9e+005

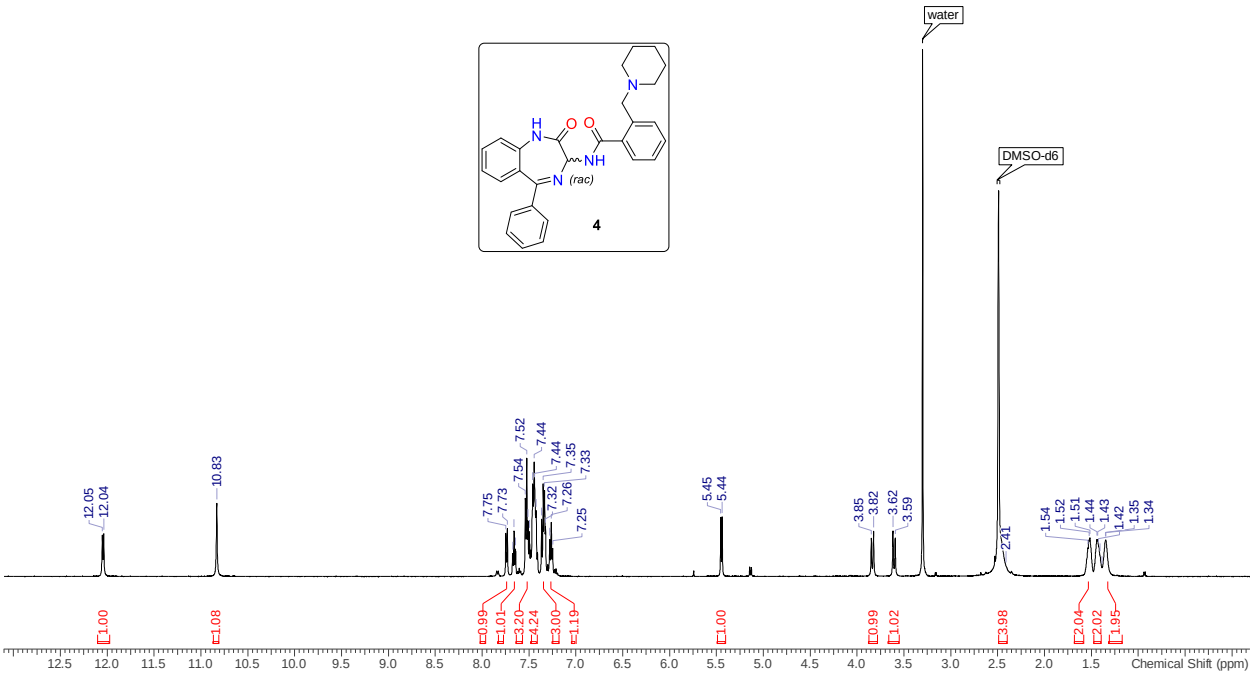


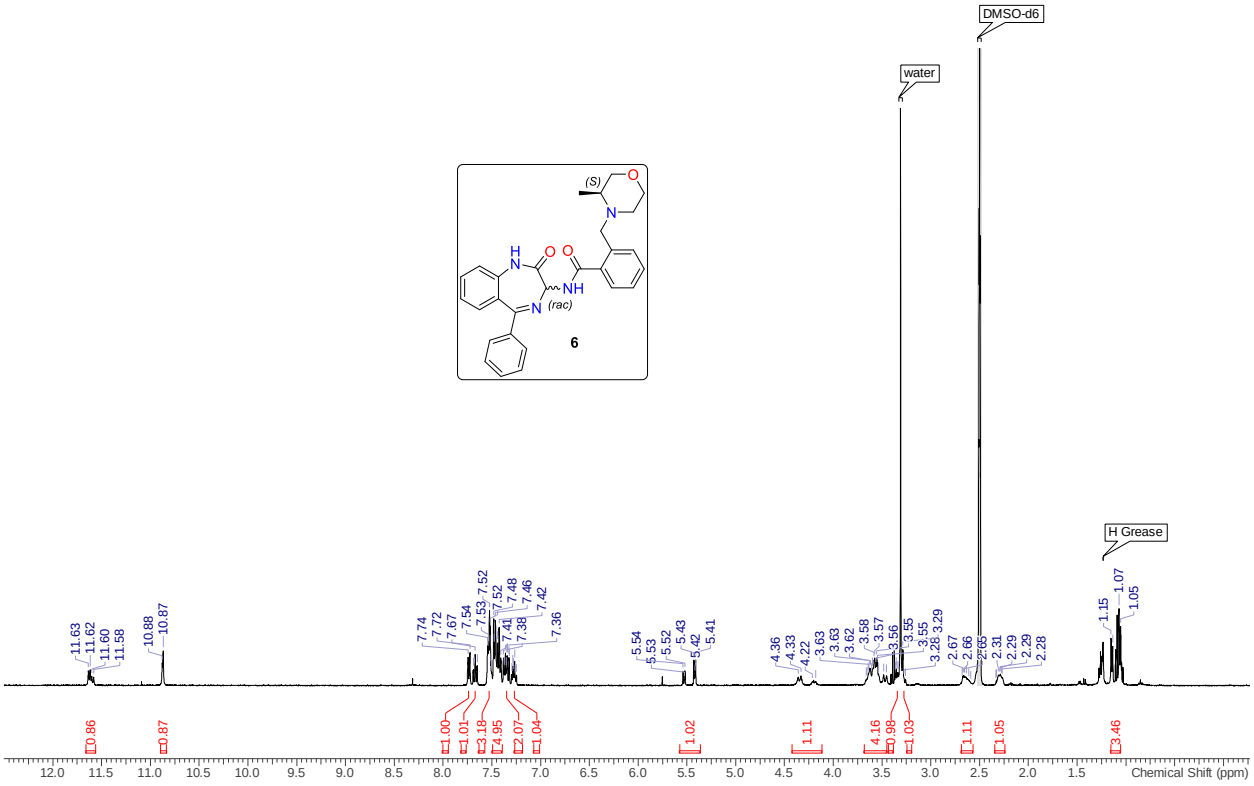
^{1}H NMR Data:

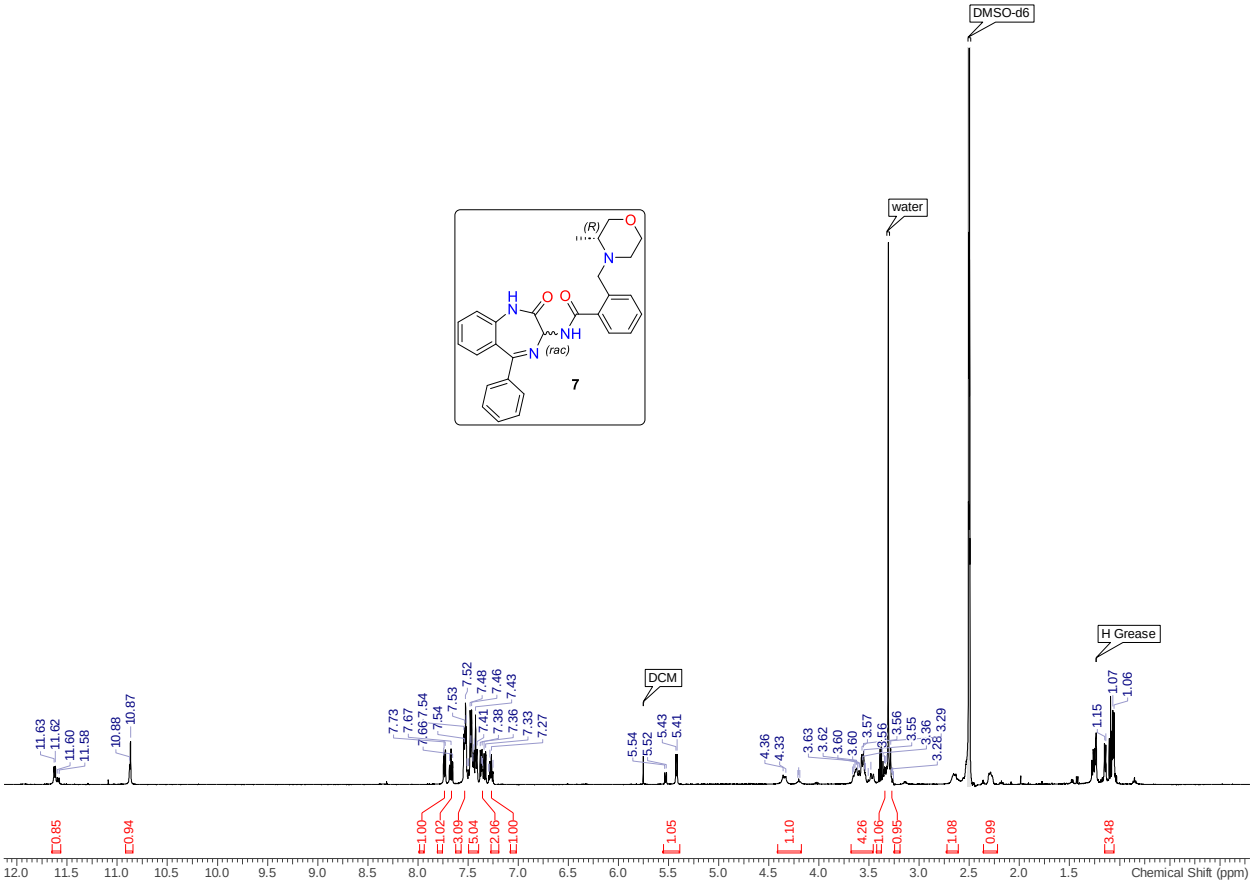

water
DMSO-d6
11.61
11.60
10.89
7.73
7.72
7.64
7.50
7.46
7.45
7.37
7.51
7.35
7.32
7.26
7.25
5.47
5.45
3.91
3.89
3.68
3.66
3.57
3.56
3.53
3.52
3.51
2.51
1.00
0.99
1.00
1.04
7.04
3.01
1.02
1.00
1.00
1.02
4.00
3.92
Chemical Shift (ppm)

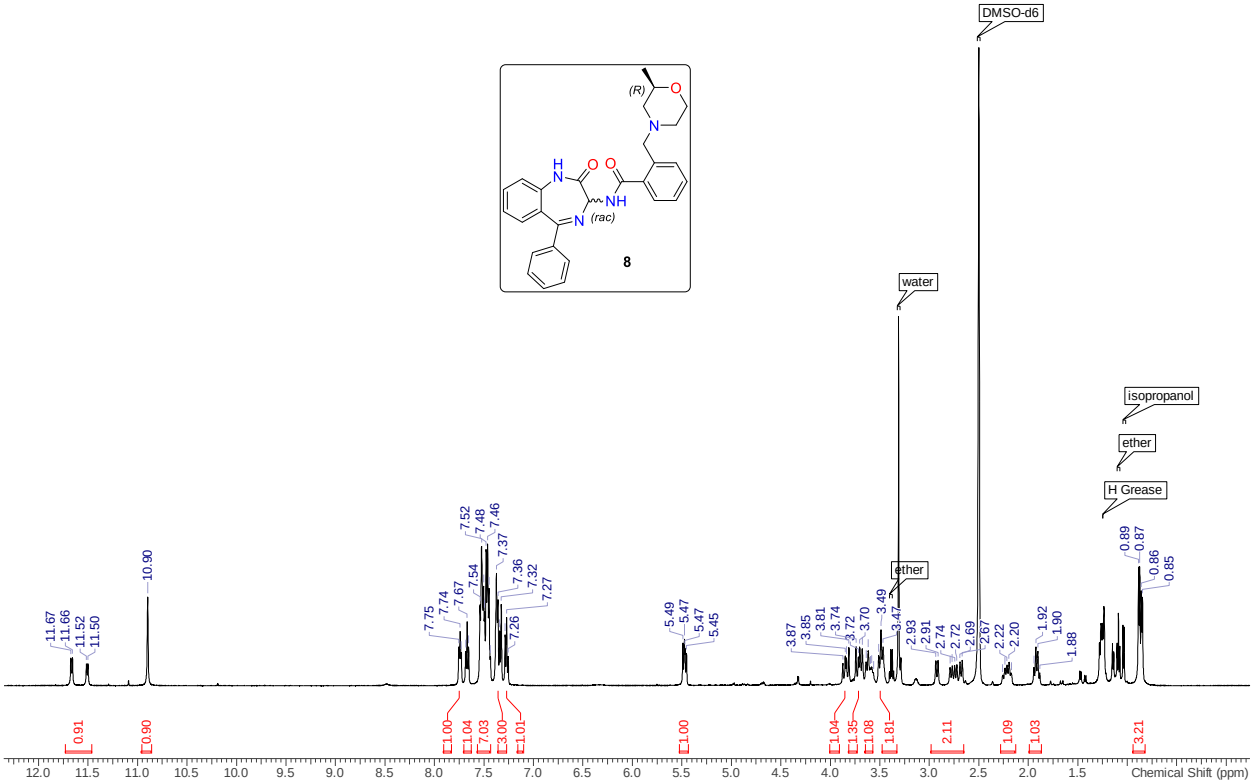

DMSO-d6
water
2
11.58
11.57
10.88
7.73
7.73
7.71
7.64
7.50
7.47
7.45
7.52
7.44
7.36
7.34
7.32
7.25
7.24
5.45
5.43
3.91
3.88
3.68
3.66
3.56
3.53
3.52
3.51
2.53
2.51
2.50
0.98
0.86
0.98
1.02
3.10
3.96
3.03
0.99
1.00
1.00
1.05
4.00
4.02
Chemical Shift (ppm)

