Supporting information

Selective A_3 Adenosine Receptor Antagonist Radioligand for Human and Rodent Species

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Chemical Synthesis:
The routes to the new antagonist analogues are shown in the main text (Schemes 1 and 2). 3,5-Dimethylbenzoyl chloride (**11a**), and 4-methylpyridine were purchased from AK Scientific, Inc. (Union City, CA). Other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). ^{1}H-NMR spectra were obtained with a Bruker 400 spectrometer using $CDCl_3$, DMSO-d_6, acetone-d_6 or a few drops of MeOH-d_4 in $CDCl_3$ as a solvent. The chemical shifts are expressed as ppm, and the coupling constants (*J*) are given in Hz. The purity of final compounds was checked using an Agilent 1260 Infinity HPLC equipped with an Agilent Eclipse 5 µm XDB-C18 analytical column (250 mm × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). Mobile phase: linear gradient solvent system, 10 mM TEAA (triethyl ammonium acetate):CH_3CN from 95:5 to 0:100 in 20 min; the flow rate was 1.0 mL/min. Peaks were detected by UV absorption with a diode array detector at 230, 254, and 280 nm. All derivatives tested for biological activity showed >95 % purity in the HPLC systems.

The previous synthesis of **9** and analogues was reported in Miwatashi et al.[1]

***N*-(4-(3,5-Dimethylphenyl)-5-(pyridin-4-yl)thiazol-2-yl)nicotinamide [DPTN, 9]:**[1]

Nicotinoyl chloride hydrochloride (55 mg, 0.293 mmol, 1.5 eq.) was added to a solution of **15a** (55 mg, 0.195 mmol, 1.0 eq.) and 4-dimethylaminopyridine (DMAP) (7.15 mg, 0.0585 mmol, 0.3 eq.) in NMP (2 mL), and the resulting mixture was stirred at 80 °C for 16 h. Half sat. $NaHCO_3$ solution (10 mL) was added to the reaction mixture and extracted with ethyl acetate (3x 20 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain the crude product, which was purified by silica column to obtain a pale yellow solid. Eluant: 80–85 % EtOAc in hexane. TLC: Rf ~ 0.3 in EtOAc. Yield: 43 mg (57%). Spectroscopic data were in agreement with the literature report.[1]

^{1}H NMR (400 MHz, DMSO-d_6) δ 13.20 (s, 1H), 9.25 (d, J = 2.3 Hz, 1H), 8.84 – 8.78 (m, 1H), 8.53 (d, J = 5.3 Hz, 2H), 8.46 (d, J = 8.0 Hz, 1H), 7.61 (dd, J = 8.0, 4.8 Hz, 1H), 7.31 (d, J = 6.1 Hz, 2H), 7.09 (s, 2H), 7.02 (s, 1H), 2.23 (s, 6H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{22}H_{19}N_4O^{32}S$: 378.1280; found 378.1279.

3-Fluoro-5-methylbenzoyl chloride (11b):
Compound **11b** was prepared by a method similar to that used for **11c**, from 3-fluoro-5-methylbenzoic acid **10b** (2.00 g, 13.0 mmol, 1.0 eq.) and thionyl chloride (2.82 mL, 38.9 mmol, 3.0 eq.) in dry toluene (~0.4 M). Yield: 89%, 2.00 g.

3-Bromo-5-methylbenzoyl chloride (11c):
To a stirred suspension of 3-bromo-5-methylbenzoic acid **10c** (3.00 g, 14.0 mmol, 1.0 eq.) in dry toluene (35 mL) at 0 °C, a solution of oxalyl chloride solution (2.5 M in DCM, 16 mL, 30.7 mmol, 2.2 eq.) was added dropwise via syringe at 0 °C, followed by the addition of 25 µL of DMF. The ice bath was removed, and the reaction mixture was stirred at room temperature for 16 h. The solvent was removed by rotary evaporation to obtain the product as an oily residue **11c**. This was carried over to the next step without any further purification. Yield: 3.14 g (96%).

Compounds **11b** and **11d** were prepared by this method with slight modifications.

3-Iodo-5-methylbenzoyl chloride (11d):
Compound **11d** was prepared was prepared by a method similar to that used for **11c**, from 3-iodo-5-methylbenzoic acid **10d** (1.00 g, 3.82 mmol, 1.0 eq.) and oxalyl chloride solution (2.5 M in DCM, 3.01 mL, 7.63 mmol, 2.0 eq.) in dry toluene (~0.25 M). Yield: 93%, 1.00 g.

*Synthesis of Weinreb-Nahm amides (**12a-d**):*

***N*-Methoxy-*N*-3,5-trimethylbenzamide (12a):**[1]
To a stirred solution of *N*,*O*-dimethylhydroxylamine hydrochloride (5.36 g, 55 mmol, 1.1eq.) and K_2CO_3 (100 mmol, 2.0 eq.) in a mixture of EtOAc (250 mL) and water (125 mL), a solution of 3,5-dimethylbenzoyl chloride (8.43 g, 50 mmol, 1.0 eq.) in EtOAc (20 mL) was added dropwise at 0 °C. The ice bath was removed, and the reaction mixture was stirred at room temperature for 18 h. The EtOAc layer was separated, and the aqueous phase was washed with ethyl acetate (2x100 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain an oily residue. This crude product was carried over to the next step without any further purification. Yield: 9.50 g (98%); TLC: Rf ~ 0.5 in 50% EtOAc in hexane.

3-Fluoro-N-methoxy-*N*,5-dimethylbenzamide (12b):
To a stirred solution of *N*,*O*-dimethylhydroxylamine hydrochloride (1.30 g, 13.2 mmol, 1.2 eq.) and K_2CO_3 (3.80 g, 27.5 mmol, 2.5 eq.) in a mixture of EtOAc (40 mL) and water (20 mL), a solution of 3-bromo-5-methylbenzoyl chloride (1.90 g, 11.00 mmol, 1.0 eq.) in EtOAc (5 mL) was added dropwise at 0°C. The ice bath was removed, and the reaction mixture was stirred at room temperature for 18 h. The EtOAc layer was separated, and the aqueous phase was washed with ethyl acetate (2x30 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain an oily residue. This was carried over to the next step without any further purification. TLC: Rf ~ 0.5 in 33% EtOAc in hexane. Yield: 2.00 g (92%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (s, 1H), 7.21 – 7.14 (m, 1H), 6.97 (dt, *J* = 9.3, 2.1 Hz, 1H), 3.56 (d, *J* = 1.9 Hz, 3H), 3.35 (s, 3H), 2.38 (s, 3H). ^{19}F NMR (377 MHz, $CDCl_3$) δ -113.98.

LCMS (ESI) m/z: [M+H]+ calculated for $C_{10}H_{13}NO_2F$: 197.09; found 198.1.

3-Bromo-*N*-methoxy-*N*,5dimethylbenzamide (12c):
To a stirred solution of *N*,*O*-dimethylhydroxylamine hydrochloride (0.922 g, 9.42 mmol, 1.1 eq.) and K_2CO_3 (2.37 g, 17.1 mmol, 2.0 eq.) in a mixture of EtOAc (40 mL) and water (20 mL), a solution of 3-bromo-5-methylbenzoyl chloride **11c** (2.00 g, 8.57 mmol, 1.0 eq.) in EtOAc (5 mL) was added dropwise at 0°C. The ice bath was removed, and the reaction mixture was stirred at room temperature for 18 h. The EtOAc layer was separated, and the aqueous phase was washed with ethyl acetate (2x20 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The

filtrate was concentrated by rotary evaporation to obtain an oily residue. This was carried over to the next step without any further purification. TLC: Rf ~ 0.75 in EtOAc. Yield: 1.92 g (87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (d, J = 1.8 Hz, 1H), 7.43 – 7.37 (m, 2H), 3.55 (s, 3H), 3.34 (s, 3H), 2.35 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{10}H_{13}NO_2{}^{79}Br$: 258.0130; found 258.0126.

3-Iodo-*N*-methoxy-*N*,5-dimethylbenzamide (12d):
To a stirred solution of *N*,*O*-dimethylhydroxylamine hydrochloride (0.411 g, 4.20 mmol, 1.1 eq.) and K_2CO_3 (1.06 g, 7.64 mmol, 2.0 eq.) in a mixture of EtOAc (20 mL) and water (10 mL), a solution of 3-iodo-5-methylbenzoyl chloride (1.07 g, 3.82 mmol, 1.0 eq.) in EtOAc (5 mL) was added dropwise via syringe at 0°C. The ice bath was removed, and the reaction mixture was stirred at room temperature for 16 h. The EtOAc layer was separated, and the aqueous phase was washed with ethyl acetate (2x20 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain an oily residue. This was carried over to the next step without any further purification. TLC: Rf ~ 0.75 in EtOAc. Yield: 1.00 g (86%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.64 – 7.59 (m, 1H), 7.41 (s, 1H), 3.55 (s, 3H), 3.33 (s, 3H), 2.33 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{10}H_{13}NO_2I$: 305.9991; found 305.9988.

*Synthesis of Weinreb–Nahm ketones (**13a-d**):*

1-(3,5-Dimethylphenyl)-2-(pyridin-4-yl)ethan-1-one (13a):[1]
To a solution of LDA (1.0 M in THF/hexane, 50 mL, 50 mmol, 4.0 eq.) in dry THF (30 mL) at -78 °C (dry ice in acetone), a solution of 4-picoline (2.43 mL, 25 mmol, 2.0 eq.) in dry THF (15 mL) was added dropwise at -78 °C. The reaction mixture was stirred at -78 to -50 °C for 2 h. To this mixture, a solution of **12a** (2.42 g, 12.5 mmol, 1.0 eq.) in dry THF (20 mL) was added dropwise at -78 °C via cannula. The ice bath was removed, and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was quenched by the addition of sat. NH_4Cl (20 mL) at 0 to 5 °C and diluted with water (50 mL). The phases were separated, and the aqueous phase was extracted with EtOAc (3x 60 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain an oily residue, which was purified by silica column to obtain a pale yellow solid. Eluant: 30-50% EtOAc in hexane. Yield: 1.41 g (50%).

1-(3-Fluoro-5-methylphenyl)-2-(pyridin-4-yl)ethan-1-one (**13b**):
To a solution of LDA (1.0 M in THF/hexane, 11 mL, 1.05 eq.) in dry THF (30 mL) at -78 °C (dry ice in acetone), a solution of 4-picoline (1.48 mL, 15.2 mmol, 1.5 eq.) was added dropwise via syringe at -78 °C. The reaction mixture was stirred at -78 °C for 45 min. To this mixture, a solution of amide **12b** (2.0 g, 10.14 mmol, 1.0 eq.) in dry THF (6 mL) was added dropwise at -78 °C via

syringe and stirring continued at -78 ºC for 3 h. The reaction mixture was quenched by the addition of sat. NH4Cl (10 mL) at 0 to 5 ºC and diluted with water (20 mL). The phases were separated, and the aqueous phase was extracted with EtOAc (3x 30 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain an oily residue, which was purified by silica column to obtain a pale yellow solid. Eluant: 40–60% EtOAc in hexane. Yield: 1.65 g (71%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 – 8.54 (m, 2H), 7.58 (s, 1H), 7.46 (d, J = 9.1 Hz, 1H), 7.22 – 7.16 (m, 2H), 7.12 (d, J = 9.1 Hz, 1H), 4.25 (s, 2H), 2.42 (s, 3H).
^{19}F NMR (377 MHz, $CDCl_3$) δ -112.46 (t, J = 9.1 Hz).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{14}H_{13}NOF$: 230.0981; found 230.0978.