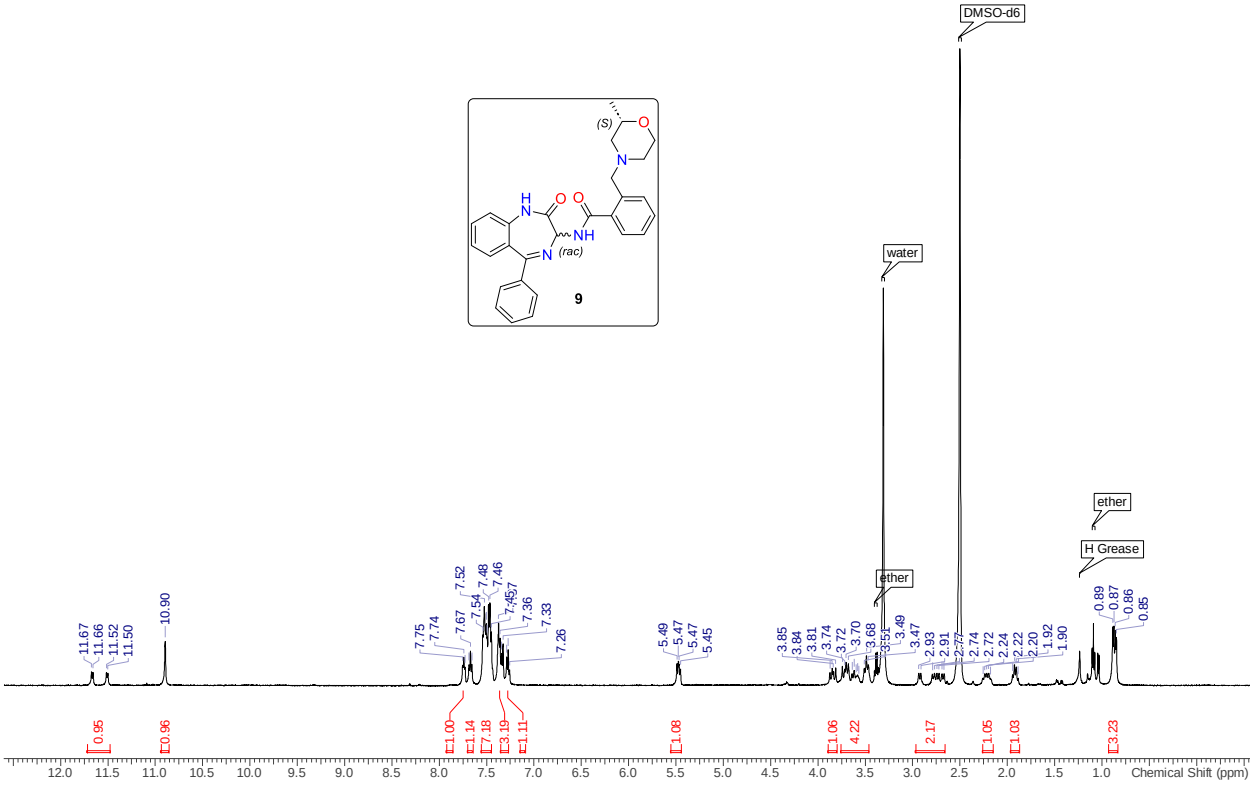

water
DMSO-d6
3
(R)
11.59
11.58
10.88
7.73
7.71
7.52
7.50
7.47
7.45
7.44
7.36
7.35
7.32
7.27
7.26
5.46
5.44
3.91
3.89
3.68
3.66
3.57
3.56
3.54
3.53
3.52
3.51
2.56
2.51
1.04
0.87
1.05
1.08
3.10
4.01
3.08
1.01
1.00
1.06
1.07
4.24
4.19
Chemical Shift (ppm)

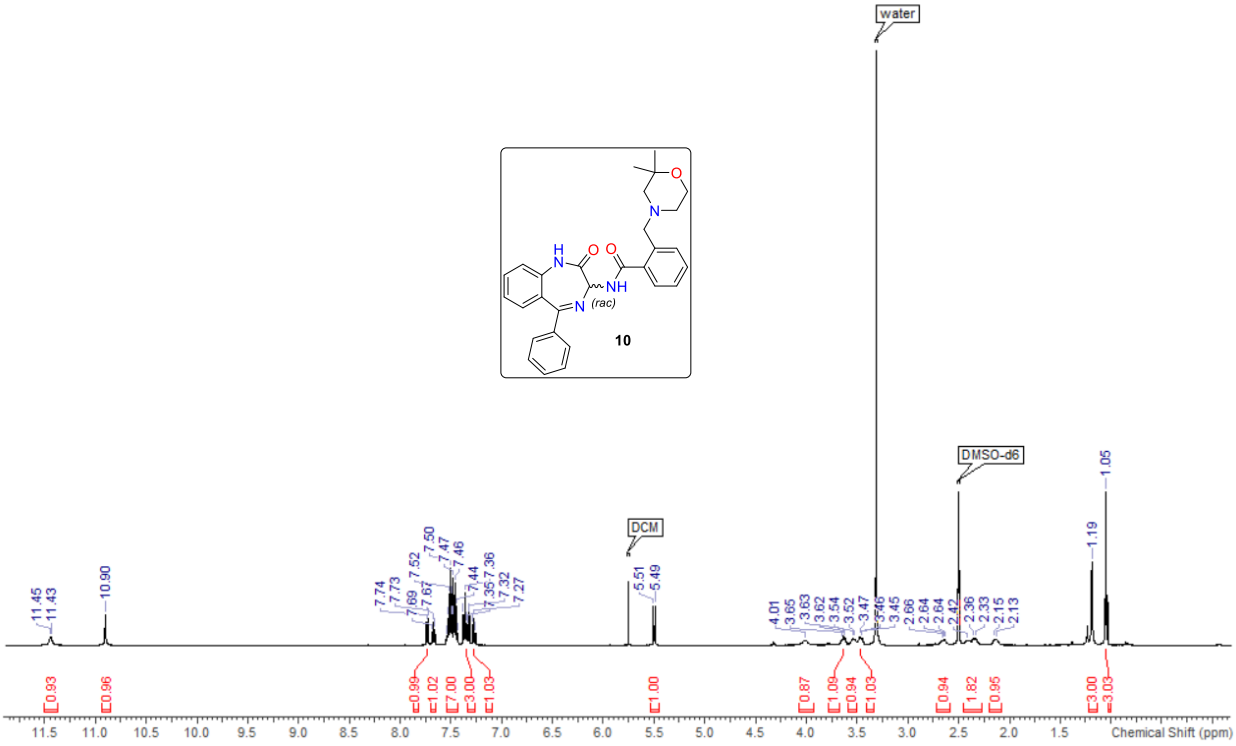

water
DMSO-d6
4
(rac)
12.05
12.04
10.83
7.75
7.73
7.54
7.52
7.44
7.44
7.35
7.33
7.32
7.26
7.25
5.45
5.44
3.85
3.82
3.62
3.59
2.41
1.54
1.52
1.51
1.44
1.43
1.42
1.35
1.34
1.00
1.08
0.99
1.01
3.20
4.24
3.00
1.19
1.00
0.99
1.02
3.98
2.04
2.02
1.95
Chemical Shift (ppm)

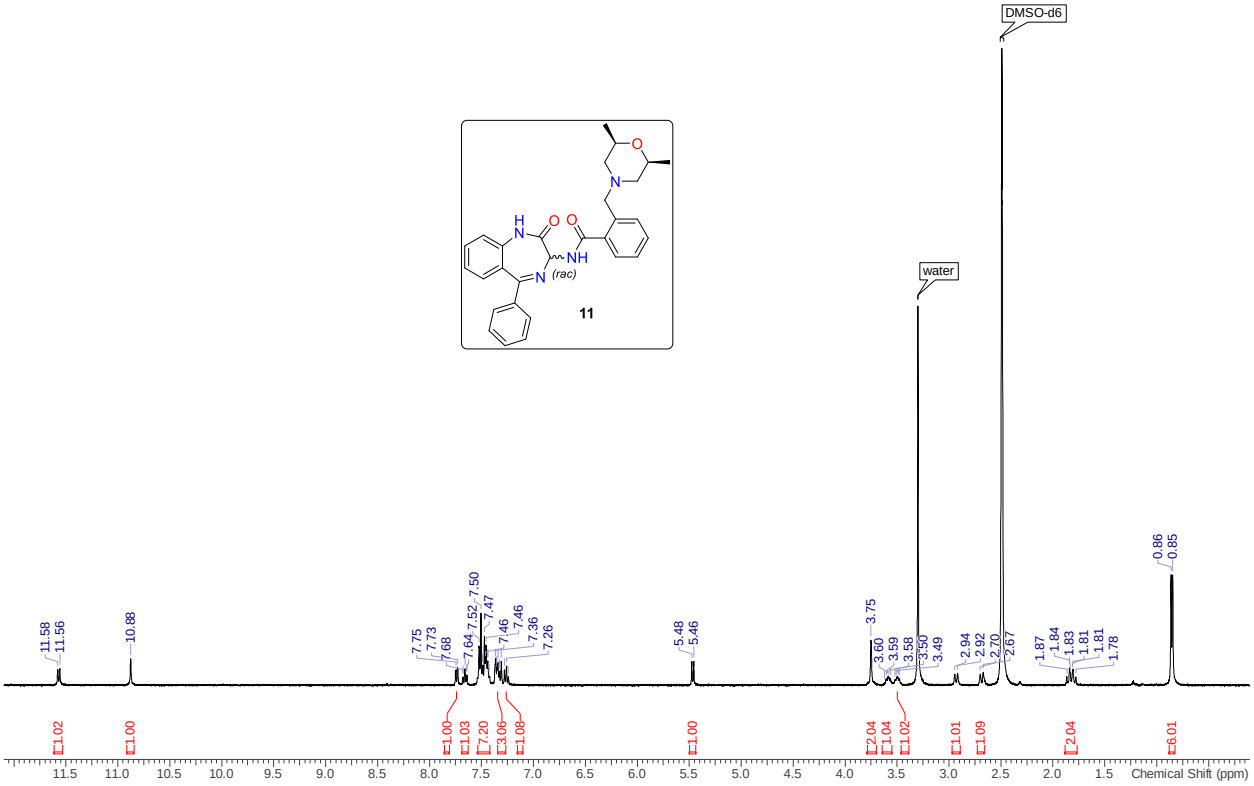

DMSO-d6
water
H Grease
(S)
O
N
H
O
O
NH
N
(rac)
6
11.63
11.62
11.60
11.58
10.88
10.87
7.74
7.72
7.67
7.54
7.53
7.52
7.52
7.48
7.46
7.42
7.41
7.38
7.36
5.54
5.53
5.52
5.43
5.42
5.41
4.36
4.33
4.22
3.63
3.63
3.62
3.58
3.57
3.56
3.55
3.55
3.28
3.29
2.67
2.66
2.31
2.29
2.29
2.28
1.15
1.07
1.05
0.86
0.87
1.00
1.01
3.18
4.95
2.07
1.04
1.02
1.11
4.16
0.98
1.03
1.11
1.05
3.46
12.0
11.5
11.0
10.5
10.0
9.5
9.0
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
Chemical Shift (ppm)

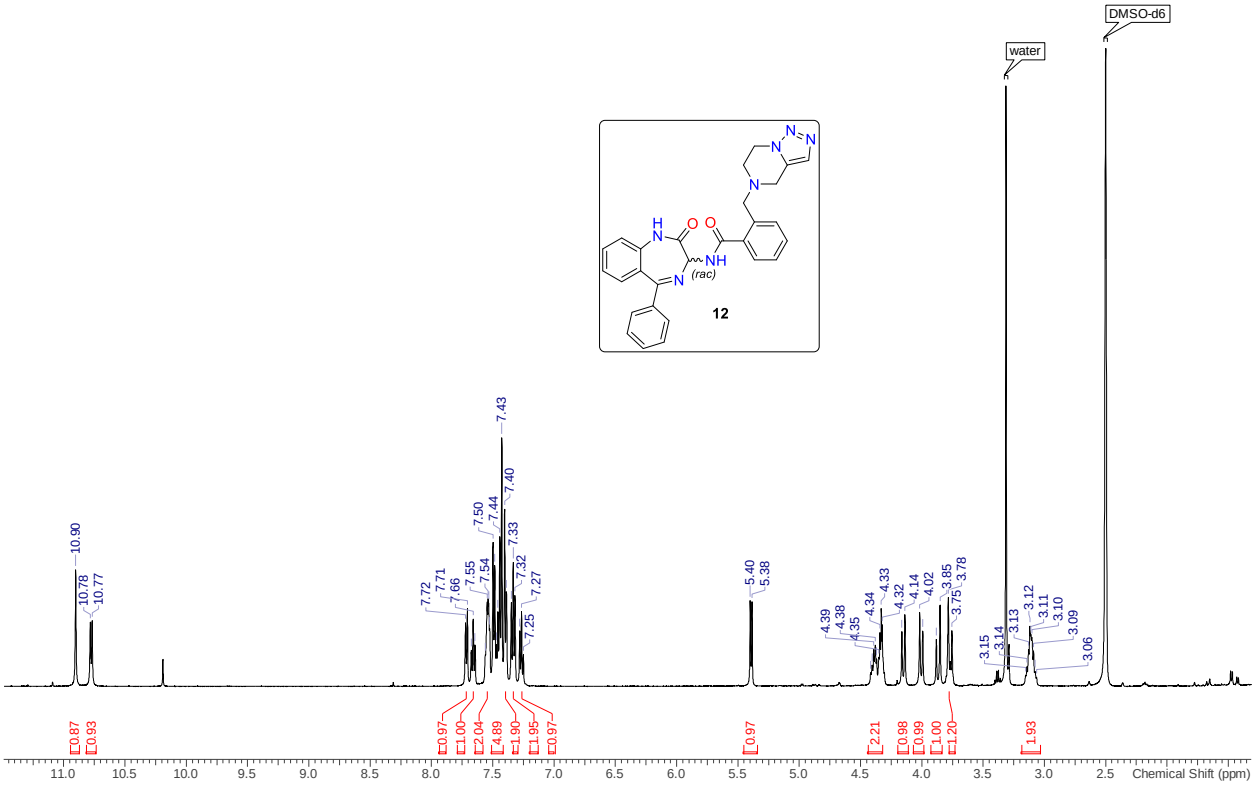

DMSO-d6
water
H Grease
DCM
(R)
(rac)
7
11.63
11.62
11.60
11.58
10.88
10.87
7.73
7.67
7.66
7.54
7.54
7.53
7.52
7.48
7.46
7.43
7.41
7.38
7.36
7.33
7.27
5.54
5.52
5.43
5.41
4.36
4.33
3.63
3.62
3.60
3.60
3.57
3.56
3.56
3.55
3.36
3.28
3.29
1.15
1.07
1.06
0.85
0.94
1.00
1.02
3.09
5.04
2.06
1.00
1.05
1.10
4.26
1.06
0.95
1.08
0.99
3.48
Chemical Shift (ppm)