1-(3-Bromo-5-methylphenyl)-2-(pyridin-4-yl)ethan-1-one (**13c**):
To a solution of LDA (1.0 M in THF/hexane, 4.30 mL, 1.05 eq.) in dry THF (15 mL) at -78 ºC (dry ice in acetone), a solution of 4-picoline (0.60 mL, 6.102 mmol, 1.5 eq.) in dry THF (15 mL) was added dropwise at -78 ºC. The reaction mixture was stirred at -78 ºC for 45 min. To this mixture, a solution of amide **12c** (1.05 g, 4.068 mmol, 1.0 eq.) in dry THF (10 mL) was added dropwise at -78 ºC via syringe and stirring continued at -78 ºC for 3 h. The reaction mixture was quenched by the addition of sat. NH_4Cl (10 mL) at 0 to 5 ºC and diluted with water (20 mL). The phases were separated, and the aqueous phase was extracted with EtOAc (3x 60 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain an oily residue, which was purified by silica column to obtain a pale yellow solid. Eluant: 30–50% EtOAc in hexane. Yield: 0.71 g (60%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (d, J = 6.1 Hz, 1H), 7.91 (s, 1H), 7.71 (s, 1H), 7.56 (s, 1H), 7.18 (d, J = 6.0 Hz, 1H), 4.24 (s, 2H), 2.40 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{14}H_{13}NO^{79}Br$: 290.0181; found 290.0181.

1-(3-Iodo-5-methylphenyl)-2-(pyridin-4-yl)ethan-1-one (**13d**):
To a solution of LDA (1.0 M in THF/hexane, 3.28 mL, 1.00 eq.) in dry THF (20 mL) at -78 ºC (dry ice in acetone), a solution of 4-picoline (479 µL, 4.92 mmol, 1.5 eq.) was added dropwise via syringe at -78 ºC. The reaction mixture was stirred at -78 o for 45 min. To this mixture, a solution of amide **12d** (1.00 g, 3.28 mmol, 1.0 eq.) in dry THF (10 mL) was added dropwise at -78 ºC via syringe and stirring continued at -78 ºC for 3 h. The reaction mixture was quenched by the addition of sat. NH_4Cl (10 mL) at 0 to 5 ºC and diluted with water (20 mL). The phases were separated, and the aqueous phase was extracted with EtOAc (3x 30 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain an oily residue, which was purified by silica column to obtain a pale yellow solid. Eluant: 40–60% EtOAc in hexane. Yield: 0.64 g (58%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71 – 8.41 (m, 2H), 8.11 (s, 1H), 7.75 (d, J = 13.2 Hz, 2H), 7.18 (d, J = 6.1 Hz, 2H), 4.23 (s, 2H), 2.38 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{14}H_{13}NOI$: 338.0042; found 338.0041.

General procedure for the synthesis of compounds 14a-d:

2-Bromo-1-(3,5-dimethylphenyl)-2-(pyridin-4-yl)ethan-1-one hydrobromide (14a):[1]
To a stirred solution of **13a** (0.66 g, 2.93mmol, 1.0 eq.) in acetic acid (3 mL), bromine (150 µL, 2.93 mmol, 1.0 eq.) was added dropwise *via* micro syringe at room temperature. The reaction mixture was stirred for 4 h at 80°C. A yellow ppt was formed after 4 h. The solvent was removed by rotary evaporation and the residue was washed with ethyl acetate (3x3 mL), filtered and dried to afford compound **14a** as a pale yellow crystalline powder. Yield: 0.592 g (52%).

2-Bromo-1-(3-fluoro-5-methylphenyl)-2-(pyridin-4-yl)ethan-1-one (14b):
To a stirred solution of **13b** (0.325 g, 1.42 mmol, 1.0 eq.) in acetic acid (3 mL), bromine (110 µL, 2.13 mmol, 1.5 eq.) was added dropwise via micro syringe at room temperature. The reaction mixture was stirred for 3 h at 80 °C. The solvent was removed by rotary evaporation and the residue was washed with ethyl acetate (10 mL), diethyl ether (20 mL), filtered and dried to afford compound **14b** as a solid in orange color. Yield: 0.24 g (43%).

^{1}H NMR (400 MHz, DMSO) δ 8.87 (d, J = 5.7 Hz, 2H), 8.01 (d, J = 5.7 Hz, 2H), 7.80 (s, 1H), 7.74 (d, J = 9.5 Hz, 1H), 7.45 (d, J = 9.4 Hz, 1H), 7.23 (s, 1H), 2.42 (s, 3H). ^{19}F NMR (377 MHz, DMSO-d_6) δ -112.80.

HRMS (ESI) m/z: [M+H]+ calculated for $C_{14}H_{12}NOF^{79}Br$: 308.009; found 0308.0086.

2-Bromo-1-(3-bromo-5-methylphenyl)-2-(pyridin-4-yl)ethan-1-one (14c):
To a stirred solution of **13c** (0.615 g, 2.12 mmol, 1.0 eq.) in acetic acid (4 mL), bromine (109 µL, 2.12 mmol, 1.0 eq.) was added dropwise via micro syringe at room temperature. The reaction mixture was stirred for 4 h at 80°C. A yellow ppt was formed after 3 h. The solvent was removed by rotary evaporation and the residue was washed with ethyl acetate (10 mL), diethyl ether (20 mL), filtered and dried to afford the compound **14c** as a pale yellow crystalline powder. Yield: 0.82 g (86%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.91 (d, *J* = 6.3 Hz, 2H), 8.10 (d, *J* = 5.4 Hz, 2H), 7.93 (s, 2H), 7.79 (s, 1H), 7.28 (s, 1H), 2.41 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{14}H_{12}NO^{79}BrI$: 415.9147; found 415.9152.

2-Bromo-1-(3-iodo-5-methylphenyl)-2-(pyridin-4-yl)ethan-1-one (14d):
To a stirred solution of **13d** (0.420 g, 1.25 mmol, 1.0 eq.) in acetic acid (3 mL), bromine (64 µL, 1.246 mmol, 1.0 eq.) was added dropwise via micro syringe at room temperature. The reaction mixture was stirred for 3 h at 80°C. The solvent was removed by rotary evaporation and the residue

was washed with ethyl acetate (10 mL), diethyl ether (20 mL), filtered and dried to afford the compound **14d** as a pale yellow solid. Yield: 0.56 g (90%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (d, *J* = 4.2 Hz, 2H), 8.25 (s, 1H), 8.17 – 8.10 (m, 2H), 7.95 (dd, *J* = 4.7, 2.7 Hz, 2H), 7.28 (s, 1H), 2.37 (s, 2H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{14}H_{12}NO^{79}BrI$: 415.9147; found 415.9152.

Compounds 15a-d:

4-(3,5-Dimethylphenyl)-5-(pyridin-4-yl)thiazol-2-amine (15a):[1]
Methylthiourea (0.119 g, 1.57 mmol, 1.1 eq.) was added to a mixture of **14a** (0.544 g, 1.49 mmol, 1.0 eq.) and triethylamine (436 µL, 3.13 mmol, 2.1 eq.) in acetonitrile (6 mL). The resulting solution was refluxed for 3 h. The solvent was removed by rotary evaporation and half sat. $NaHCO_3$ solution (5 mL) was added to the residue. The precipitate was collected by filtration, and the resulting solid was washed with water and ether successively to afford **15a** as a pale yellow powder. Yield: 0.270 g (64%).

4-(3-Fluoro-5-methylphenyl)-5-(pyridin-4-yl)thiazol-2-amine (15b):
Methylthiourea (12 mg, 0.155 mmol, 1.5 eq.) was added to a mixture of **14b** (40 mg, 0.103 mmol, 1.0 eq.) and triethylamine (43 µL, 0.310 mmol, 3.0 eq.) in acetonitrile (2 mL). The resulting solution was refluxed for 3h in a sealed tube. The reaction mixture was cooled to the room temperature and diluted with EtOAc (10 mL). Then, the crude mixture was washed with half sat. $NaHCO_3$ solution (5 mL) and separated the layers. The aqueous phase was washed with EtOAc (2x10 mL) and the combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain the crude product, which was purified by silica column to obtain a pale yellow solid. Yield: 22 mg (76%).

^{1}H NMR (400 MHz, a few drops of MeOH-d_4 in $CDCl_3$) δ 8.47 (d, *J* = 5.3 Hz, 2H), 7.65 (s, 1H), 7.54 (d, *J* = 8.5 Hz, 1H), 7.41 (d, *J* = 5.2 Hz, 2H), 6.96 (d, *J* = 9.3 Hz, 1H), 2.31 (s, 3H). 19F NMR (377 MHz) δ -114.61.

HRMS (ESI) m/z: [M+H]+ calculated for $C_{15}H_{13}N_3F^{32}S$: 286.0814; found 286.0811.

4-(3-Bromo-5-methylphenyl)-5-(pyridin-4-yl)thiazol-2-amine (15c):
Methylthiourea (0.13 g, 1.71 mmol, 1.1 eq.) was added to a mixture of **14c** (0.70 g, 1.56 mmol, 1.0 eq.) and triethylamine (652 µL, 4.68 mmol, 3.0 eq.) in acetonitrile (10 mL). The resulting solution was refluxed for 3 h. The solvent was removed by rotary evaporation, and half sat. $NaHCO_3$ solution (15 mL) was added to the residue. The reaction mixture was extracted with ethyl acetate (3x30 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain the crude product, which was purified by silica column to obtain a pale yellow solid. Yield: 0.43 g (80%). TLC: Rf ~ 0.25 in hexane-EtOAc (1:1).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.47 (d, J = 6.5 Hz, 1H), 7.73 (s, 2H), 7.43 (s, 1H), 7.28 – 7.22 (m, 3H), 2.26 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{15}H_{13}N_3{}^{79}Br^{32}S$: 346.0014; found 346.0010.

4-(3-Iodo-5-methylphenyl)-5-(pyridin-4-yl)thiazol-2-amine (15d):
Methylthiourea (62 mg, 0.818 mmol, 1.2 eq.) was added to a mixture of **15c** (339 mg, 0. 682 mmol, 1.0 eq.) and triethylamine (285 µL, 2.05 mmol, 3.0 eq.) in acetonitrile (10 ml). The resulting solution was refluxed for 4 h. The solvent was removed by rotary evaporation, and half sat. $NaHCO_3$ solution (15 mL) was added to the residue. The reaction mixture was extracted with ethyl acetate (3x30 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain the crude product, which was purified by silica column to obtain a pale yellow solid. Yield: 102 mg (38%). TLC: Rf ~ 0.3 in 33% Acetone in hexane (1:2).

^{1}H NMR (400 MHz, Acetone-d_6) δ 8.54 (d, J = 6.1 Hz, 2H), 8.31 (s, 1H), 7.95 (s, 1H), 7.73 (s, 1H), 7.49 (d, J = 6.1 Hz, 2H), 4.56 (s, 2H), 2.33 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{15}H_{13}N_3{}^{32}SI$: 393.9875; found 393.9877.

Compounds 16b-d and 17:

***N*-(4-(3-Fluoro-5-methylphenyl)-5-(pyridin-4-yl)thiazol-2-yl)nicotinamide (16b):**
Nicotinoyl chloride hydrochloride (37 mg, 0.210 mmol, 3.0 eq.) was added to a solution of **15b** (20 mg, 0.0701mmol, 1.0 eq.) and 4-dimethylaminopyridine (DMAP) (9 mg, 0.0585 mmol, 0.0701 mmol, 1.0 eq.) in DMA (2 mL), and the resulting mixture was stirred at 80 °C for 16 h. Half sat. $NaHCO_3$ solution (10 mL) was added to the reaction mixture and the mixture extracted with ethyl acetate (3x 15 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain the crude product, which was purified by silica column to obtain a pale yellow solid. Eluant: 40–50% acetone in hexane. TLC: Rf ~ 0.3 in 66 % EtOAc in hexane (2:1). Yield: 16 mg (59%).