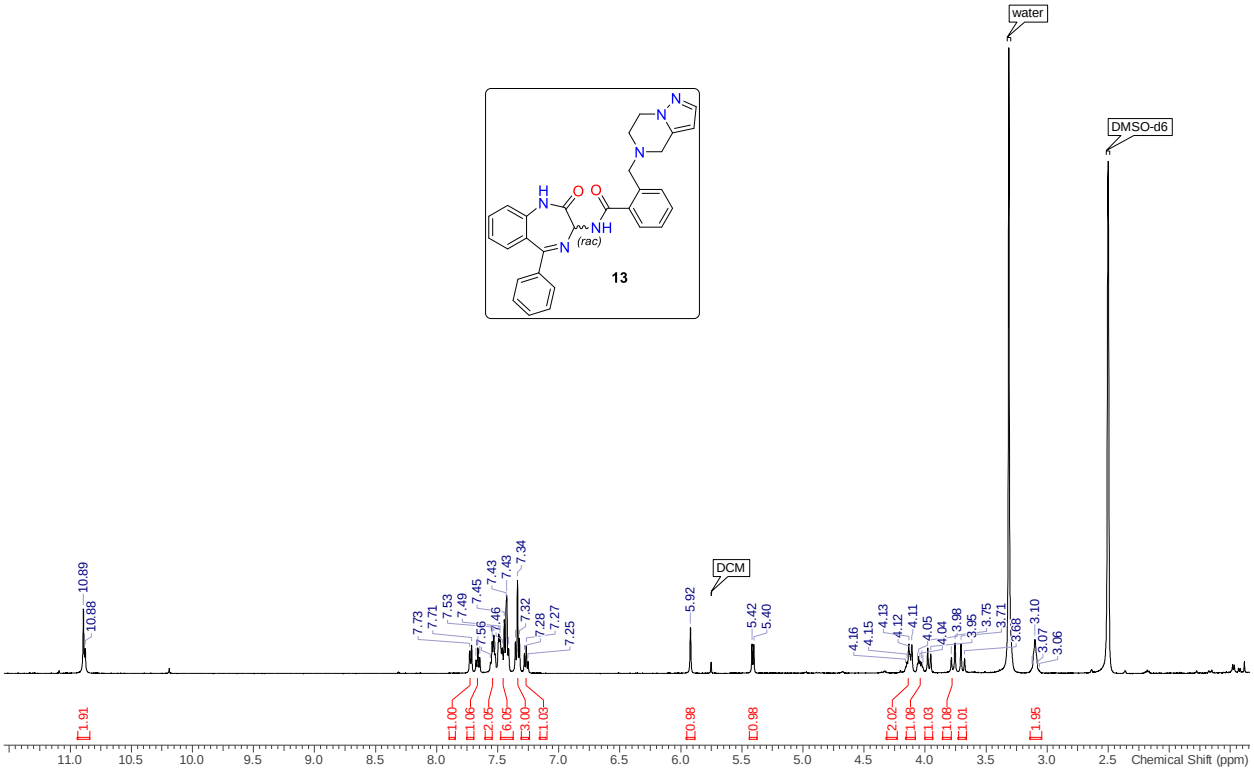

DMSO-d6
water
isopropanol
ether
H Grease
ether
(R)
(rac)
8
11.67
11.66
11.52
11.50
10.90
7.75
7.74
7.67
7.54
7.52
7.48
7.46
7.37
7.36
7.32
7.27
7.26
5.49
5.47
5.47
5.45
3.87
3.85
3.81
3.74
3.72
3.70
3.49
3.47
2.93
2.91
2.74
2.72
2.69
2.67
2.22
2.20
1.92
1.90
1.88
0.89
0.87
0.86
0.85
0.91
0.90
1.00
1.04
7.03
3.00
1.01
1.00
1.04
1.35
1.08
1.81
2.11
1.09
1.03
3.21
Chemical Shift (ppm)

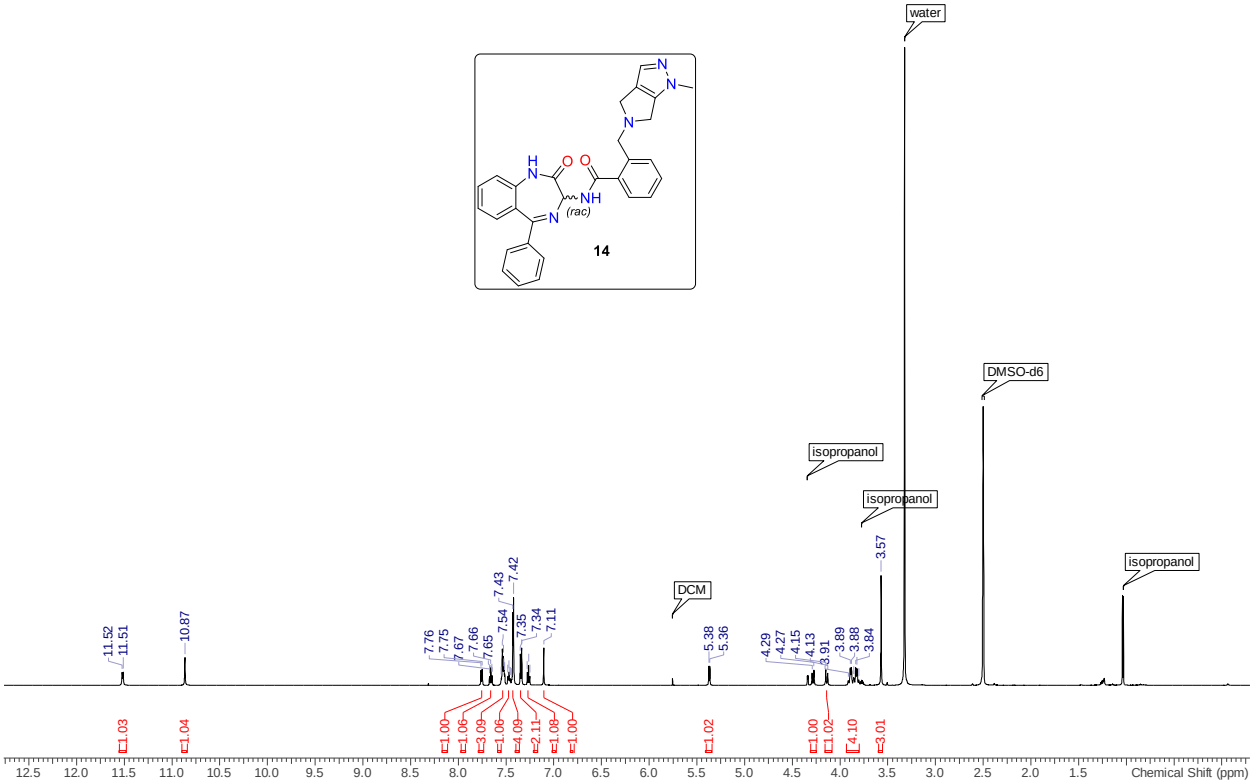

DMSO-d6
water
ether
H Grease
ether
9
Chemical Shift (ppm)

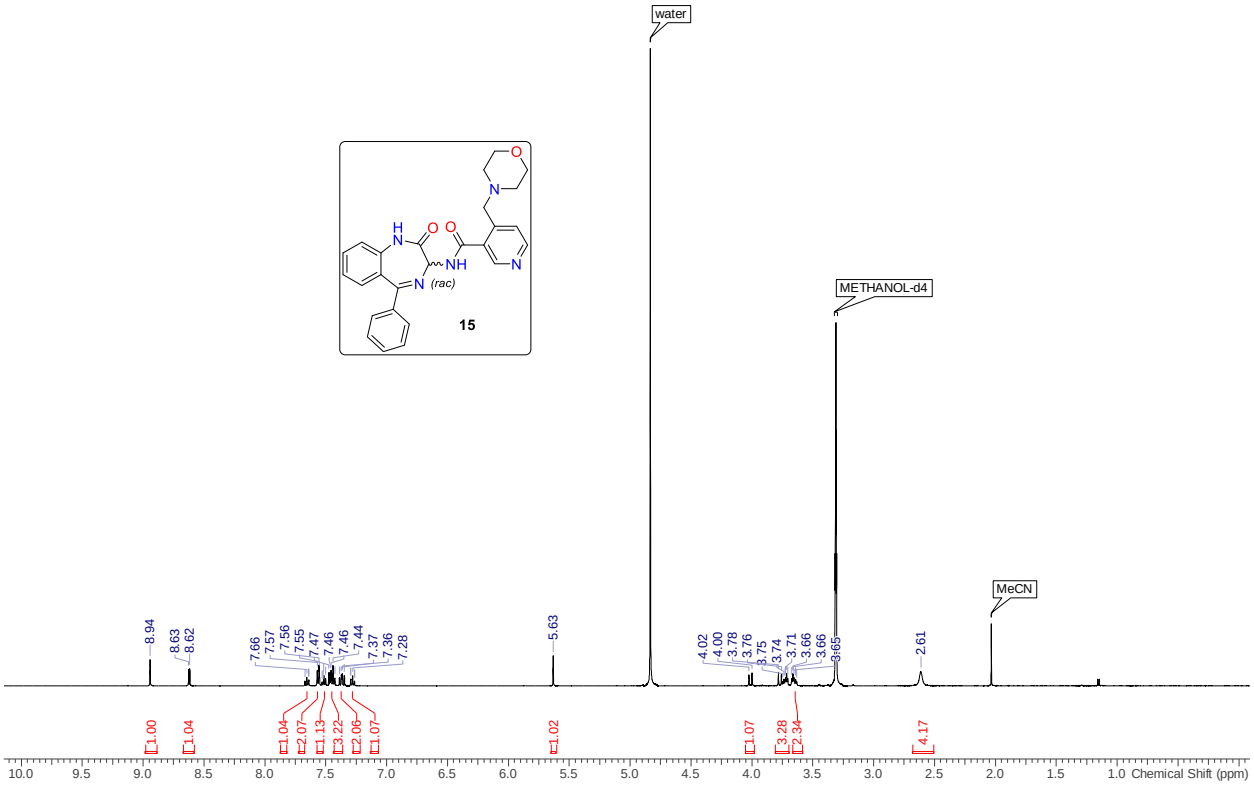

water
DMSO-d6
DCM
10
(rac)
Chemical Shift (ppm)

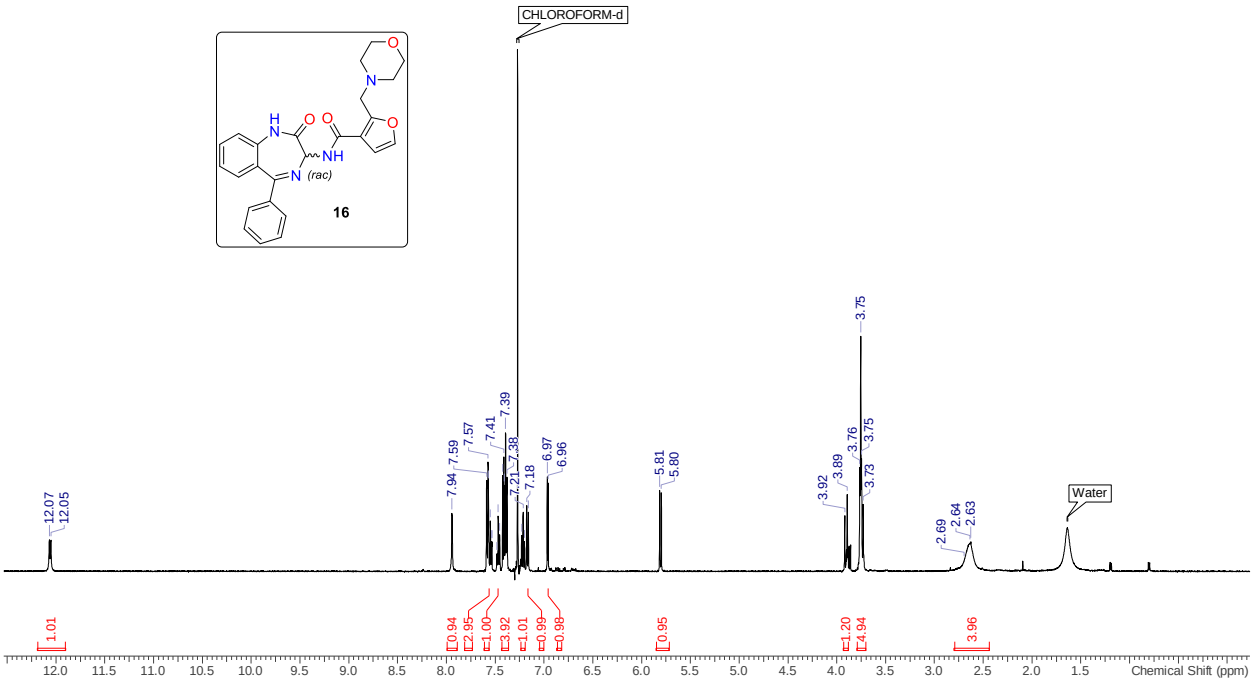

DMSO-d6
water
(rac)
11
11.58
11.56
10.88
7.75
7.73
7.68
7.64
7.52
7.50
7.47
7.46
7.46
7.36
7.26
5.48
5.46
3.75
3.60
3.59
3.58
3.50
3.49
2.94
2.92
2.70
2.67
1.87
1.84
1.83
1.81
1.81
1.78
0.86
0.85
1.02
1.00
1.00
1.03
7.20
3.06
1.08
1.00
2.04
1.04
1.02
1.01
1.09
2.04
6.01
Chemical Shift (ppm)

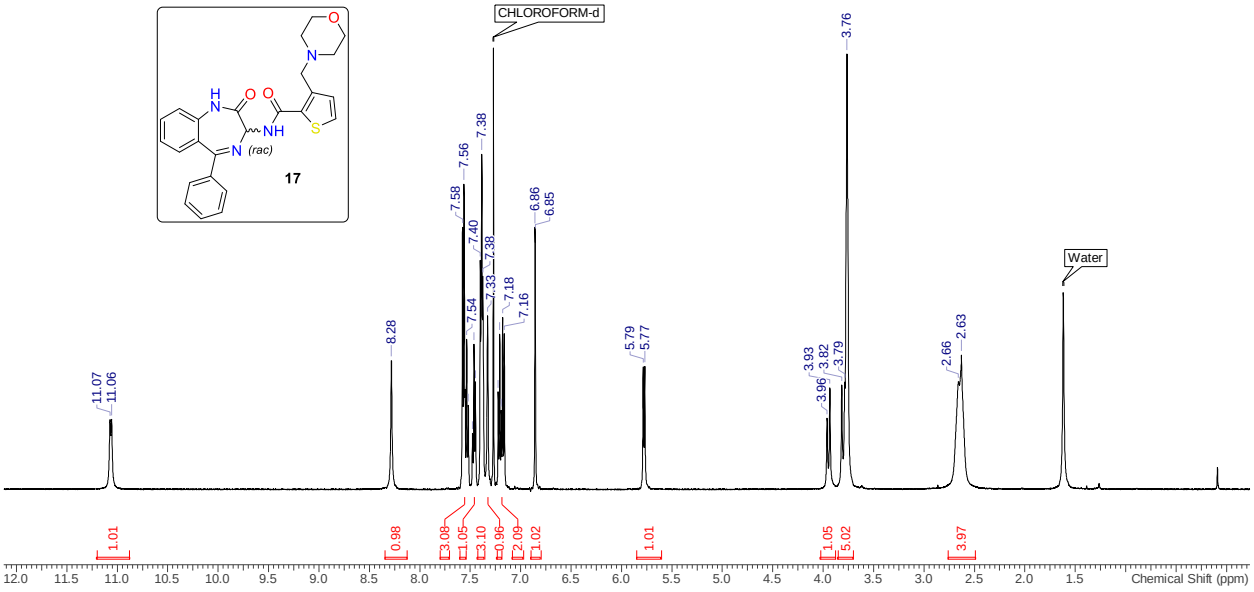

DMSO-d6
water
12
(rac)
10.90
10.78
10.77
7.72
7.71
7.66
7.55
7.54
7.50
7.44
7.43
7.40
7.33
7.32
7.27
7.25
5.40
5.38
4.39
4.38
4.35
4.34
4.33
4.32
4.14
4.02
3.85
3.78
3.75
3.15
3.14
3.13
3.12
3.11
3.10
3.09
3.06
0.87
0.93
0.97
1.00
2.04
4.89
1.90
1.95
0.97
0.97
2.21
0.98
0.99
1.00
1.20
1.93
11.0
10.5
10.0
9.5
9.0
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
Chemical Shift (ppm)

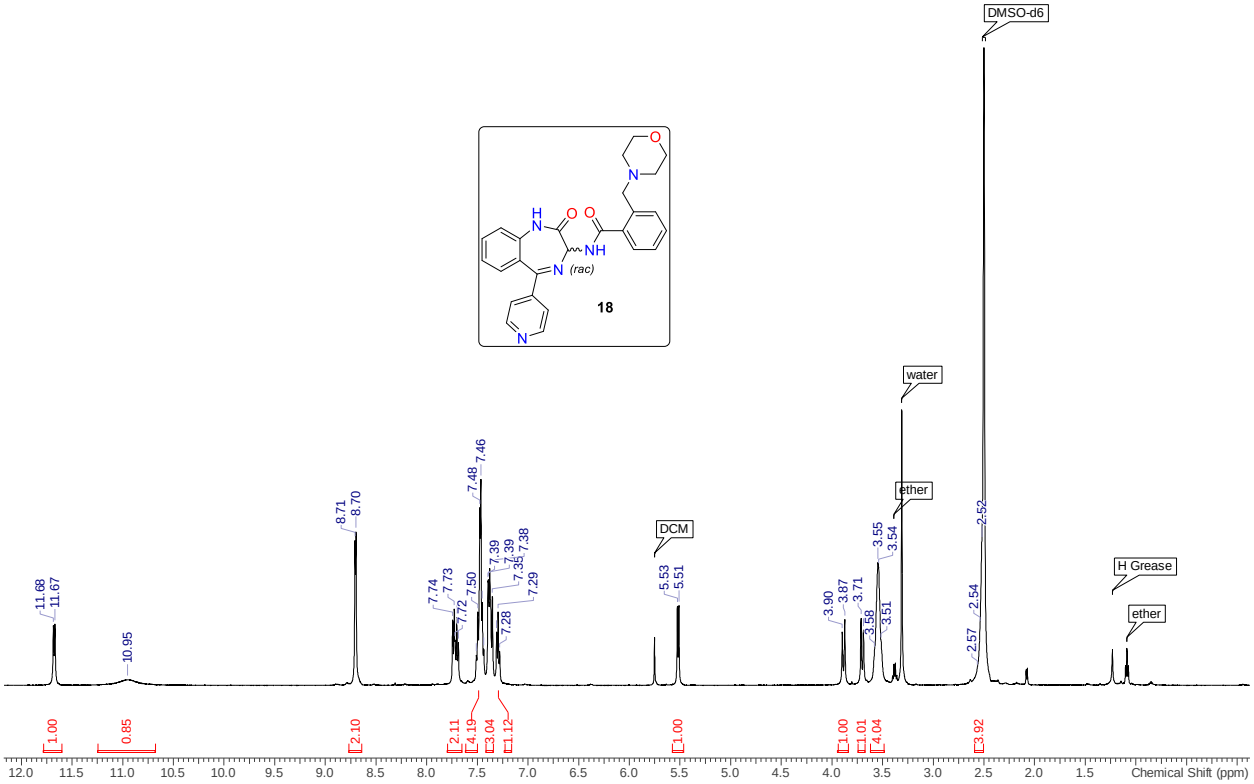

water
DMSO-d6
DCM
13
(rac)
10.89
10.88
7.73
7.71
7.53
7.49
7.45
7.43
7.43
7.34
7.56
7.46
7.32
7.28
7.27
7.25
5.92
5.42
5.40
4.16
4.15
4.13
4.12
4.11
4.05
4.04
3.98
3.95
3.75
3.71
3.68
3.10
3.07
3.06
1.91
1.00
1.06
2.05
6.05
3.00
1.03
0.98
0.98
2.02
1.09
1.03
1.08
1.01
1.95
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
Chemical Shift (ppm)

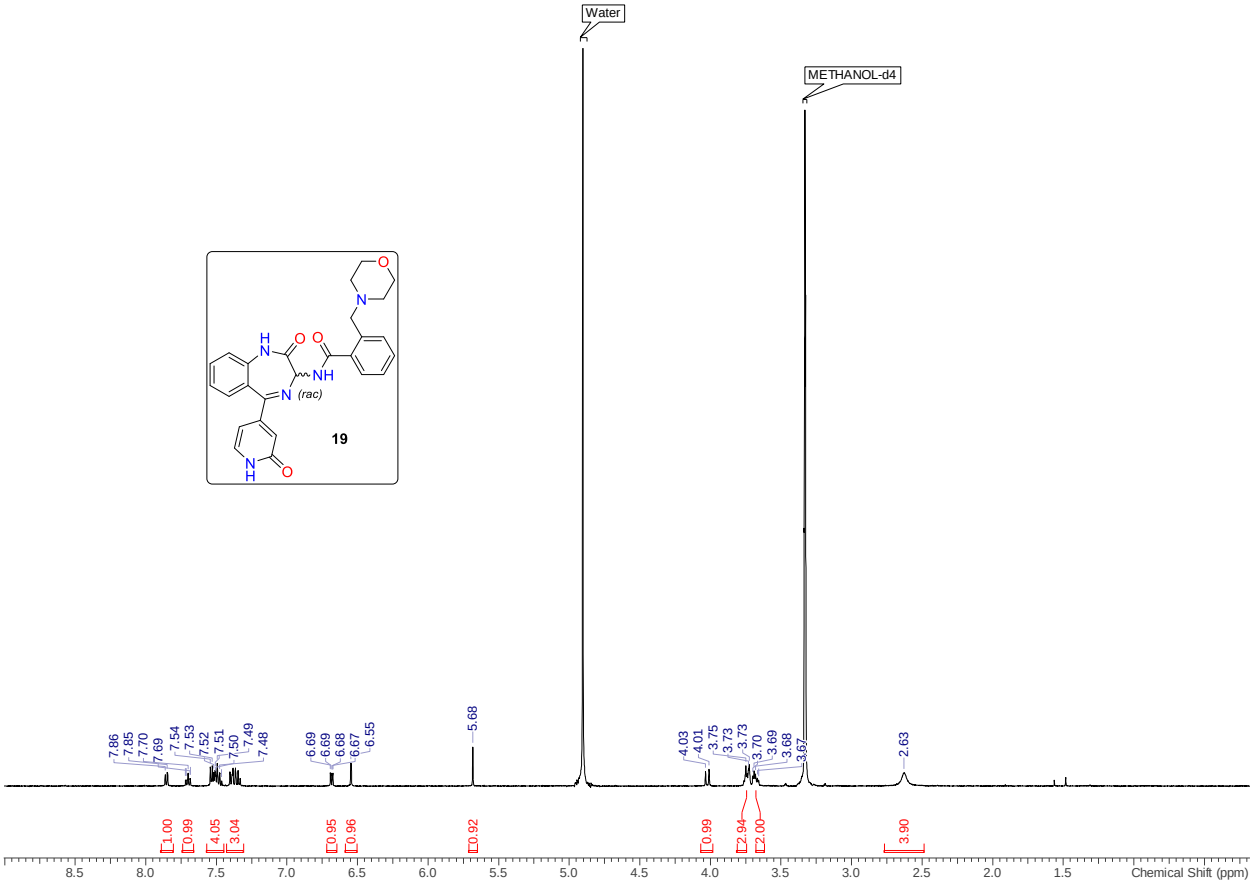

water
DMSO-d6
isopropanol
isopropanol
isopropanol
DCM
3.57
11.52
11.51
10.87
7.76
7.75
7.67
7.66
7.65
7.54
7.43
7.42
7.35
7.34
7.11
5.38
5.36
4.29
4.27
4.15
4.13
3.91
3.89
3.88
3.84
1.03
1.04
1.00
1.06
3.09
1.06
4.09
2.11
1.08
1.00
1.02
1.00
1.02
4.10
3.01
12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 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
Chemical Shift (ppm)
14
(rac)