^{1}H NMR (400 MHz, acetone-d_6) δ 9.37 (s, 1H), 8.85 (d, J = 4.2 Hz, 1H), 8.63 – 8.51 (m, 3H), 7.62 (dd, J = 8.0, 4.8 Hz, 1H), 7.36 (d, J = 6.2 Hz, 2H), 7.17 (s, 1H), 7.00 (dd, J = 16.1, 9.9 Hz, 2H), 2.31 (s, 3H). On the spectrum, peaks at 3.00, 2.83, and 1.97 ppm were from DMA solvent traces.

HRMS (ESI) m/z: [M+H]+ calculated for $C_{21}H_{16}N_4O^{32}SF$: 391.1029; found 391.1031.

***N*-(4-(3-Bromo-5-methylphenyl)-5-(pyridin-4-yl)thiazol-2-yl)nicotinamide (16c):**
Nicotinoyl chloride hydrochloride (121 mg, 0.679 mmol, 5.0 eq.) was added to a solution of **15c** (47 mg, 0.136 mmol, 1.0 eq.) and 4-dimethylaminopyridine (DMAP) (66 mg, 0.272 mmol, 2.0 eq.) in NMP (2 mL), and the resulting mixture was stirred at 80 °C for 20 h. Half sat. $NaHCO_3$

solution (10 mL) was added to the reaction mixture slowly at RT and the mixture extracted with ethyl acetate (3x 20 mL). Extracts were washed with brine, dried and concentrated to give a solid. The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain the crude product, which was purified by silica column to obtain a pale yellow solid. Eluant: 70-80% EtOAc in hexane. TLC: Rf ~ 0.4 in 66 % EtOAc in hexane (2:1). Yield: 53 mg (87%).

^{1}H NMR (400 MHz, a few drops of MeOH-d_4 in $CDCl_3$) δ 9.16 (d, *J* = 2.3 Hz, 1H), 8.74 (dd, *J* = 4.9, 1.6 Hz, 1H), 8.48 – 8.42 (m, 2H), 8.34 (dt, *J* = 8.1, 2.0 Hz, 1H), 7.49 (dd, *J* = 8.0, 4.9 Hz, 1H), 7.39 (d, *J* = 1.9 Hz, 1H), 7.28 – 7.16 (m, 3H), 7.12 (s, 1H), 2.23 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{21}H_{16}N_4O^{79}Br^{32}S$: 451.0228; found 451.0235.

***N*-(4-(3-Iodo-5-methylphenyl)-5-(pyridin-4-yl)thiazol-2-yl)nicotinamide (16d):**
Nicotinoyl chloride hydrochloride (84 mg, 0.473 mmol, 3.0 eq.) was added to a solution of **15d** (62 mg, 0.158 mmol, 1.0 eq.) and 4-dimethylaminopyridine (DMAP) (19 mg, 0.158 mmol, 1.0 eq.) in DMA (2 mL), and the resulting mixture was stirred at 80 °C for 20 h. Half sat. $NaHCO_3$ solution (10 mL) was added to the reaction mixture slowly at RT and the mixture extracted with ethyl acetate (3x 20 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain the crude product, which was purified by silica column to obtain a pale yellow solid. Eluant: 65–80% EtOAc in hexane. TLC: Rf ~ 0.4 in 66 % EtOAc in hexane (2:1). Yield: 50 mg (39%).

^{1}H NMR (400 MHz, a few drops of MeOH-d_4 in $CDCl_3$) δ 9.27 – 9.07 (m, 1H), 8.85 – 8.70 (m, 1H), 8.49 (s, 2H), 8.33 (dt, J = 8.1, 2.0 Hz, 1H), 7.62 (s, 1H), 7.55 – 7.44 (m, 2H), 7.24 (d, J = 5.1 Hz, 2H), 7.16 (s, 1H), 2.22 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{21}H_{16}N_4OI^{32}S$: 499.0089; found 499.0092.

5-Bromo-*N*-(4-(3,5-dimethylphenyl)-5-(pyridin-4-yl)thiazol-2-yl)nicotinamide (17):
5-Bromo-nicotinoyl chloride (39 mg, 0.178 mmol, 2.0 eq.) was added to a solution of **15a** (25 mg, 0.089 mmol, 1.0 eq.) and 4-dimethylaminopyridine (DMAP) (11 mg, 0.089 mmol, 1.0 eq.) in DMF (1 mL), and the resulting mixture was stirred at 80 °C for 16 h. Half sat. $NaHCO_3$ solution (10 mL) was added to the reaction mixture slowly at RT and the mixture extracted with ethyl acetate (3x 10 mL). The combined EtOAc layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporation to obtain the crude product, which was purified by silica column to obtain a pale yellow solid. Eluant: 65–80% EtOAc in hexane. TLC: Rf ~ 0.4 in 50% EtOAc in hexane (1:1). Yield: 24 mg (59%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 13.24 (s, 1H), 9.20 (d, J = 1.9 Hz, 1H), 8.96 (d, J = 2.2 Hz, 1H), 8.72 (d, J = 2.1 Hz, 1H), 8.56 – 8.50 (m, 2H), 7.34 – 7.28 (m, 2H), 7.09 (s, 2H), 7.03 (s, 1H), 3.27 (s, 0H), 2.23 (s, 6H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{22}H_{18}N_4OS^{79}Br$: 465.0385; found 465.0390.

Methyl (E)-3-(3-methyl-5-(2-(nicotinamido)-5-(pyridin-4-yl)thiazol-4-yl)phenyl)acrylate (19):

In an oven dried 10 mL microwave vial, compound **16d** (6 mg, 12 µmol, 1.0 eq.), $Pd(OAc)_2$ (0.3 mg, 1.2 µmol, 0.1 eq.), P(*o*-tol)$_3$ (0.73 mg, 2.4 µmol, 0.2 eq.) were combined and dissolved in dry DMA (1.0 mL). To this vial, Et_3N (7 µL, 4 eq.), and methyl acrylate (4 µL, 0.212 mmol, 2.0 eq.) were added, and the reaction mixture was purged with nitrogen for 2 min. The solution was stirred under nitrogen at 80 °C for 20 h. The solvent was removed by rotary evaporation to obtain the crude product, which was purified by silica column to obtain the pure product (**19**). Yield: 1.8 mg (33%). Eluent: 50–60% EtOAc in hexane.

^{1}H NMR (400 MHz, DMSO-d_6) δ 13.24 (s, 1H), 9.26 (d, J = 2.3 Hz, 1H), 8.84 – 8.78 (m, 1H), 8.54 (d, J = 5.1 Hz, 2H), 8.50 – 8.43 (m, 1H), 7.65 – 7.51 (m, 4H), 7.39 (s, 1H), 7.35 – 7.29 (m, 2H), 6.48 (d, J = 16.0 Hz, 1H), 3.71 (s, 3H), 2.31 (s, 3H).

HRMS (ESI) m/z: [M+H]+ calculated for $C_{25}H_{21}N_4O_3S$: 457.1334; found 457.1328.

N-(4-(3-((5-chlorothiophen-2-yl)ethynyl)-5-methylphenyl)-5-(pyridin-4-yl)thiazol-2-yl)nicotinamide (20):

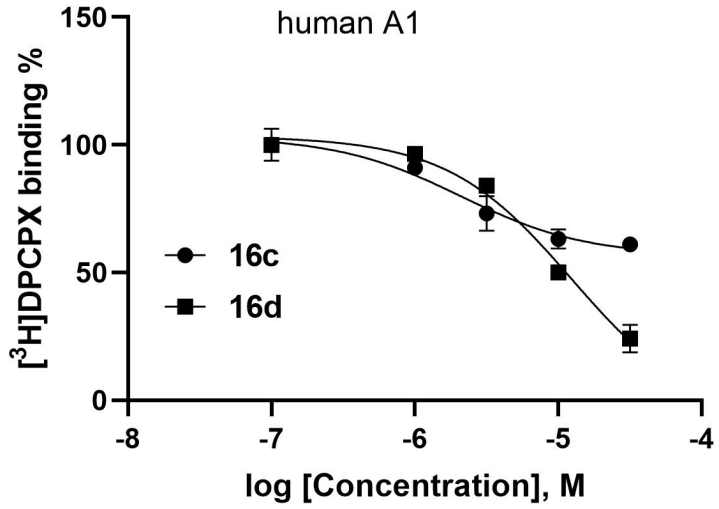
In an oven dried 10 mL microwave vial, compound **16d** (7.2 mg, 14.5 µmol, 1.0 eq.), $PdCl_2(PPh_3)_2$ (2.4 mg, 3.46 µmol, 0.24 eq.), CuI (1.0 mg, 3.64 µmol mmol, 0.4 eq.) were combined and dissolved in dry DMF (1.0 mL). To this vial, Et_3N (50 µL, 36.1 µmol, 2.5 eq.), and 2-chloro-5-ethynylthiophene (10 µL, 91 µmol, 6.3 eq.) were added successively, and the reaction mixture was purged with nitrogen for 2 min. The solution was stirred under nitrogen at 80 °C for 16 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (5 mL), and passed through a short silica plug. The solvent was removed under vacuum to obtain the crude product, which was purified by silica column to obtain the pure product (**20**). Yield: 4.4 mg (59%). Eluent: 50–55% EtOAc in hexane.

^{1}H NMR (400 MHz, DMSO-d_6) δ 13.21 (s, 1H), 9.25 (s, 1H), 8.82 (s, 1H), 8.57 (s, 2H), 8.46 (d, *J* = 8.2 Hz, 1H), 7.65 – 7.51 (m, 3H), 7.41 (d, *J* = 4.6 Hz, 2H), 7.35 (s, 2H), 7.30 (d, *J* = 4.0 Hz, 1H), 7.17 (d, *J* = 4.0 Hz, 1H), 2.29 (s, 3H).

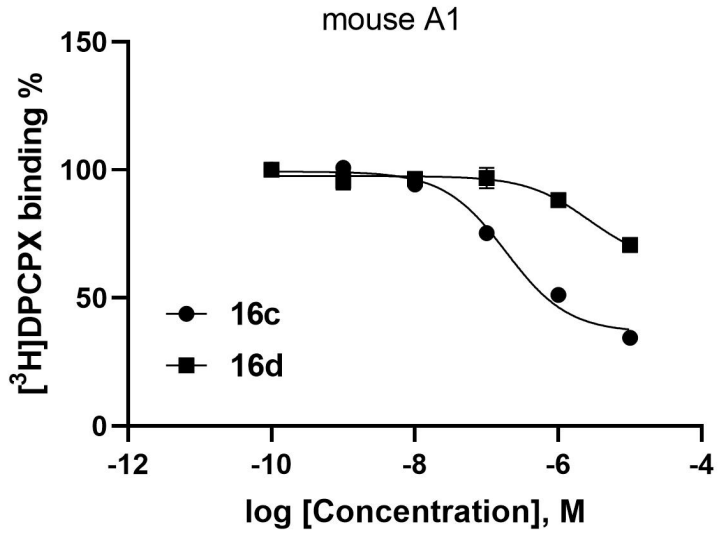
HRMS (ESI) m/z: [M+H]+ calculated for $C_{27}H_{18}N_4OS_2Cl$: 513.0611; found 513.0608.

Figure S1. Binding curves of **16c** and **16d** (MRS7907) at A_1AR.