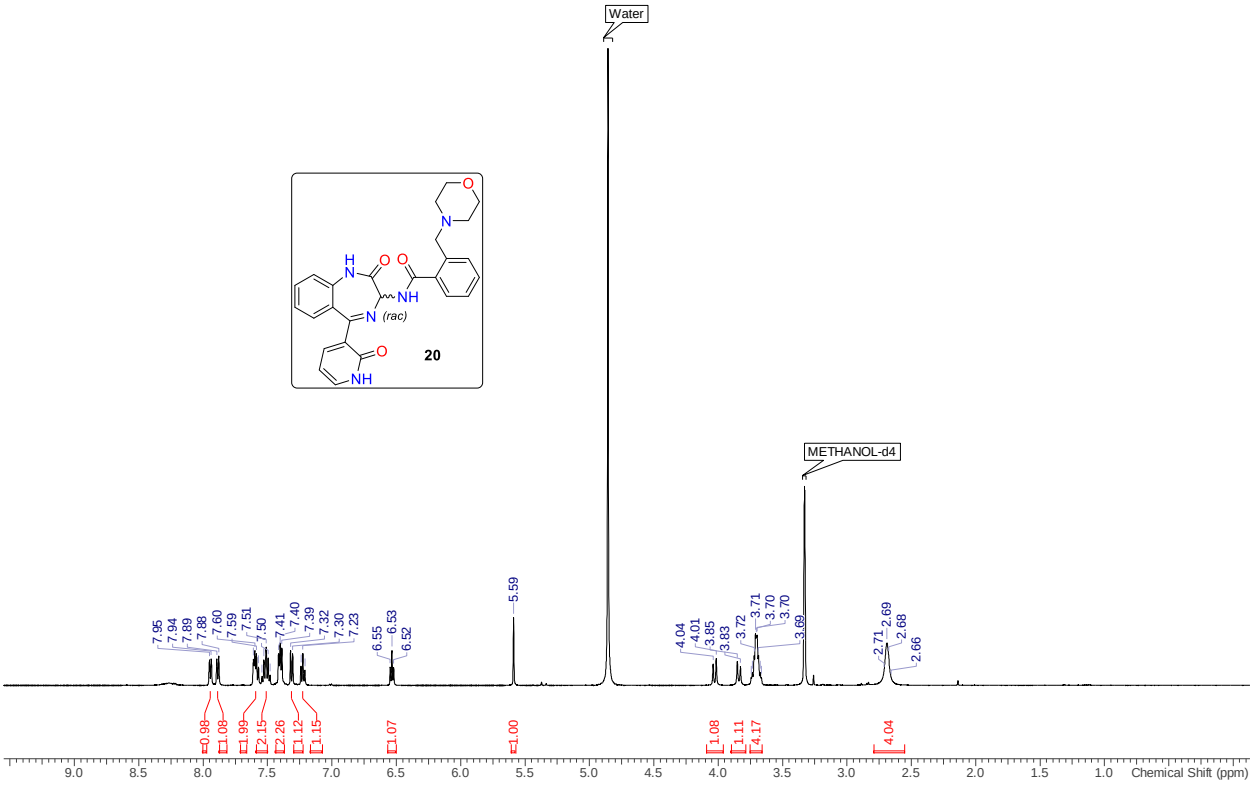

water
METHANOL-d4
MeCN
15
(rac)
8.94
8.63
8.62
7.66
7.57
7.56
7.55
7.47
7.46
7.46
7.44
7.37
7.36
7.28
5.63
4.02
4.00
3.78
3.76
3.75
3.74
3.71
3.66
3.66
3.65
2.61
1.00
1.04
1.04
2.07
1.13
3.22
2.06
1.07
1.02
1.07
3.28
2.34
4.17
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

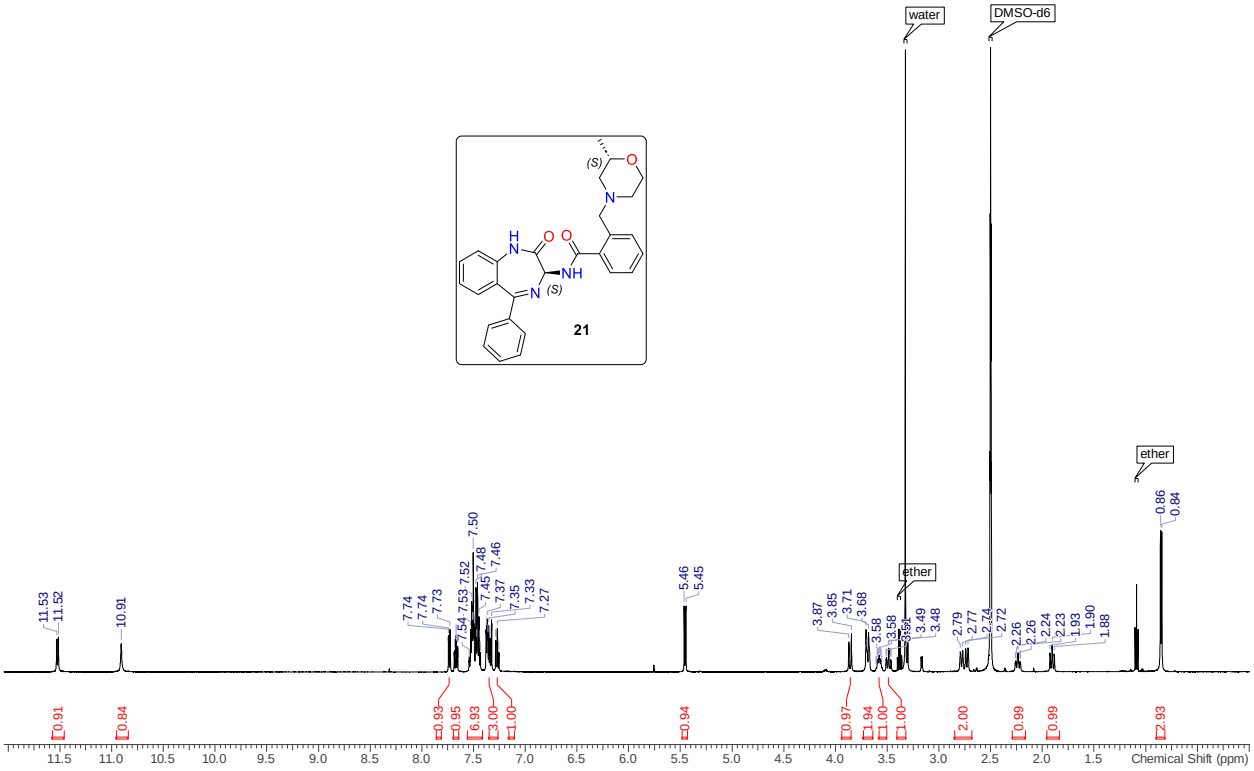

CHLOROFORM-d
16
(rac)
12.07
12.05
7.94
7.59
7.57
7.41
7.39
7.38
7.21
7.18
6.97
6.96
5.81
5.80
3.92
3.89
3.76
3.75
3.75
3.73
2.69
2.64
2.63
Water
1.01
0.94
2.95
1.00
3.92
1.01
0.99
0.98
0.95
1.20
4.94
3.96
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
Chemical Shift (ppm)

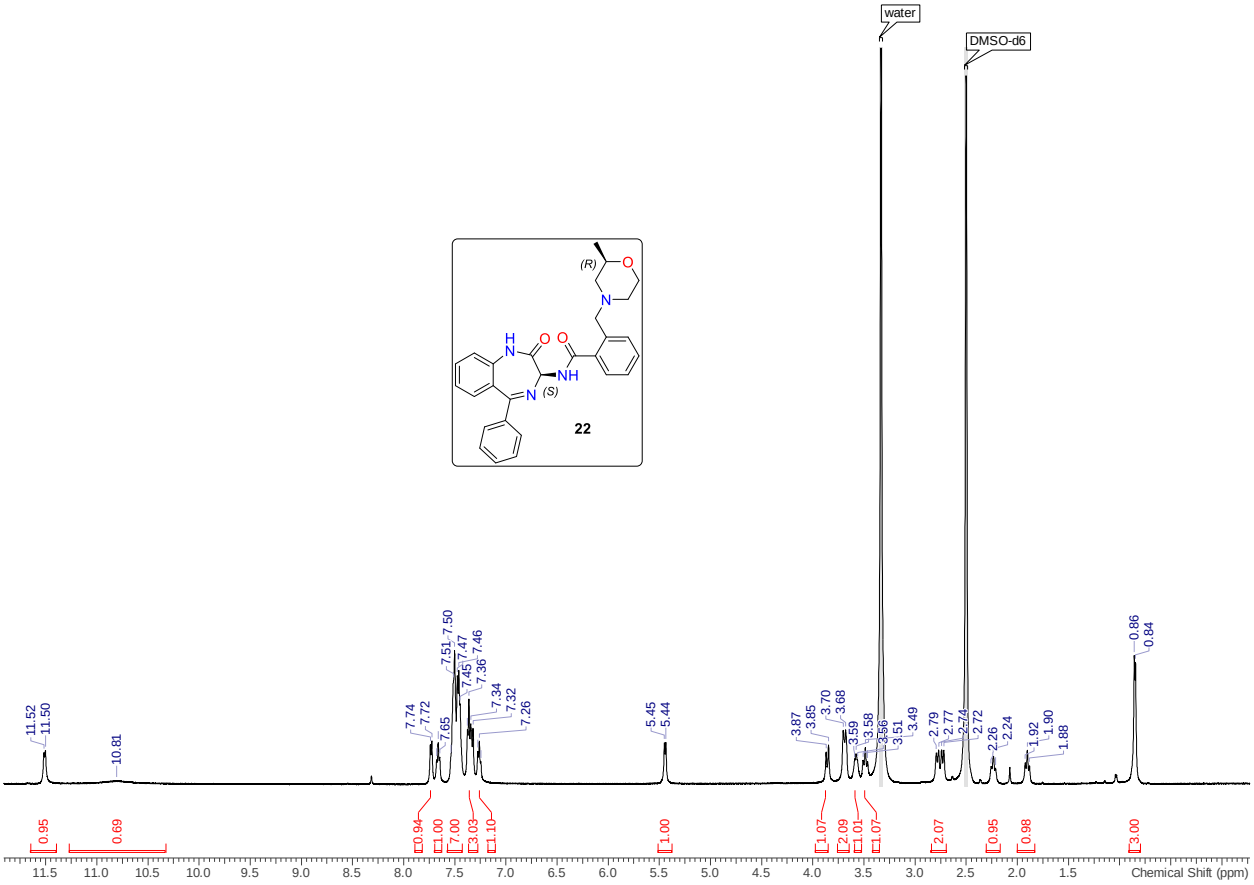

CHLOROFORM-d
Water
(rac)
17
11.07
11.06
8.28
7.58
7.56
7.54
7.40
7.38
7.33
7.18
7.16
6.86
6.85
5.79
5.77
3.96
3.93
3.82
3.79
3.76
2.66
2.63
1.01
0.98
3.08
1.05
3.10
0.96
2.09
1.02
1.01
1.05
5.02
3.97
Chemical Shift (ppm)

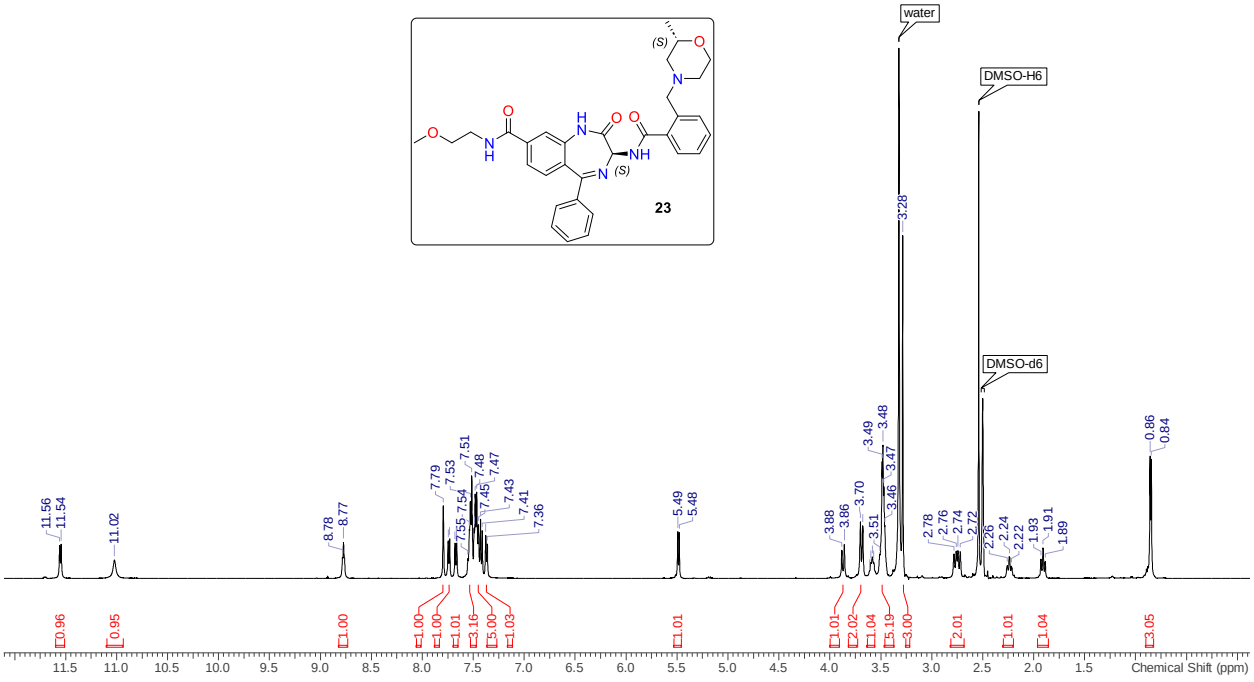

DMSO-d6
water
ether
DCM
H Grease
ether
18
(rac)
11.68
11.67
10.95
8.71
8.70
7.74
7.73
7.72
7.50
7.48
7.46
7.39
7.39
7.38
7.38
7.29
7.28
5.53
5.51
3.90
3.87
3.71
3.58
3.55
3.54
3.51
2.57
2.54
2.52
1.00
0.85
2.10
2.11
4.19
3.04
1.12
1.00
1.00
1.01
4.04
3.92
Chemical Shift (ppm)

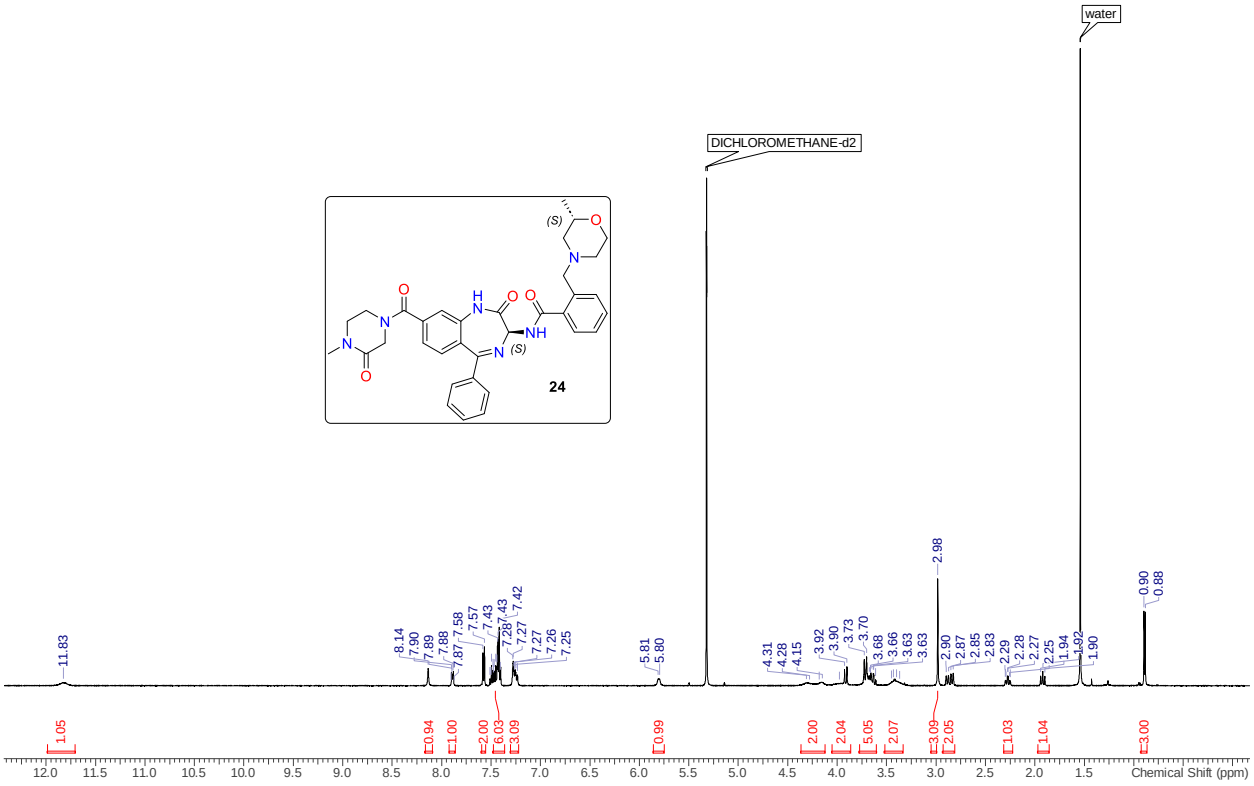

Water
METHANOL-d4
19
(rac)
7.86
7.85
7.70
7.69
7.54
7.53
7.52
7.51
7.50
7.49
7.48
6.69
6.69
6.68
6.67
6.55
5.68
4.03
4.01
3.75
3.73
3.73
3.70
3.69
3.68
3.67
2.63
1.00
0.99
4.05
3.04
0.95
0.96
0.92
0.99
2.94
2.00
3.90
Chemical Shift (ppm)

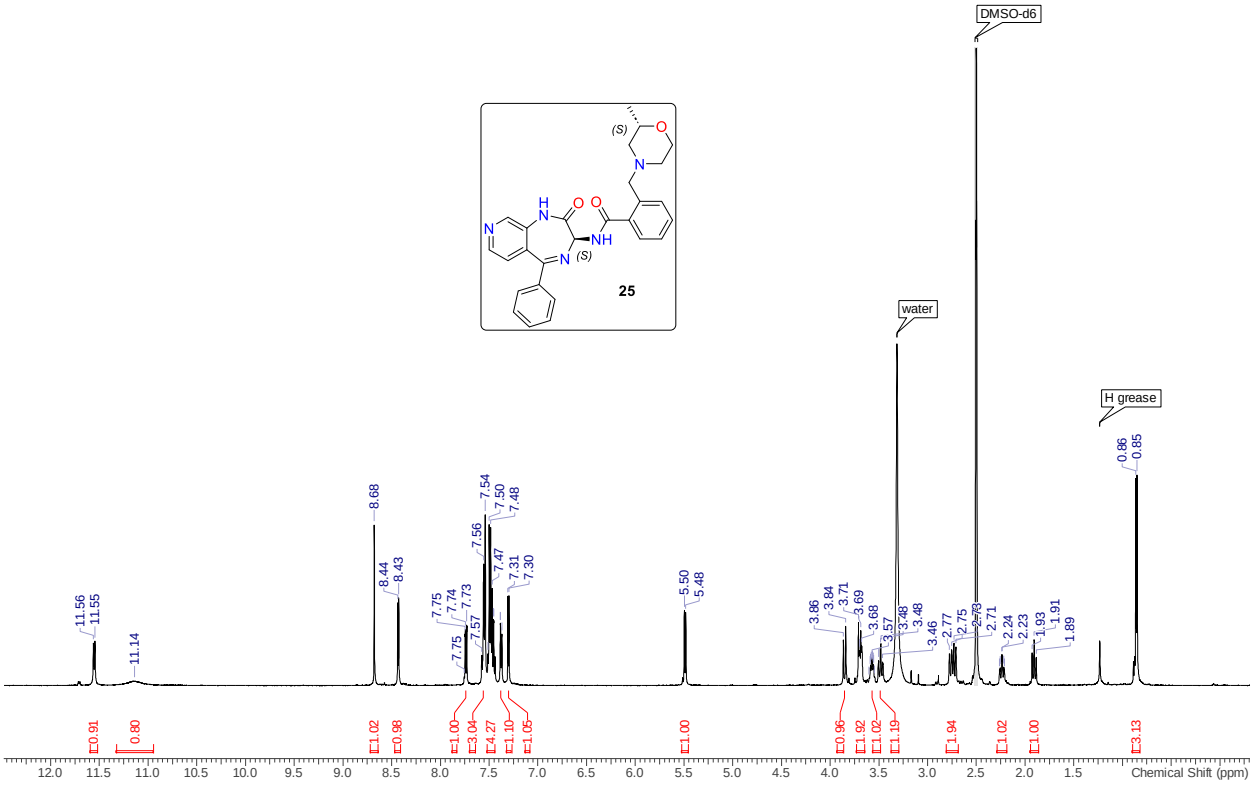

Water
METHANOL-d4
20
(rac)
7.95
7.94
7.89
7.88
7.60
7.59
7.51
7.50
7.41
7.40
7.39
7.32
7.30
7.23
6.55
6.53
6.52
5.59
4.04
4.01
3.85
3.83
3.72
3.71
3.70
3.70
3.69
2.71
2.69
2.68
2.66
0.98
1.08
1.99
2.15
2.26
1.12
1.15
1.07
1.00
1.08
1.11
4.17
4.04
Chemical Shift (ppm)


water
DMSO-d6
ether
ether
21
11.53
11.52
10.91
7.74
7.74
7.73
7.54
7.53
7.52
7.50
7.48
7.46
7.45
7.37
7.35
7.33
7.27
5.46
5.45
3.87
3.85
3.71
3.68
3.58
3.49
3.48
2.79
2.77
2.74
2.72
2.26
2.26
2.24
2.23
1.93
1.90
1.88
0.86
0.84
0.91
0.84
0.93
0.95
6.93
3.00
1.00
0.94
0.97
1.94
1.00
1.00
2.00
0.99
0.99
2.93
Chemical Shift (ppm)


water
DMSO-d6
(R)
(S)
22
11.52
11.50
10.81
7.74
7.72
7.65
7.51
7.50
7.47
7.45
7.46
7.36
7.34
7.32
7.26
5.45
5.44
3.87
3.85
3.70
3.68
3.59
3.58
3.56
3.51
3.49
2.79
2.77
2.74
2.72
2.26
2.24
1.92
1.90
1.88
0.86
0.84
0.95
0.69
0.94
1.00
7.00
3.03
1.10
1.00
1.07
2.09
1.01
1.07
2.07
0.95
0.98
3.00
Chemical Shift (ppm)


23
water
DMSO-H6
DMSO-d6
11.56
11.54
11.02
8.78
8.77
7.79
7.53
7.51
7.55
7.54
7.48
7.47
7.45
7.43
7.41
7.36
5.49
5.48
3.88
3.86
3.70
3.51
3.49
3.48
3.47
3.46
3.28
2.78
2.76
2.74
2.72
2.26
2.24
2.22
1.93
1.91
1.89
0.86
0.84
0.96
0.95
1.00
1.00
1.00
1.01
3.16
5.00
1.03
1.01
1.01
2.02
1.04
5.19
3.00
2.01
1.01
1.04
3.05
Chemical Shift (ppm)


water
DICHLOROMETHANE-d2
24
11.83
8.14
7.90
7.89
7.88
7.87
7.58
7.57
7.43
7.28
7.42
7.27
7.27
7.26
7.25
5.81
5.80
4.31
4.28
4.15
3.92
3.90
3.73
3.70
3.68
3.66
3.63
3.63
2.98
2.90
2.87
2.85
2.83
2.29
2.28
2.27
2.25
1.94
1.92
1.90
0.90
0.88
1.05
0.94
1.00
2.00
6.03
3.09
0.99
2.00
2.04
5.05
2.07
3.09
2.05
1.03
1.04
3.00
Chemical Shift (ppm)


DMSO-d6
water
H grease
25
11.56
11.55
11.14
8.68
8.44
8.43
7.75
7.74
7.73
7.57
7.56
7.54
7.50
7.48
7.47
7.31
7.30
5.50
5.48
3.86
3.84
3.71
3.69
3.68
3.57
3.48
3.46
2.77
2.75
2.73
2.71
2.24
2.23
1.93
1.91
1.89
0.86
0.85
0.91
0.80
1.02
0.98
1.00
3.04
4.27
1.10
1.05
1.00
0.96
1.92
1.02
1.19
1.94
1.02
1.00
3.13
Chemical Shift (ppm)

IV: HTS Hit Finding Methods

A high-throughput screen on a library of 1.8 million AstraZeneca small molecules was performed against Cbl-b using a Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) assay. A FAM probe based on a patent compound[5] was used to monitor compound binding through FRET with terbium labelled Cbl-b. (See Section V, TR-FRET Probe Displacement Assay.)

The HTS was performed in single point at room temperature using white 1536-well microtiter plates (Greiner, 782075). DMSO-solubilized compounds were acoustically dispensed to assay-ready plates using an Echo 555 (Labcyte) giving a final compound concentration of 10 μM or 100 μM (dependent on compound properties) and a final DMSO concentration of less than 1%. Unlabeled patent compound **Ex336**[5] was used as an inhibitor control and DMSO as a neutral control.

Biotinylated Cbl-b (36-427, 2.6 nM) was incubated for 15 min with 1 nM Streptavidin Terbium Cryptate (Cisbo) and 65 nM FAM-probe, in final assay buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM TCEP, 0.01 % Tween-20 and 1 mM EDTA). 3 μL reagent mix was dispensed using a Thermo Multidrop Combi into microtiter plates containing compounds. Assay plates were incubated for 75 min before the reading fluorescent emission using a Pherastar FSX (BMG) with a TRF 337-620-520 module. Compound responses were analyzed using Genedata Screener (Genedata, Basel, CH) and calculated as a TR-FRET ratio of acceptor (520 nm) emission / donor (620 nm) emission. Robust Z-score normalization was then performed, where the median of compounds on the plate was centered to zero. Approximately 9000 compounds with Z-score ≤ -25 were progressed.

Ten-point concentration response curves were carried out in both the TR-FRET assay and a orthogonal fluorescent polarization (FP) assay. For the FP assay, 250 nM biotinylated Cbl-b (36-427) was incubated with 65 nM FAM-based probe for 15 min in final assay buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM TCEP, 0.01 % Tween-20 and 1 mM EDTA). 3 μL reagent mix was dispensed using a Thermo Multidrop Combi into microtiter plates containing compounds. Assay plates were incubated for 75 min before the reading fluorescent emission using a Pherastar FSX (BMG) with a FP 485-520-520 module. Polarization was calculated using the equation; 1000 x ((Parallel – Perpendicular) / (Parallel + Perpendicular)). Percentage inhibition for concentration response curves in both the TR-FRET and FP assays were calculated as a percentage of inhibitor control (10 μM NX-1607) minus neutral control (DMSO). Data was then fitted using the nonlinear regression analysis; 4 parameter logistic smart fit method in Genedata Screener (Genedata, Basel, CH).

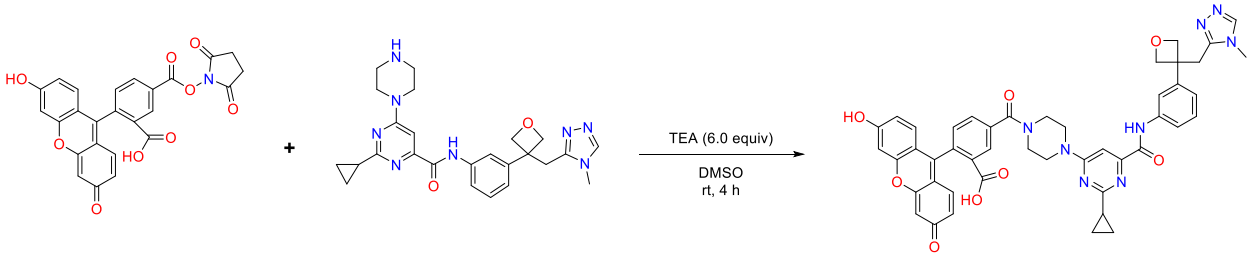
V. Biological Assay Methods

SPR:

SPR experiments were performed at 25 °C using Biacore 4000, T200, or 8K instruments (Cytiva). A SA sensor chip (series S, Cytiva) was docked and primed into the system in 50 mM Hepes pH 7.5, 100 mM NaCl, 1 mM TCEP, 1% DMSO, 0.05% Tween20. To immobilize Cbl-b on the surface, the sensor chip surface was first conditioned with 3 × 60/120 s injections of 50 mM NaOH/1 M NaCl at 10 μL/min after which Cbl-b (36-427) Avidin-Biotinylated protein (200 nM) was then captured on the active flow cells. The remaining biotin binding sites were then blocked on all (active and reference) surfaces with a 60/120 s injection of 50 μM amine-PEG2-biotin. The binding of compounds was tested with multi/ single cycle injections over a suitable concentration−response range at a flow rate of 30 μL/min. The obtained sensorgrams were processed by reference and blank subtraction and adjusted by solvent correction. Dose response sensorgrams were analyzed in Biaevaluation and Insight software.