A



B

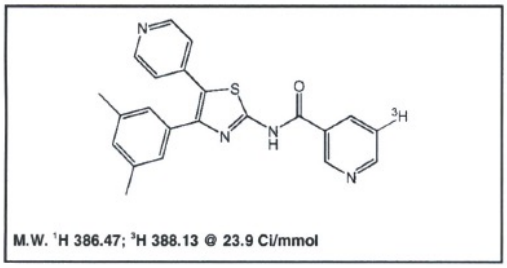


Radiochemical purity

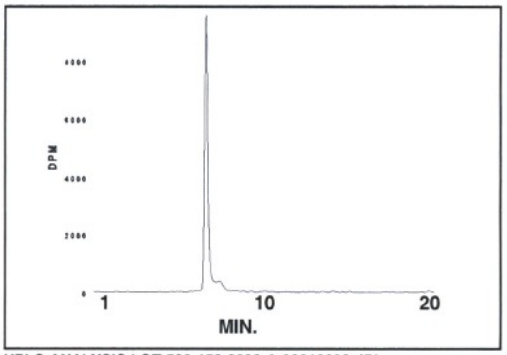
Certificate of Analysis

MT-1003183

MRS7799, [^{3}H]-



M.W. ^{1}H 386.47; ^{3}H 388.13 @ 23.9 Ci/mmol



HPLC ANALYSIS LOT 592-150-0239-A-20210622-JPL
File Name: int4g13O Date and Time: 6/22/2021 2:27:14 PM
HPLC-04 - Radio

Peak #	Area %	Time	Area
1	97.45	6.63000	13838.44863
2	0.20	7.13000	28.52257
3	2.34	7.43330	332.95790
Totals	100.00		14199.92910

Lot #: 592-150-0239-A-20210622-JPL

**Molar Activity by LC-MS:* 23.9 Ci/mmol

Concentration: 1.0 mCi/ml; 16.24 µg/ml

Packaged in: Ethanol solution

Date of Analysis: June 22, 2021

**Radiochemical Purity by HPLC:* 97%

Column: Phenomenex Kinetex XB-C18 3.0 x 150mm, 5µm

Flow Rate: 1 ml/min.

Mobile Phase: A: Water with 0.1% TFA
B: Acetonitrile
0-15min 0-100% B; Hold to 20min.

**ID by HPLC:* The retention time is consistent with that of the standard
**ID by LC-MS:* Conforms to reference standard
**ID by ^{3}H NMR:* Results confirm label position indicated in structure

Storage Recommendation: Store at -20°C.

Off-target Screening

Determined by the Psychoactive Drug Screening Program (PDSP) at the University of North Carolina. We thank Dr. Bryan L. Roth (Univ. North Carolina at Chapel Hill) and National Institute of Mental Health's Psychoactive Drug Screening Program (Contract # HHSN-271-2008-00025-C) for screening data.

Procedures are described in Besnard et al.[2] and at: https://pdsp.unc.edu/pdspweb/content/UNC-CH%20Protocol%20Book.pdf

Unless noted in the text, no significant interactions (<50% inhibition at 10 μM) for any of the compounds were found at the following sites (human unless noted): $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, $5HT_{1E}$, $5HT_{2A}$, $5HT_{2B}$, $5HT_{2C}$, $5HT_3$, $5HT_{5A}$, $5HT_6$, $5HT_7$, α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, β_3, BZP rat brain site, D_1, D_2, D_3, D_4, D_5, delta opioid receptor (DOR), kappa opioid receptor (KOR), $GABA_A$, H_1, H_2, H_3, H_4, M_1, M_2, M_3, M_4, M_5, mu opioid receptor (MOR), σ_1, σ_2, DAT, NET, SERT, TSPO.

Table S1.

Off-target binding of **16d** at 10 μM

Protein	Mean (% inhibition at 10 μM)	
5-HT1A	5.63	
5-HT1B	7.01	
5-HT1D	11.76	
5-HT1E	8.03	
5-HT2A	6.53	
5-HT2B	5.63	
5-HT2C	14.95	
5-HT3	29.37	
5-HT5A	-10.31	
5-HT6	19.33	
5-HT7A	5.47	
Alpha1A	-23.28	
Alpha1B	-10.83	
Alpha1D	29	
Alpha2A	-2.96	
Alpha2B	-19.58	
Alpha2C	17.74	
Beta1	-27.85	
Beta2	-9.18	
Beta3	-69.4	*
BZP Rat Brain Site	26.47	
D1	11.49	
D2	49.25	
D3	64.85	
D4	61.68	
D5	-16	
DAT	-7.75	
DOR	12.05	
GABAA	12.94	
H1	-24.32	
H2	15.41-	
H3	-29.13	
H4	5.24	
KOR	15.75	
M1	15.16	
M2	17.67	
M3	6.01	
M4	8.36	
M5	-6.3	
MOR	10.6	
NET	12.29	
PBR	77.36	
SERT	-5.95	
Sigma 1	40.06	
Sigma 2	70.29	

*K_i >10 μM, determined in secondary binding

Determined by the NIMH Psychoactive Drug Screening Program, Univ. of North Carolina

Pharmacokinetics of 9 in Male Sprague-Dawley Rats

Compound **9** was formulated in 10% DMSO/30% PEG400 in phosphate-buffered saline (pH 8.5). Compound **9** was administered as an intravenous bolus at a dose of 1 mg/kg to male Sprague-Dawley rats (n=2 per timepoint). Plasma and brain samples were obtained at 0.083-, 0.25-, 0.5-, 1-, 2-, 4- and 8-h post-dose. At each timepoint, animals were euthanized, blood and brain samples were obtained. Blood samples were transferred to heparinized tubes for preparation of plasma, and brain samples were obtained, rinsed in ice-cold phosphate-buffered saline. Samples were then flash-frozen and stored at -80 ˚C until bioanalysis. Concentration-time data for **9** were analyzed using Phoenix WinNonLin (Certara, Princeton, NJ. USA) to generate pharmacokinetic data for brain and plasma C_{max}, T_{max}, AUC, clearance (CL), volume of distribution (V_d), half-life ($t_{1/2}$) and extrapolated initial plasma and brain concentration (C_0).

Plasma and Brain Binding of 9

The binding of **9** was determined in ex vivo plasma samples from **9**-dosed rats, and rat brain homogenates spiked with **9**. Plasma samples obtained at 0.083-, 0.25- and 0.5-h post dose were pooled for binding studies. Brain homogenates from naïve animals were spiked with 1 μM **9** for binding studies. Antipyrene, sulfamethoxazole and (-)-warfarin were employed as positive controls for low, moderate and high binding to validate the integrity of the binding studies. Plasma and brain homogenates were incubated for 5 h at 37 ˚C in rapid equilibrium dialysis (RED) devices (ThermoFisher Scientific, Waltham, MA. USA) (n=3). At the end of the incubation period, aliquots from the sample and buffer side of the RED devices were analyzed by LC-MS/MS for quantitation of 9 concentrations and calculation of percent bound and free fraction in each tissue matrix.

Bioanalytical Analysis of 9

Concentrations of **9** in rat plasma and brain samples were obtained using a LC-MS/MS bioanalytical method developed for the compound. The method is summarized in Table 1. Briefly, a gradient liquid-chromatography method was employed (Sciex Exion, Sciex, Framingham, MA. USA), following by MS/MS monitoring of the **9** (Q1 and Q3 masses of 387.10 and 266.10 Daltons, respectively, AB Sciex 5500, Sciex, Framingham, MA. USA). Tolbutamide was employed as an internal standard. The lower limits of quantitation of **9** in plasma and brain samples were 1 ng/mL and 1 ng/g, respectively. The respective upper limits of quantitation were 1000 ng/mL and 1000 ng/g.

Table S2.
Summary of bioanalytical methods for quantitation of 9

Bioanalysis Methods		
System Components		
Module	Manufacturer	Model
LC	Sciex	Exion
Autosampler	Sciex	AD Multiplate
MS Detection	AB Sciex	5500 Triple Quad_6
HPLC Method		
Column	Restek Force Biphenyl 1.8 µm (30 x 2.1 mm)	
Elution	Gradient, 0.6 mL/min	
	Mobile Phase A: 0.1% Formic Acid in Water	
	Mobile Phase B: 0.1% Formic Acid in Acetonitrile	
MS Detection and Calibration for MRS7799 in Rat Plasma		
Peak Name: Tolbutamide		
Use as Internal Standard		
Q1/Q3 Masses: 271.00/155.00 Da		
Peak Name: MRS7799		
Internal Standard: Tolbutamide		
Q1/Q3 Masses: 387.10/266.10 Da		
Fit	Quadratic	Weighting 1 / x
A	-0.000000427	
B	0.0173	
C	0.00959	
Correlation coefficient	0.9967	
Use Area		
MS Detection and Calibration for MRS7799 in Rat Brain Homogenate		
Peak Name: Tolbutamide		
Use as Internal Standard		
Q1/Q3 Masses: 271.00/155.00 Da		
Peak Name: MRS7799		
Internal Standard: Tolbutamide		
Q1/Q3 Masses: 387.10/266.10 Da		
Fit	Quadratic	Weighting 1 / x
A	-0.00000228	
B	0.0170	
C	0.00484	
Correlation coefficient	0.9963	
Use Area		

Estimation of Plasma and Brain A_3 Adenosine Receptor Occupancy of 9

Unbound plasma and brain concentrations of compounds can be used to estimate peripheral and central receptor occupancy using receptor affinity data and simple mass action equations.[3,4] Using unbound plasma and brain concentrations of **9** obtained from the rat pharmacokinetic study and the experimentally determined K_i of **9** for the rat A_3 receptor (our value of 1.6 nM, rather than 0.36

nM as determined previously[1]), estimations of peripheral and brain receptor occupancy (%RO) were determined using **Equation 1**:

$$\%RO = \frac{(C)_{ub}}{Ki + (C)_{ub}} * 100$$

where $(C)_{ub}$ is the unbound matrix molar concentration of **9** and K_i is the affinity constant for **9** for rat A_3R.

Table S3. Estimated Brain A_3AR Occupancy.

Dose (mg/kg)	K_i (nM)	F_{ub} Brain	Total [Brain] (nM, Cmax)	Unbound [Brain] (nM, Cmax)	%RO Max	Total [Brain] (nM, Avg)	Unbound [Brain] (nM, Avg)	%RO Avg
1	1.6	0.03	1968	59	97	246	7.4	82
3	1.6	0.03	5907	177	99	738	22	93
10	1.6	0.03	17721	531	100	2215	66	98
30	1.6	0.03	53163	1594	100	6645	199	99

Figure S2. Estimated A_3AR Occupancy as Function of Time 3 mg/kg Intravenous Dose, No Residence Time Assumptions.