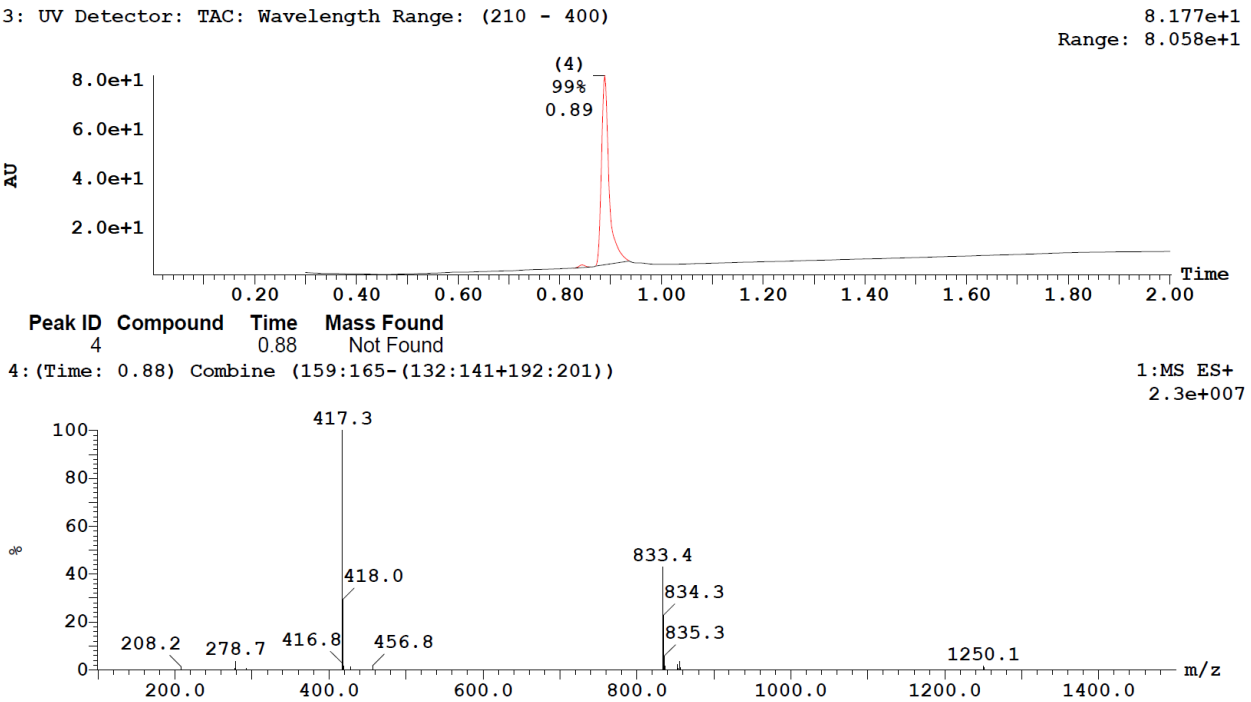
TR-FRET Probe Displacement Assay:

FAM-labelled probe synthesis:



In a 1-dram vial equipped with a stir bar, 5-(((2,5-dioxopyrrolidin-1-yl)oxy)carbonyl)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (5.0 mg, 0.011 mmol, 1.0 equiv) was added. To the vial was added DMSO (0.43 mL), followed by the addition of 2-cyclopropyl-*N*-(3-(3-((4-methyl-4*H*-1,2,4-triazol-3-yl)methyl)oxetan-3-yl)phenyl)-6-(piperazin-1-yl)pyrimidine-4-carboxamide[5] (5.5 mg, 0.012 mmol, 1.1 equiv). The reaction was stirred at rt for 1 min before the addition of triethylamine (8.8 µL, 0.63 mmol, 6.0 equiv), which turned the solution to a deep orange color. The reaction was stirred at rt for 4 h, at which point it was directly purified by C18 column chromatography eluting with a 10-100% MeCN in water gradient (0.1% TFA buffer). The desired fractions were combined and concentrated to afford 5-(4-(2-cyclopropyl-6-((3-(3-((4-methyl-4*H*-1,2,4-triazol-3-yl)methyl)oxetan-3-yl)phenyl)carbamoyl)pyrimidin-4-yl)piperazine-1-carbonyl)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (1.8 mg, 20%) as a yellow solid. LC-MS (ES+): 833.4 m/z [M+H], tR = 0.89 min

LCMS of Probe:



Protein expression and purification:
Cbl-b (N-term His-TEV-Cbl-b(36-427)-C-term Avi) and ZAP70 (N-term-GST-TEV-AVI-ZAP70(1-606)-Cterm_pFastBac) were separately expressed in Escherichia coli BL21(DE3) Tuner cells in Terrific Broth media. In brief, expression was induced at A600 = 1.0 with 50 μM IPTG and expression occurred for 20 h at 18 °C. Cells were collected and stored at −80 °C. Cell pellets were resuspended in 750 ml per 100 g pellet of Cbl-b or ZAP70 lysis buffer (50 mM Hepes pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP, DNase (0.1 μg/mL), 0.1 mM PMSF). The solution was homogenized and the clarified lysate loaded into a column for nickel affinity purification preequilibrated in buffer A (50 mM Hepes pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP, 20 mM imidazole). Protein was eluted in buffer A supplemented with 300 mM imidazole. Fractions containing Cbl-b or ZAP70 were pooled and buffer exchanged by dialysis into biotinylation buffer (25 mM Hepes pH=7.5, 300 mM potassium glutamate, 0.5 mM TCEP). Biotinylation was achieved using the BirA kit (Avidity) as per manufacturer's instructions and biotinylation confirmed by mass spectrometry. Size exclusion chromatography was used to further purify the Cbl-b or ZAP70 into Storage buffer (25 mM Hepes pH 7.5, 500 mM NaCl, 0.5 mM TCEP), and fractions containing pure Cbl-b or ZAP70 were pooled and concentrated to 9.95 and 3.08 mg/mL respectively and snap frozen on liquid nitrogen.

FRET displacement assay:
The FAM-labelled probe molecule was synthesised, Tb-labelled streptavidin was purchased from Perkin Elmer (Waltham, MA, USA), and recombinant Cbl-b protein was expressed and purified as described above. FRET probe displacement assays were performed in black medium bind low volume Greiner plates at room temperature in sterile filtered assay buffer consisting of 50 mM TRIS HCl (pH 7.5), 0.01% Tween P20, 100 mM NaCl, 1 mM TCEP, and 1 mM EDTA. For all experiments with test compounds, a dilution series of test compound was by direct dispense of compounds in 100% DMSO into an assay plate using the ECHO 655 acoustic dispenser with a backfill of DMSO to 50 nL (1% final assay concentration). To each concentration of inhibitor to be tested, an assay master mix of appropriate assay reagents (2.6 nM biotinylated Cbl-b, 65 nM FAM-probe, 1 nM Tb-labelled Streptavidin) were added to bring the final volume to 5 μL and the final DMSO concentration to 1%. In general, all assays were performed with 3 replicates of each inhibitor concentration. Assay plates were read using a PHERAstar FSX plate reader from BMG LabTech (Ortenberg, Germany) together with standard Tb-based TR-FRET settings with 337 nm excitation wavelength and emission monitored at 520 nm (donor) and 490 nm (acceptor). Emission intensities were measured over a 100 μs window following a 60 μs post excitation delay. A ratio of raw acceptor/donor was calculated and normalized relative to wells containing fully bound or fully competed FAM-probe (results reported in relative percent FAM-probe displaced). Curve fitting and data analysis were carried out using Genedata Screener (Genedata, Basel, Switzerland).

*Determination of FRET-Probe K_d and K_i values for compounds **1-25**:*

The plot below describes the titration of the FRET-probe in a fixed concentration of Cbl-b protein to determine the FRET-probe K_d.

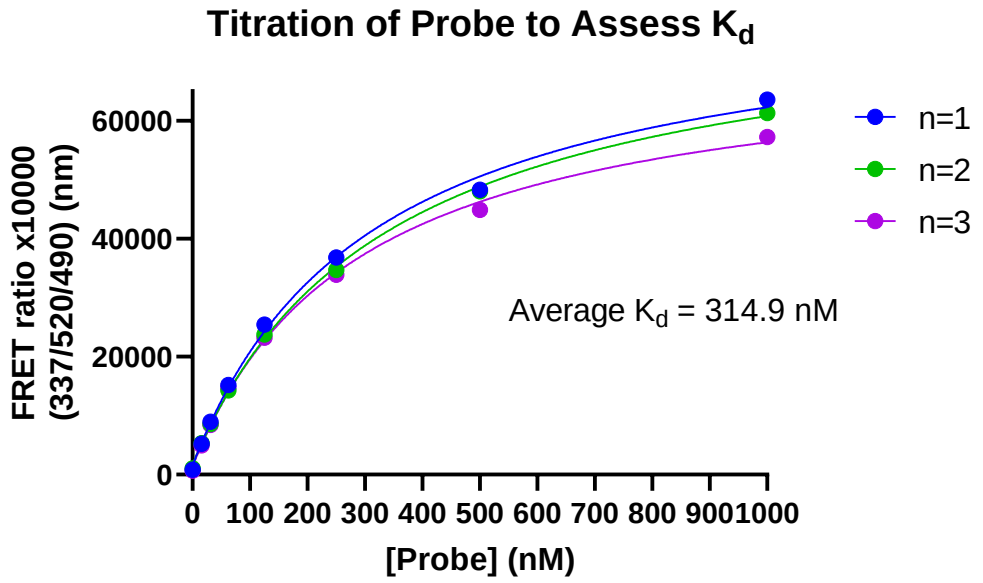


Figure S1: Determination of FRET-Probe K_d.

To calculate the Ki values for compounds **1-25**, the Cheng-Prusoff equation[6] is used:

$$K_i = \frac{IC_{50}}{1 + \frac{[R]}{K_d}}$$

[R] = Concentration of FRET-probe in the assay = 0.065 µM

K_d = Dissociation constant of FRET-Probe = 0.3149 µM

IC_{50} = Experimentally determined IC_{50} of test compound in µM

Table S1: Experimental IC_{50} values and Calculated K_i values for Compounds 1-25.

Compound Number	IC_{50} (μM)	K_i (μM)
1	0.21	0.17
2	0.094	0.078
3	8.0	6.6
4	>100	N.D.
5	>100	N.D.
6	1.9	1.6
7	4.8	4.0
8	0.031	0.026
9	0.026	0.022
10	3.9	3.2
11	1.8	1.5
12	0.30	0.25
13	0.71	0.59
14	0.35	0.29
15	1.3	1.1
16	1.5	1.2
17	0.36	0.30
18	2.0	1.7
19	11	9.1
20	20	17
21	0.013	0.011
22	0.010	0.0083
23	0.013	0.011
24	0.013	0.011
25	0.015	0.012

T-Cell IL-2 Secretion Assay:

Reagents:

RPMI 1640 (Sigma R7509); Heat inactivated FBS (Gibco 10270-106); Glutamax 100X (Thermo Fisher 35050061); HEPES 1M (Thermo Fisher 15630080); Dulbecco's PBS (Sigma D8537); DMSO (Sigma D5879) MultiCyt® QBeads® Human PlexScreen (2) Plex for 1 x 384 plate (Sartorius 90602); CD3 Monoclonal Antibody (OKT3), Functional Grade, eBioscience (Thermo Fisher 16-0037-85); Propidium Iodide (Abcam AB14083); Pen/Strep (sigma P0781); Non-essential amino acids (Sigma M7145); Sodium Pyruvate (Sigma S8636)

Method:

On Day 1, thaw the desired number of cryo-preserved human CD3+T-cells in warm T-cell medium (RPMI 1640, FBS 10%, Glutamax 1%, Pen/Strep 1%, Non-essential amino acids 1%, HEPES 100mM, Sodium Pyruvate 1%) without stimulation. Plate approximately $2x10^6$ cells/mL in a T25 flask. Incubate at 37 ˚C overnight to recover the T-cells.

Coat inner 308 wells of a Greiner 781090 flat bottom 384-well plate with αCD3 antibody (stimulation plate) using 20 µL αCD3 antibody diluted in cold PBS. Put the plate onto a plate shaker for 1 min at 900 rpm and then incubate overnight at 4 ˚C. Optimal concentration of αCD3 antibody needs to be determined by titration.

On Day 2, spin down T-cells at 400 r.c.f. for 5 min. Remove the supernatant and resuspend the cells with fresh T-cell medium. Count cells using a haemocytometer. Resuspend cells to a concentration of $1.7x10^6$/mL in T-cell medium (75000 cells in the final volume). Pour cells into a sterile reservoir. Using a ThermoFisher multichannel pipette, add 30 µL into each well of the inner 308 wells of Corning 384-well Black/Clear Round Bottom Ultra-Low Attachment Spheroid Microplate. Bravo liquid handler is used to transfer 10 µL of test compound in T-cell medium from a pre-made assay ready 384-well compound plate (AstraZeneca, compound management) to the T-cell plate. After 1 hour incubation with compounds at 37 ˚C, T-cells (40 µL) are transferred to αCD3 coated stimulation plate using Bravo liquid handler. Incubate the stimulation plate at 37 ˚C for additional 24 h.