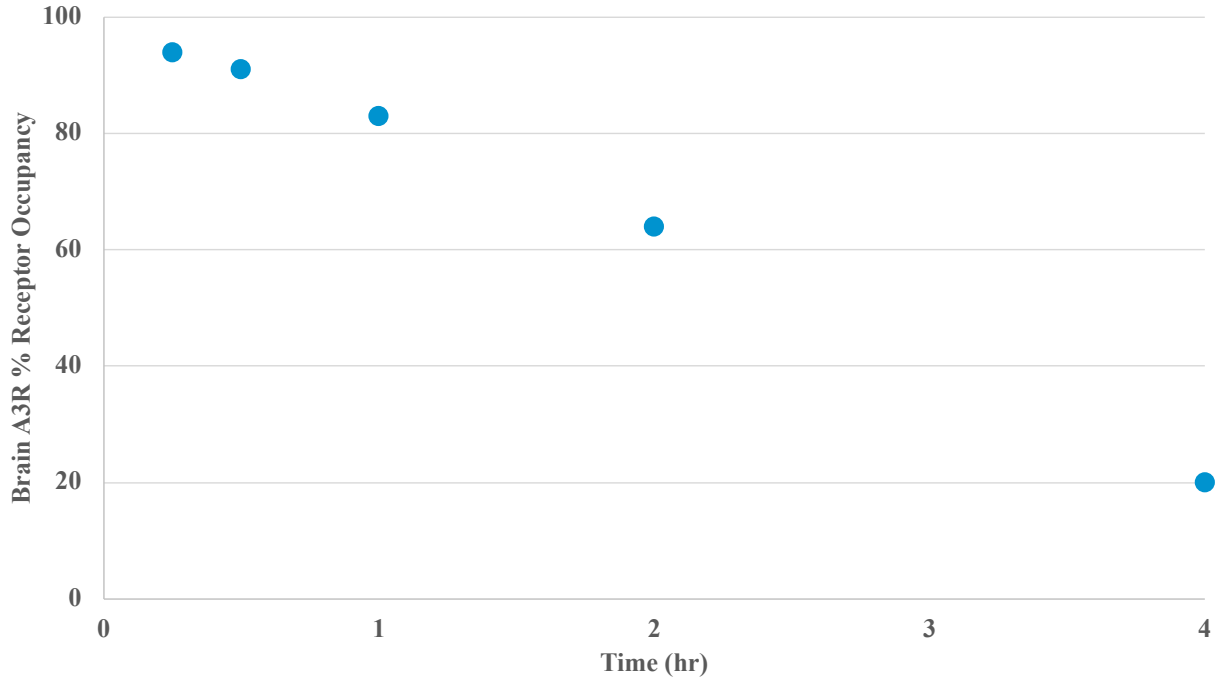
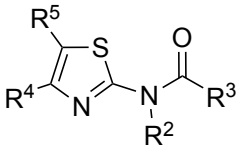
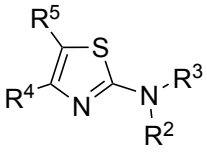


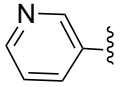
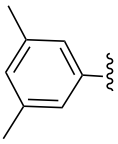
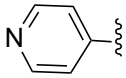
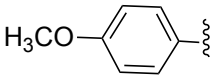
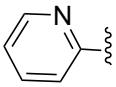
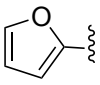
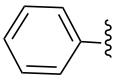
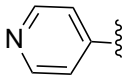
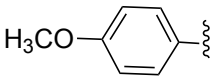
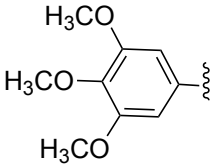
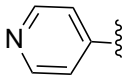
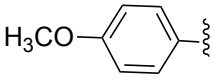
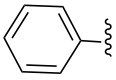
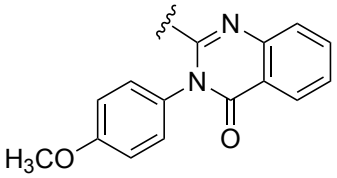
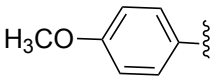
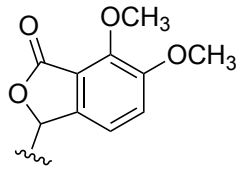
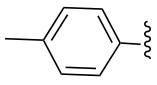
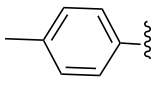
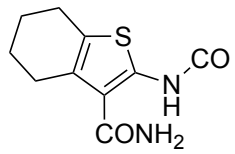
Table S4. Other thiazole-2-amines in the literature that have relatively high A_3AR affinity. R^2 is H.

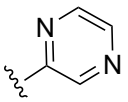
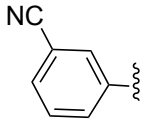
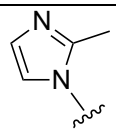


9, 26 – 30



31 – 33

Compound	R^3	R^4	R^5	K_i (hA_3AR, nM) [reference]
9, DPTN, MRS7799				0.36,[1] 1.65[5]
26			H	1.8 [compound **17e**[6]]
27				9.36 [compound **5d**[7]]
28	CH_2CH_3		H	2.4 [compound **20**[8]]
29	CH_3			0.4 [compound **3f**[9]]
30				4.63 [compound **17**[10]]
31, ID-3	H			1300 [library hit, [11]]
32				330 [compound **8n**[9,12]]

33, QAF805[a]				10.2 [various numbers[12-14]]

a. QAF805 **33**, a mixed A_{2B}/A_3AR antagonist, was in a Phase 1 clinical trial for asthma but has since been discontinued.[14]

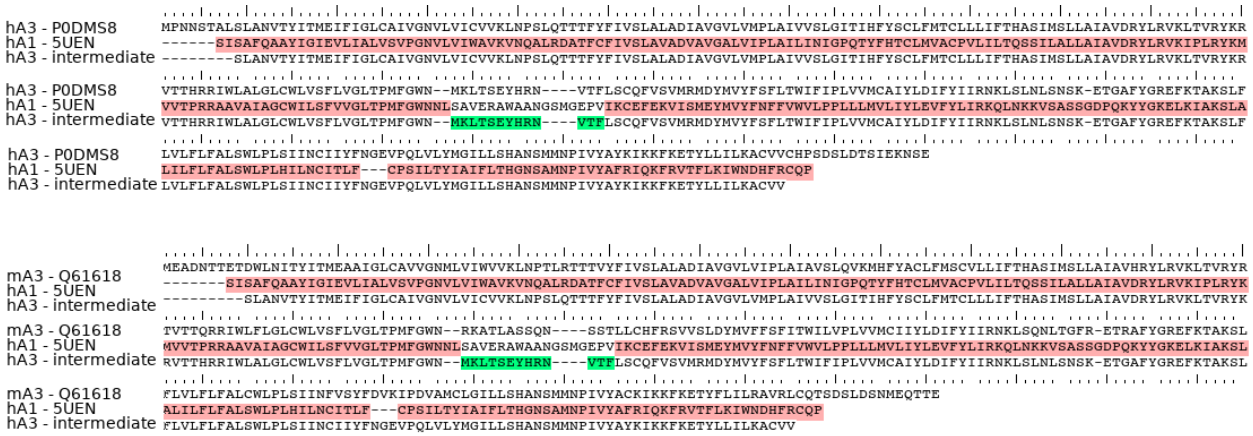
Molecular Modeling Methods

Homology Modeling and Protein Preparation

A model for hA_3AR and mA_3AR was obtained by means of homology modeling using hA_1AR as a template. In particular, the hA_1AR's antagonist-bound X-ray structure with PDB ID 5UEN[15] was employed. The structure was prepared using the Protein Preparation Wizard tool[16] of the Schrödinger suite (Maestro 2021-2[17]), and the missing intracellular loop (IL) 3 was reconstructed with Prime[18,19] and minimized using OPLS4[20] force field and water environment.

The prepared structure was used as a template for homology modeling of hA_3AR and mA_3AR. A previously generated intermediate-state, agonist-bound hA_3AR model[21] was used as a template for extracellular loop (EL) 2 (M151-F163), to be consistent with previous works.[21] In addition, EL3 (P261-K265) was removed from the hA_1AR template, because it is 2 residues longer than A_3AR's sequences. The alignment is reported in the figure below (Figure S3). The first 6 (hA_3AR) and 7 (mA_3AR) N-terminal residues and the last 13 C-terminal residues of both receptors were not modelled.

Figure S3. Sequence alignment of hA_3AR (UNIPROT ID: P0DMS8) to hA_1AR (PDB ID: 5UEN[15]), and of mA_3AR (UNIPROT ID: Q61618) to hA_1AR (PDB ID: 5UEN) and hA_3AR (agonist-bound, intermediate state model[21]). The portions of template sequences that were used for modeling are highlighted.



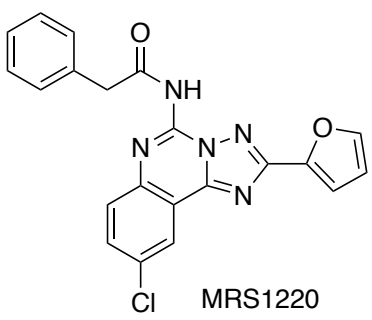
The models were obtained with Prime[18,19] (Schrödinger suite (Maestro 2021-2[17])), and then prepared with the Protein Preparation Wizard tool.[16] The predicted tautomeric state of histidines was HIE (hydrogen at Nε nitrogen) for H79, H95, H124, H158 and H304, and HID (hydrogen at Nδ nitrogen) for H272 in the case of hA_3AR, and HIE for H80, H96 and H108 and HID for H168 and H273 in the case of mA_3AR.

The models were optimized by minimizing the non-conserved residues with the OPLS4 force field and VSGB solvation model. Furthermore, a conformational search of F80 (hA_3AR) and F81 (mA_3AR) (with the Conformational Search tool of the Schrödinger suite (Maestro 2021-2[17])) was included to relieve steric clashes.

The models were finally refined by including an Induced Fit Docking step of known antagonists, i.e. MRS1220 (hA_3AR $K_i \sim 0.7$ nM) and MRS1523 (mA_3AR $K_i \sim 349$ nM) (Figure S4). The Induced Fit Docking[22] tool (Schrödinger suite (Maestro 2021-2[3])) was employed, using a box centered on

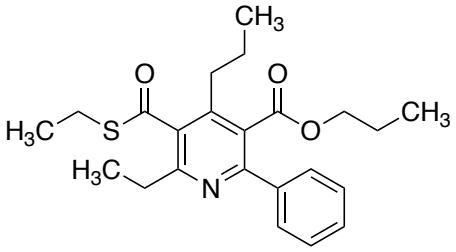
F168 (for hA$_3$AR, and F169 for mA$_3$AR) and N250 (for hA$_3$AR, and N251 mA$_3$AR) and with inner and outer dimensions of 10 Å and 30 Å, and with Glide-XP[23,24,25] scoring function. Residues within 3 Å of the ligands were refined, excluding F168/F169 and N250/N251 (hA$_3$AR/mA$_3$AR). Maximum 20 poses were generated, and the top scoring model was selected for each receptor (Figure S5).

Figure S4. Structures of MRS1220 and MRS1523.



MRS1220

Ki (hA$_3$AR) = 0.65 nM

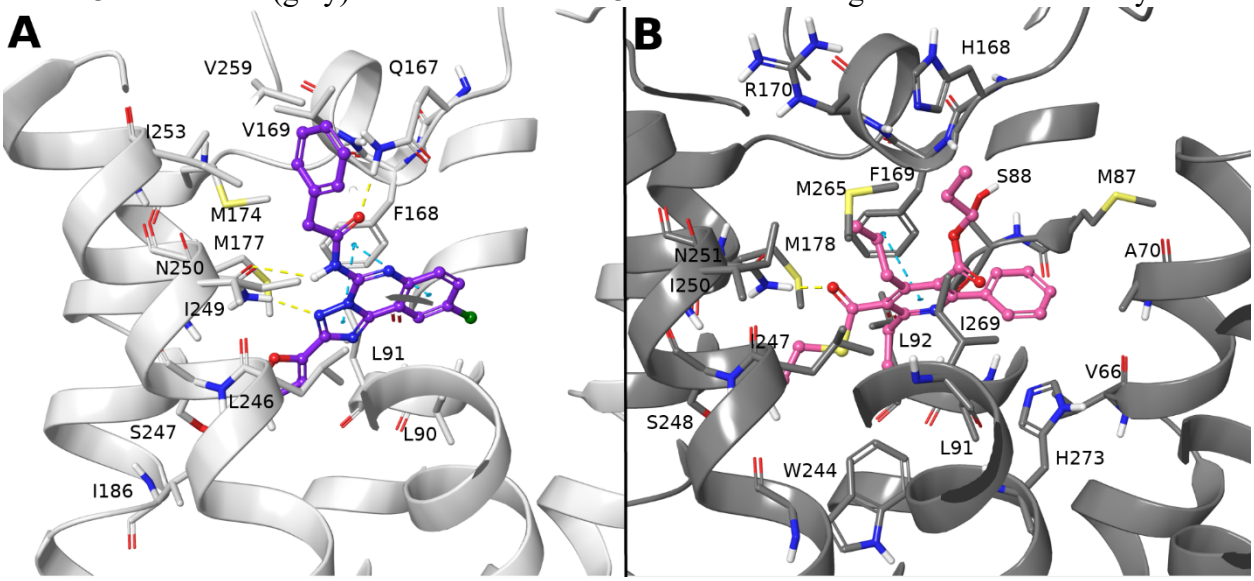


MRS1523

Ki (hA$_3$AR) = 43.9 nM

Ki (mA$_3$AR) = 349 nM

Figure S5. Docking poses of MRS1220 (purple) at hA3AR model (white), and of MRS1523 (pink) at mA3AR model (grey). Residues within 3 Å from the ligand are rendered by sticks.



Molecular Docking

Compounds **9** and **19** were docked to the hA$_3$AR model obtained as described above, using Glide-XP scoring function.[23,24,25] In particular, the Induced Fit Docking[22] tool was employed to take into account the flexibility of Q167/H168 and V169/R170 (hA$_3$AR/mA$_3$AR). In particular, Q167/H168 and V169/R170 were trimmed and selected for optimization, while all the other residues were not refined. A grid with inner and outer boxes of 10 Å and 30 Å and centered on F168/F169 and

N250/N251(hA_3AR/mA_3AR) was used. A maximum of 20 poses was retained for each ligand, and one pose per ligand was selected by visual inspection among the top ranked conformations.

Figure S6. Superposition of the new antagonist-bound/inactive state hA_3AR model (white) to a previously obtained agonist-bound/intermediate state hA_3AR model[21] (cyan).

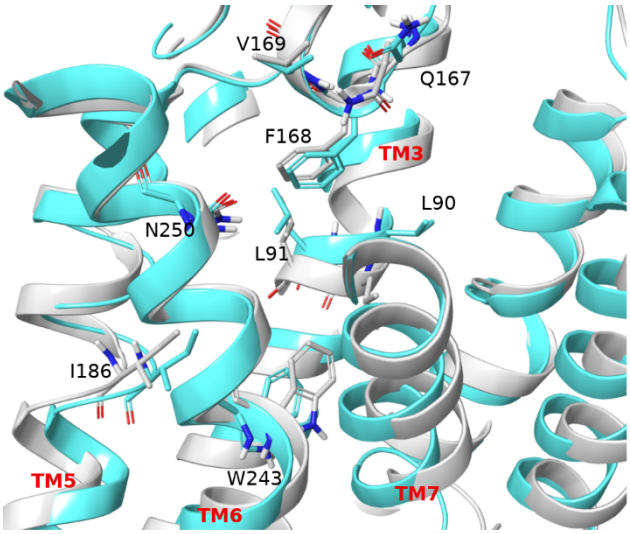
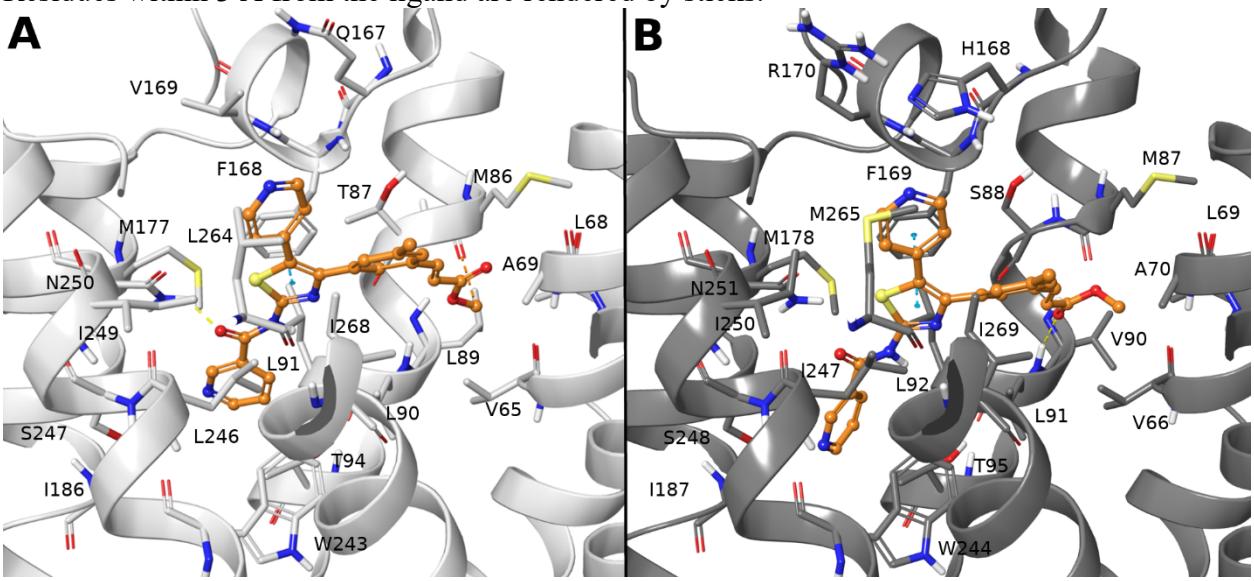


Figure S7. Docking poses of compound **19** (orange) at hA_3AR (A) and mA_3AR (B) models. Residues within 3 Å from the ligand are rendered by sticks.



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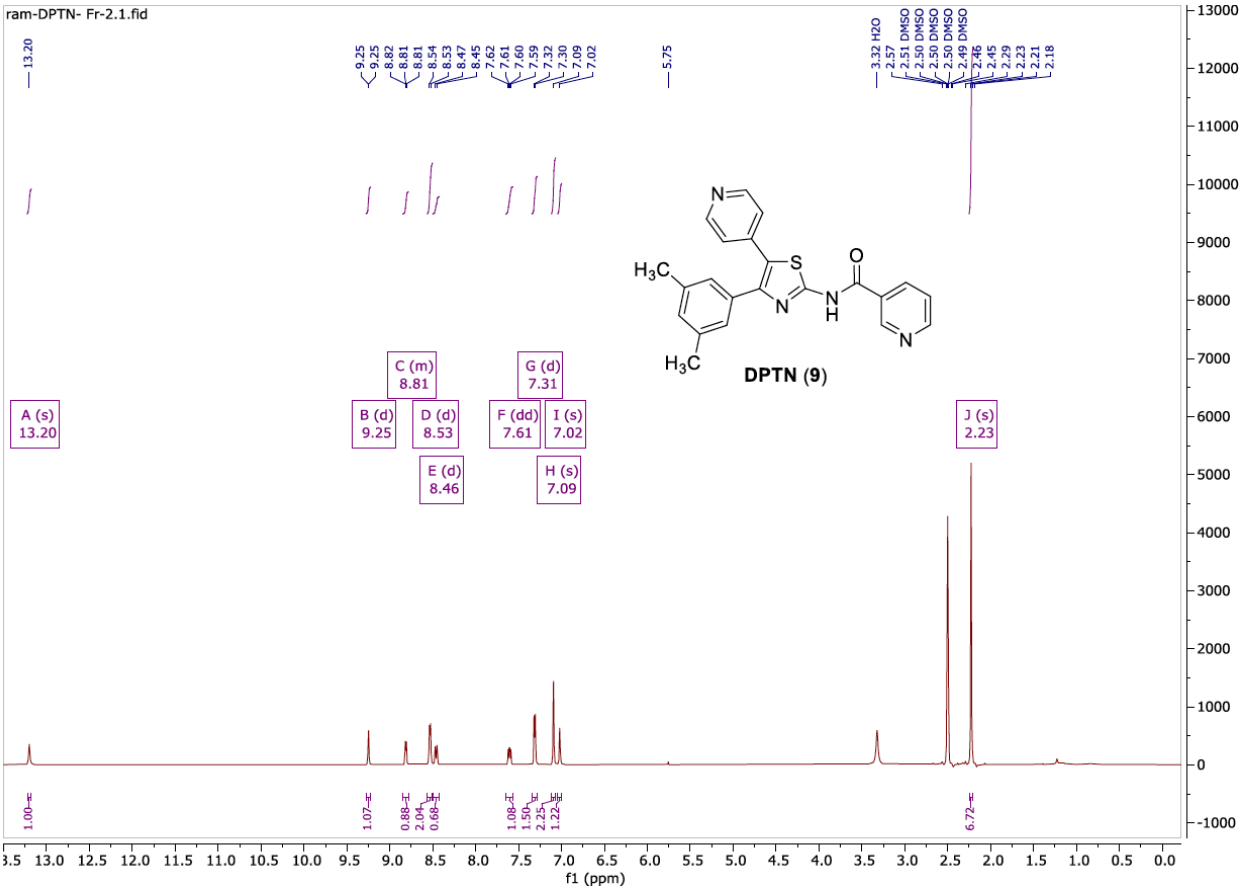
Representative NMR and mass spectra



Elemental Composition Report **Page 1**

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
62 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-200 N: 4-4 O: 0-50 32S: 1-1
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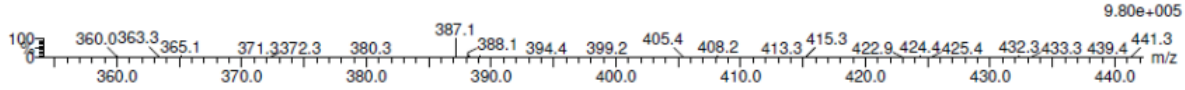
TOF MS ES+