On Day 3, using the Bravo liquid handler to remove 10 µL of cell supernatant from the stimulation plate and add it to a Greiner 781280 v-bottom 384 well plate for cytokine analysis. Replace the removed supernatant with 10 µL of compound-containing media from the pre-made compound plate. Freeze supernatant plate at -20 ˚C or use immediately in cytokine assay. Place stimulation plate at 37 ˚C in the incubator up to 3 days for viability assay.

Cytokine assay using supernatant is performed on iQue Advanced Flow Cytometry (Satorius) with IL2 MultiCyt QBeads (Satorius) according to supplier's protocol, but with a 50x capture beads dilution. The remaining T-cells are treated with propidium iodide (1:500 final dilution) for 30 min before cell viability data is acquired using iQue. For cytokine release, median fluorescence intensity of IL-2 is used. Viable T-cells are defined as propidium iodide negative singlets/total singlets. Data is analyzed by Genedata using automatrix parser. Graphs in the

publication were plotted by GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) using data exported from Genedata.

CCKA Binding Assay:

Evaluation of the affinity of compounds for the agonist site of the human CCKA receptor in transfected CHO cells were determined in a radioligand binding assay. Assays were performed at Eurofins CEREP, and details are described at https://www.eurofinsdiscoveryservices.com. Specifically, CHO cell membrane homogenates expressing CCKA receptor (4 µg protein) were incubated for 60 min at 22°C with 0.08 nM [^{125}I]CCK-8 in the absence or presence of the test compound in a buffer containing 10 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$ and 0.002% bacitracin. Nonspecific binding was determined in the presence of 1 µM CCK-8. Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) and rinsed several times with ice-cold incubation buffer containing 50 mM Tris-HCl and 0.5% BSA using a 96-sample cell harvester (Unifilter, Packard). The filters were dried, then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results were expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound was CCK-8. Assays were run in eight point concentration response mode with half log dilutions and a Ki value determined according to Cheng and Prusoff[6], 1973.

Ubiquitin Transfer FRET Assay:

The Cbl-b and ZAP70 recombinant proteins were expressed and purified in-house, Ube1 (E1), Ubiquitin fluorescein, and UbcH5b (E2) were purchased from R&D Systems (Minneapolis, MN, USA), and Tb-labelled streptavidin was purchased from Perkin Elmer (Waltham, MA, USA). For all experiments with test compounds, a dilution series of test compound was by direct dispense of compounds in 100% DMSO into an 'assay-ready plate' using the ECHO 655 acoustic dispenser with a backfill of DMSO to 100 nL (1% final assay concentration). For control experiments to ensure that the FRET ubiquitination signal was irreversible, 10 µM E2-Ub conjugate (R&D Systems) was added to the assay plate after the reaction had completed. The FRET *in vitro* ubiquitination assays were prepared in black medium bind low volume Greiner plates and run in sterile filtered assay buffer consisting of 20 mM Tris HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. The Cbl-b auto-ubiquitination reaction mixture was prepared by mixing 10 nM Cbl-b pre-labelled with Tb-labelled streptavidin (10:1), 30 nM ZAP70 kinase, 100 nM Ube1, 500 nM Ubiquitin Fluorescein and 500 nM UbcH5b. The reaction mixture was then dispensed (7.5 µL / well) into the assay-ready plate and the assay was then initiated upon the addition of 1 mM ATP (2.5 µL / well – final assay volume 10 µL), plate sealed and then incubated at 37 °C for 60 min. The ZAP70 ubiquitination reaction mixture was prepared by mixing 10 nM Cbl-b, 30 nM ZAP70 kinase pre-labelled with Tb-labelled streptavidin (10:1), 100 nM Ube1, 500 nM Ubiquitin Fluorescein and 500 nM UbcH5b. The reaction mixture was then dispensed (7.5 µL / well) into the assay-ready plate and the assay was then initiated upon the addition of 1 mM ATP (2.5 µL / well – final assay volume 10 µL), plate sealed and then incubated at 37 °C for 60 min. Assay plates were read using a PHERAstar FSX plate reader from BMG LabTech (Ortenberg, Germany) together with standard Tb-based TR-FRET settings with 337 nm excitation wavelength and emission monitored at 520 nm (donor) and 490 nm (acceptor). Emission intensities were measured over a 100 µs window following a 60 µs post excitation delay. A ratio of raw acceptor/donor was calculated and normalized relative to control wells without ATP added, without ZAP70, and/or a fully-inhibitory test compound (results reported in relative to min and max (1% DMSO) controls). Curve fitting and data analysis were carried out using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

pPLC-γ Assay and Western Blot:

Human T-cells were isolated from healthy donor leukopaks (Stemcell Technologies) and cultured overnight at 37 ^{o}C and 5% CO_2. Cells were plated (200,000 cells per well) in a 96-well round bottom plate and treated with compounds at various concentrations at 37 ^{o}C for 15 min. 1.2µL/well of Dynabeads human T-activator CD3/CD28 (ThermoFisher Scientific, Waltham, MA, Cat# 11131D) was added to each well to stimulate the cells and incubated for 1 hour. Cells were lysed using PhosphoSafe™ Extraction Reagent (Milipore Sigma, cat # 71296). Protein extracts were separated by SDS-PAGE, transferred onto Nitricellulose membranes, and probed with primary antibodies against phospho-PLCγ1 (Y783) (CST #14008), PLCγ1 (CST # 5690) or GAPDH (CST #2118) followed by secondary HRP-conjugated antibody, and visualized with the Pierce ECL Western blotting substrate (ThermoFisher Scientific, cat # 34076).

VI: Pharmacokinetic Methods (LogD):

$LogD_{7.4}$ by shake flask determination:

Equal parts of 10 mM sodium phosphate buffer solution pH7.4 and 1-octanol was mixed vigorously in a separating funnel three times to saturate the solutions. The mixture was left overnight to separate the octanol and buffer phase prior to the assay. Test compounds were prepared to 1 mM DMSO. Incubations were carried out in 96 deep well plates where 500 µL of 1-octanol (saturated with sodium phosphate buffer pH7.4), 500 µL of 10 mM sodium phosphate buffer pH7.4 (saturated with 1-octanol) and 10 µL of pooled test compounds in DMSO was added to each well with a final DMSO concentration of 1% v/v, using a Hamilton STAR liquid Handler (Hamiltion Bonaduz AG, Switzerland). The plate was sealed and vortexed at 2200 rpm for at least 30 seconds followed by at least 3 h equilibration on a horizontal shaker. The samples were then centrifuged at 3,220 g at room temperature for 30 min. Aliquots from the octanol phase (5 µL) were diluted 100-fold with a 1:1 mixture of deionized water and ACN and shaken for 5 min at 1000 rpm. The samples were then further diluted 10-fold (1000-fold dilution) with a 1:1 mixture of deionized water and ACN and shaken for 5 min at 1000 rpm. The 1000-fold octanol diluted samples are serially diluted to 10,000-, 100,000-, and 1,000,000-fold with a 1:1 mixture of deionized water and ACN. Aliquots from the buffer phase (50 µL) were transferred to a 96-well plate, followed by addition of 450 µL of 1:1 mixture of deionized water and ACN (10-fold dilution), and shaken for 5 min at 1000 rpm. The 10-fold buffer samples were serially diluted into 100-, 1000-, and 10,000-fold with a 1:1 mixture of deionized water and ACN and shaken for 5 min at 1000 rpm. Four dilutions of the octanol and buffer samples are analysed to provide a dynamic range of detection, comparable concentrations where possible were used in buffer and octanol to calculate $LogD_{7.4}$ to reduce risk of saturation. The samples were analyzed by LC–MS/MS. The LogD value was calculated using the following equation:

$$LogD_{7.4} = Log\ (\frac{MS^A\ in\ octanol\ phase\ x\ DF}{MS^A\ in\ buffer\ phase\ x\ DF})$$

Where MS^A is the peak area of test compound and DF is the dilution factor.

VII: X-ray Crystal Structure Data

A plasmid construct encoding SUMO-6His-Ulp1-Cbl-b (36-427) was expressed in E.coli strain Gold BL21 (DE3). Recombinant protein was purified by Ni^{2+}-IMAC, followed by tag cleavage with Ulp1 protease, subtractive Ni^{2+}-IMAC and size exclusion chromatography.
Cbl-b (36-427) was diluted to 10 mg/mL in 20 mM HEPES pH 7.5, 200 mM NaCl, 0.5 mM TECP and incubated with 1 mM inhibitor (from 100 mM stock in DMSO) for 15-45 min on ice. Sitting-drop vapor diffusion crystallization experiments were carried out with protein and precipitant (8-11 % PEG8000, 5 % MPD, 0.05 M magnesium acetate, 0.05 M propionate-cacodylate-bis-tris propane (PCTP) buffer pH 7.0-8.5) in 1:1 ratio. Crystals belonging to space group P212121 formed after 24 h. Crystallization was greatly facilitated by micro-seeding and by the presence of inhibitors, but once formed, crystals tolerated soaking to displace the inhibitor used during co-crystallization with other compounds (10 mM with final DMSO concentration of 10 %). Diffraction data were collected at Diamond Light Source beamline I04. Data collection and refinement details and statistics are given in supplementary table **S2**.

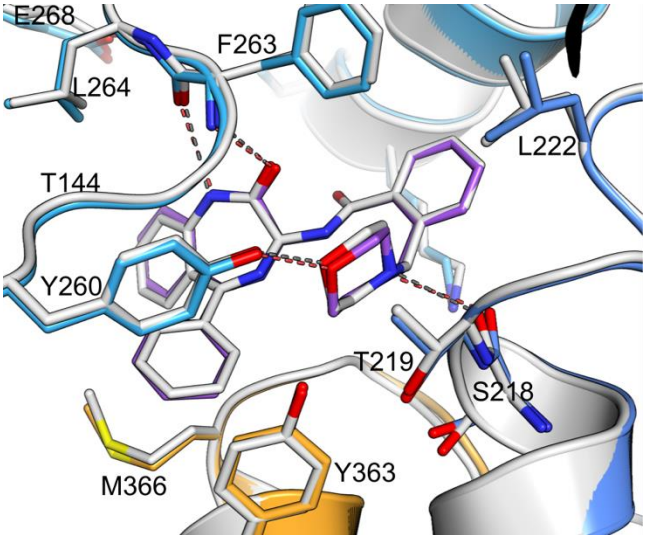


Figure S2. Crystal structure of 1 (colors as in Fig. 1) and 2 (grey) bound to Cbl-b. Compound **2** is the purified active enantiomer of **1**, consequently the structures and bound ligands are essentially identical.

Table S2. Crystallographic data collection and refinement statistics.
Values in parentheses are for the highest resolution shell.

Protein **Compound**	**Cbl-b 36-427** **1**	**Cbl-b 36-427** **Ex23**	**Cbl-b 36-427** **25**
PDB ID	8QNG	8QNH	8QNI
Crystallization condition	9 % PEG8000 0.05 M Mg Acetate 0.05 M PCTP pH 7.5	9 % PEG8000 5 % MPD 0.05 M Mg Acetate 0.05 M PCTP pH 8	11 % PEG8000 5 % MPD 0.05 M Mg Acetate 0.05 M PCPT pH 8.0
Cryoprotection	15 % MPD	10 % MPD	10 % MPD
Data collection			
DLS beamline	I04	I04	I04
Detector	DECTRIS PILATUS 6M	DECTRIS PILATUS 6M	DECTRIS PILATUS 6M
Processing Software	XDS/STARANISO	XDS/STARANISO	XDS/STARANISO
Ligand restraints	GRADE	GRADE	GRADE
Wavelength (Å)	0. 9795	0. 9999	0.9795
Space group	P212121	P212121	P212121
Cell dimensions			
a, *b*, *c* (Å)	55.58 72.05 94.79	57.04 73.60 93.97	57.12 73.36 102.74
α, β, γ (°)	90 90 90	90 90 90	90 90 90
Resolution (Å)	47.944—2.197 (2.458—2.197)	19.802—2.001 (2.126—2.001)	51.370—2.483 (2.747—2.483)
I / σI	13.3 (1.6)	10.3 (1.4)	14.5 (1.2)
CC 1/2	0.999 (0.634)	0.998 (0.430)	1.000 (0.467)
Completeness, spherical (%)	59.7 (10.6)	85.7 (23.6)	75.2 (14.7)
Completeness, ellipsoidal (%)	92.9 (72.3)	94.5 (58.0)	86.7 (29.5)
Redundancy	6.7 (6.2)	6.3 (6.6)	6.5 (6.9)
Refinement			
Resolution (Å)	47.944—2.197	19.802—2.001	51.370—2.483
No. reflections	79871	148573	79046
R_{work} / R_{free}	0.193 / 0.260	0.190 / 0.225	0.189/0.222
No. atoms			
Protein	3092	3102	3044
Inhibitor	34	39	35
Metal ions	3	3	3
Water	20	38	12
B-factors			
Protein	62.0	52.1	72.7
Ligand	29.4	32.4	50.1
Metal ions	69.2	40.9	71.3
Water	35.6	43.4	52.5
R.m.s. deviations			
Bond lengths (Å)	0.0101	0.0100	0.0084
Bond angles (°)	1.10	0.99	0.96

VIII: References

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