Minimum:				-1.5				
Maximum:		5.0	3.0	100.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
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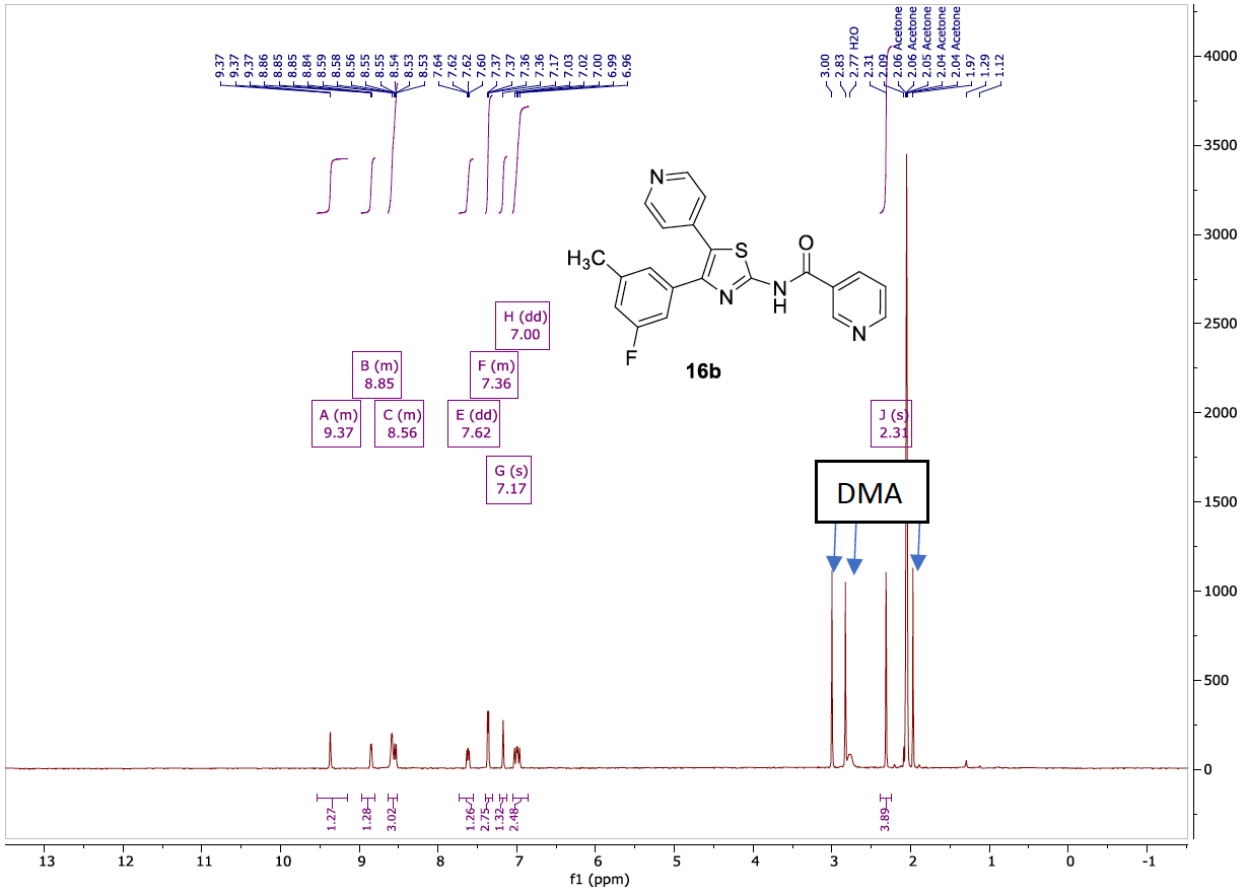

Elemental Composition Report **Page 1**

Single Mass Analysis
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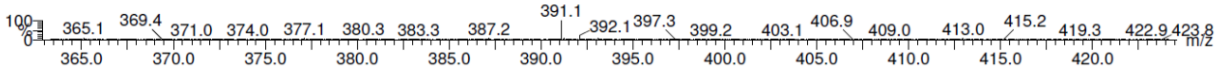
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
54 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-250 N: 4-4 O: 0-50 32S: 1-1 F: 1-1
RAM-04MAY21-458-CC 111 (1.894) AM2 (Ar,25000.0,0.00,0.00); ABS
TOF MS ES+



Minimum:				-1.5				
Maximum:		5.0	5.0	100.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
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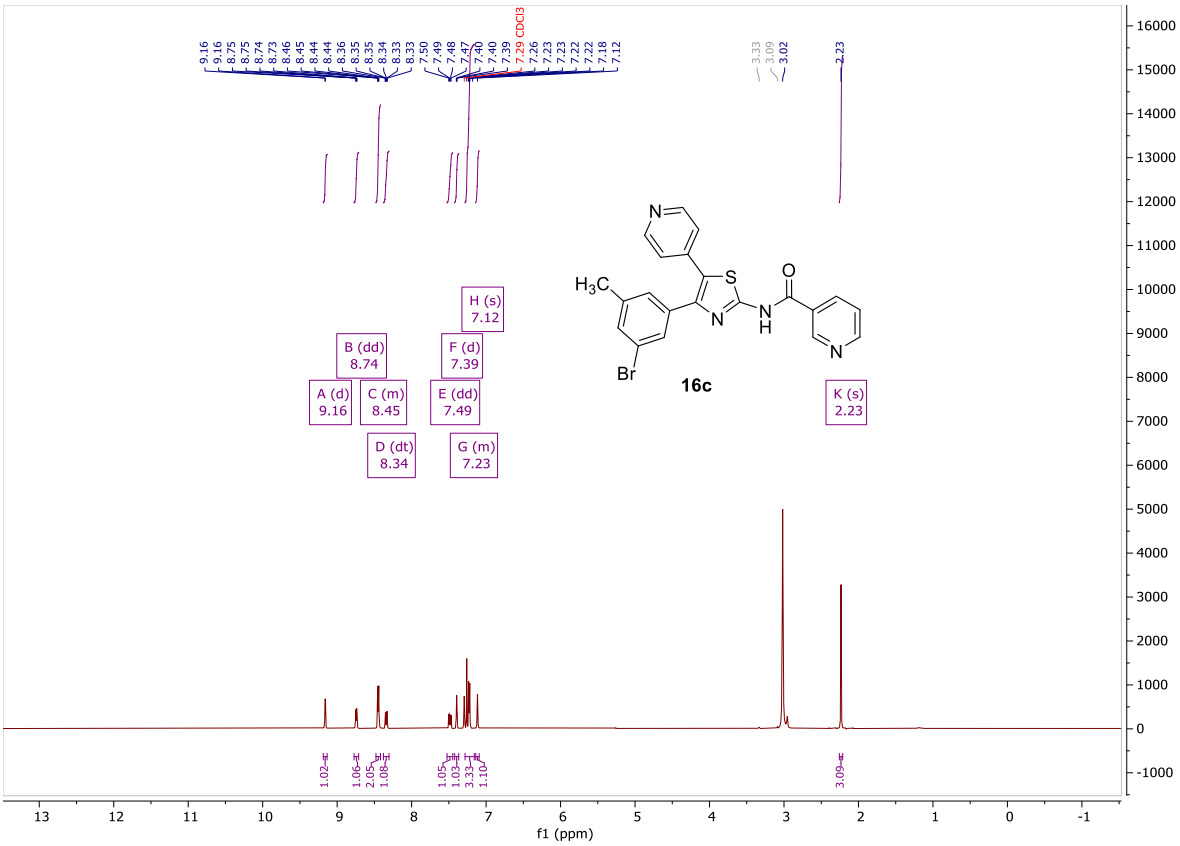


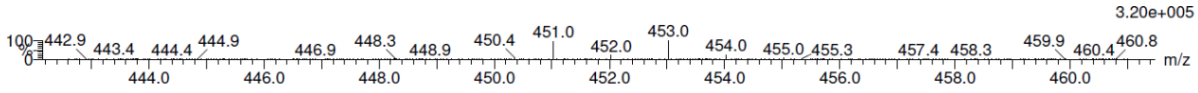
Elemental Composition Report

Single Mass Analysis

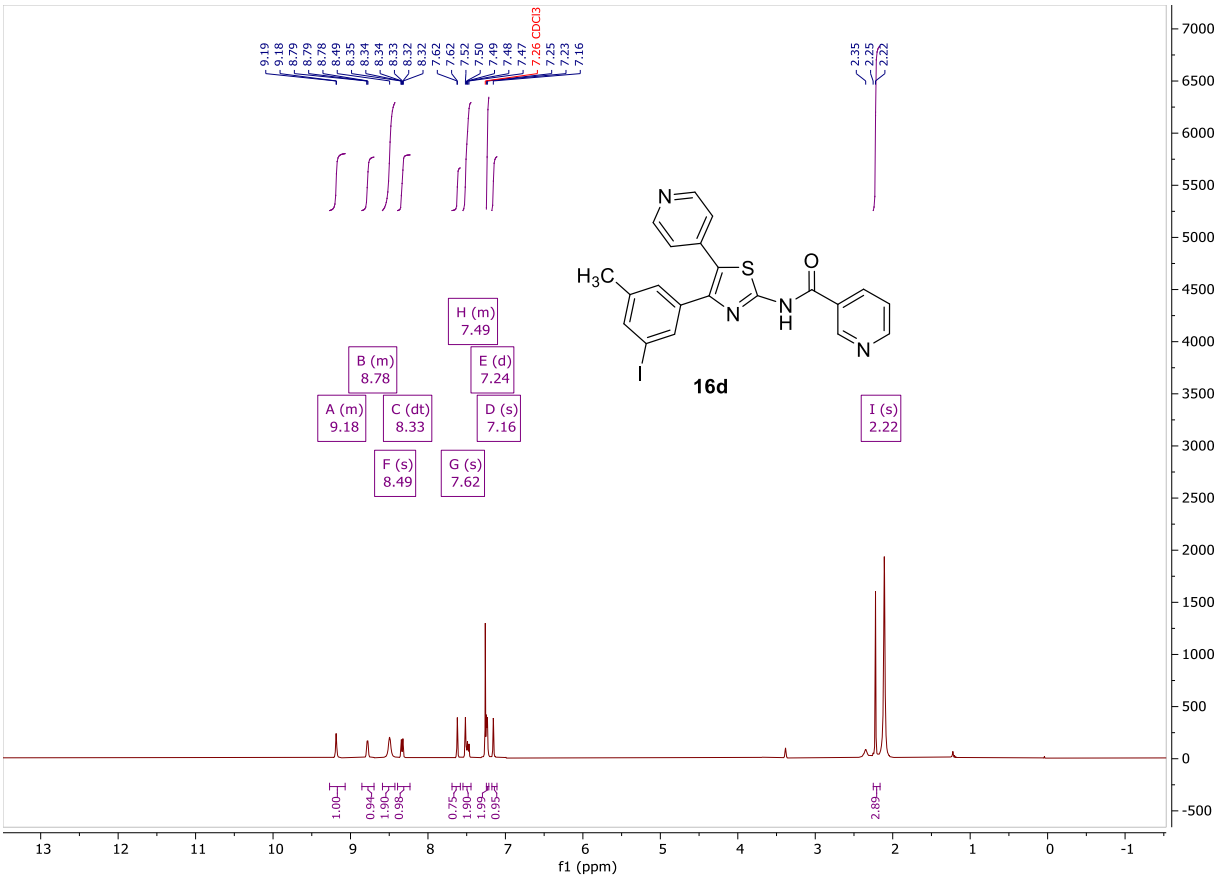
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
54 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-250 N: 4-4 O: 0-60 79Br: 1-1 32S: 1-1

RAM-13FEB20-400-F-HPLC 93 (1.590) AM2 (Ar,25000.0,0.00,0.00); ABS
TOF MS ES+



Minimum:				-1.5				
Maximum:		5.0	5.0	100.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
451.0235	451.0228	0.7	1.6	15.5	417.3	n/a	n/a	C21 H16 N4 O 79Br 32S



Elemental Composition Report

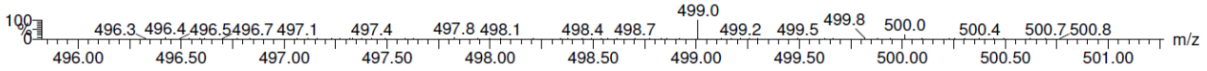
Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

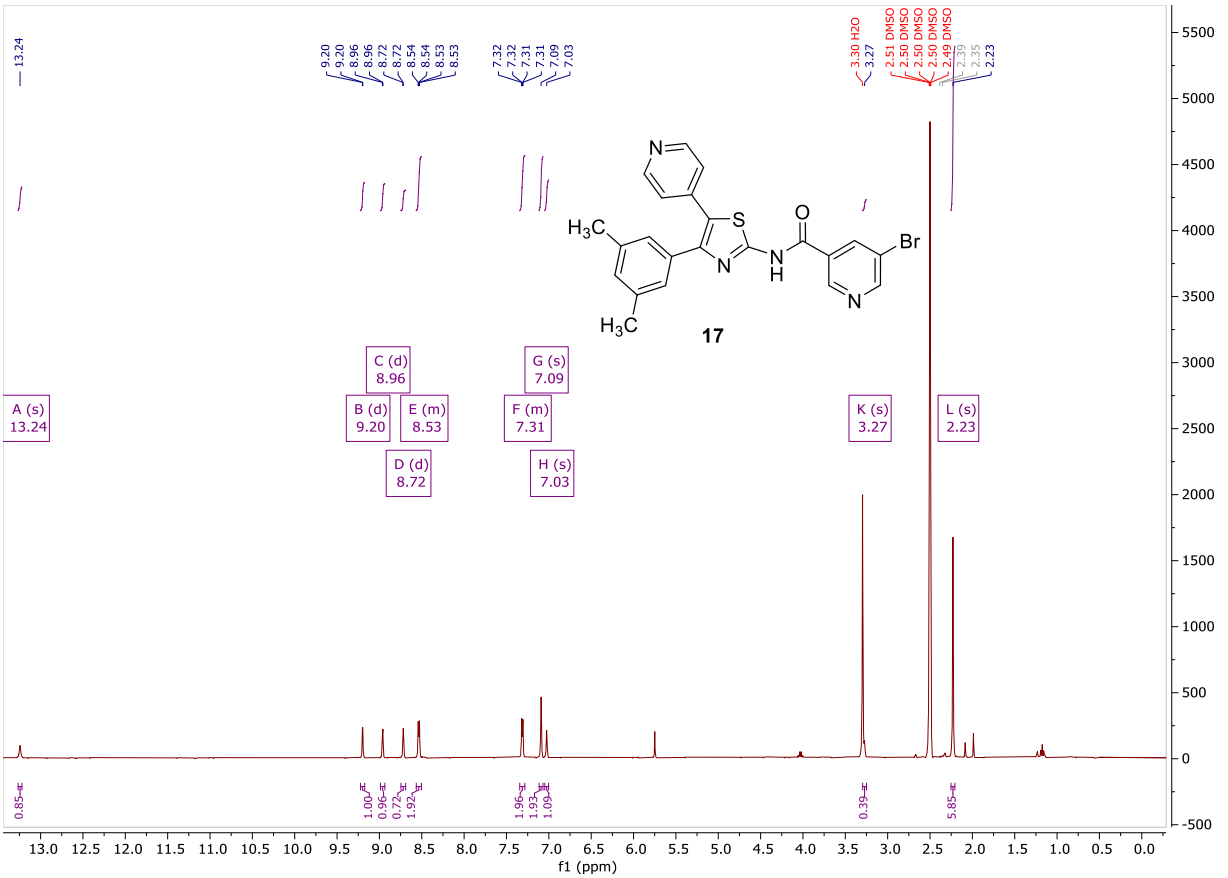
Monoisotopic Mass, Even Electron Ions
54 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-250 N: 4-4 O: 0-50 I: 1-1 32S: 1-1

RAM-01OCT20-415-RM-30MIN-PW 165 (2.808) AM2 (Ar,25000.0,0.00,0.00); ABS
TOF MS ES+

7.16e+005



Minimum:			-1.5				
Maximum:		5.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
499.0092	499.0089	0.3	0.6	15.5	300.9	n/a	n/a	C21 H16 N4 O I 32S



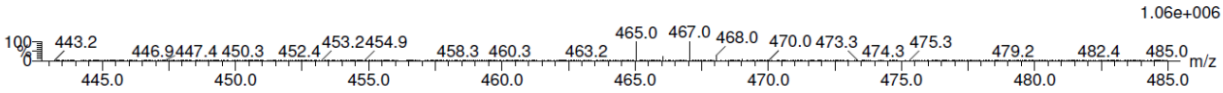
Elemental Composition Report

Single Mass Analysis

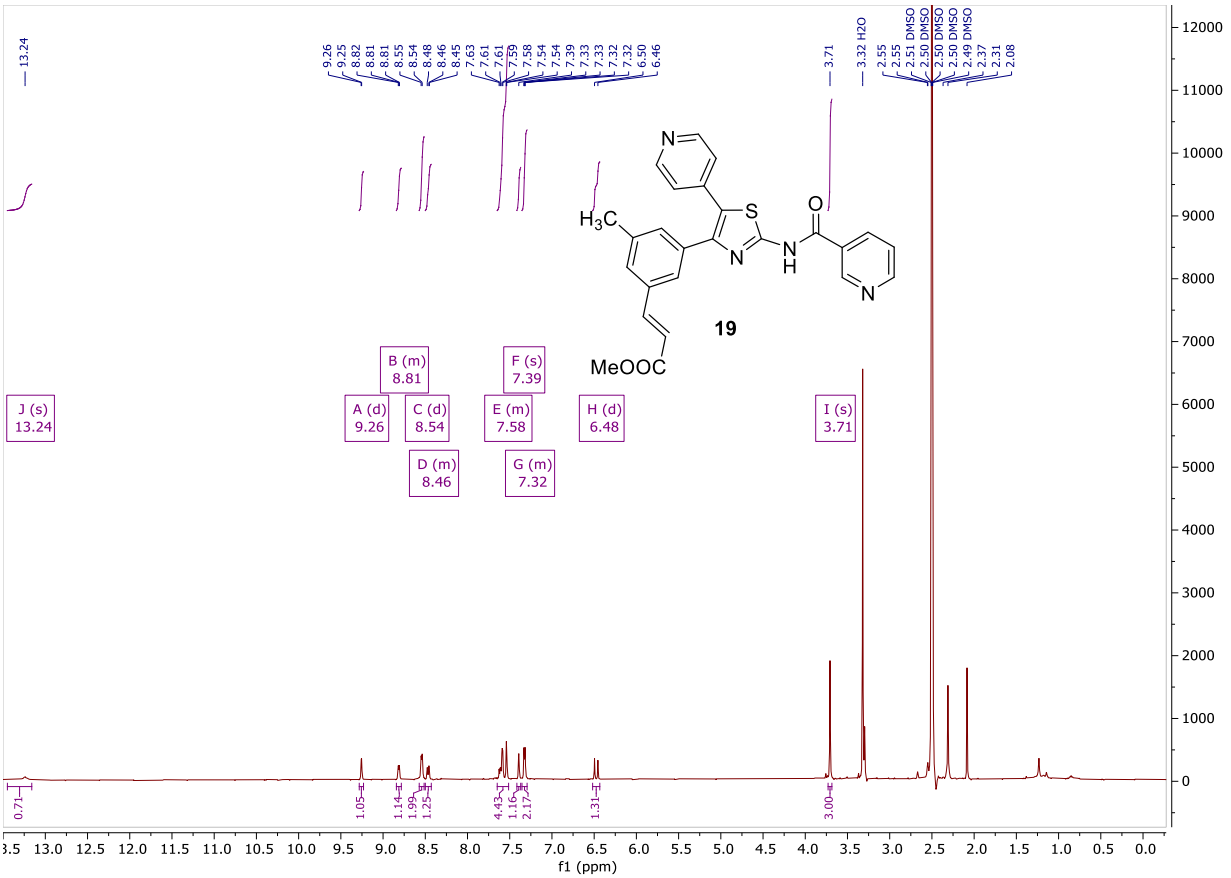
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
55 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-250 N: 4-4 O: 0-50 32S: 1-1 79Br: 1-1

RAM-25MAY21-459-RM-16H 306 (5.193) AM2 (Ar,25000.0,0.00,0.00); ABS
TOF MS ES+



Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%) Formula
465.0390	465.0385	0.5	1.1	15.5	540.4	n/a	n/a C22 H18 N4 O 32S 79Br

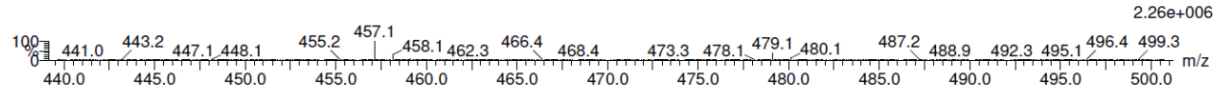


Elemental Composition Report

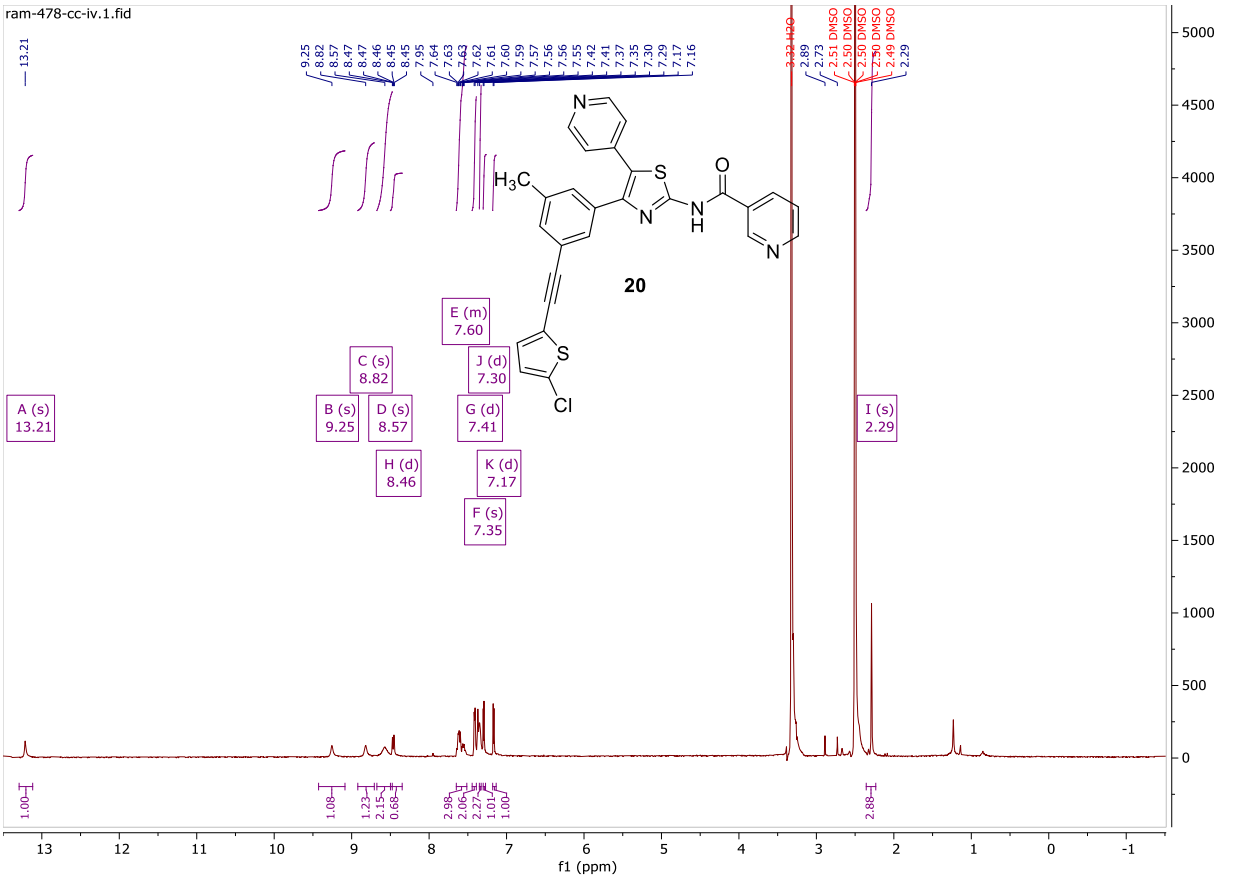
Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
78 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-250 N: 4-4 O: 0-20 32S: 1-1
RAM-08SEP21-479-CC 299 (5.075) AM2 (Ar,25000.0,0.00,0.00); ABS
TOF MS ES+



Minimum:				-1.5			
Maximum:		5.0	3.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
457.1328	457.1334	-0.6	-1.3	17.5	452.7	n/a	n/a	C25 H21 N4 O3 32S



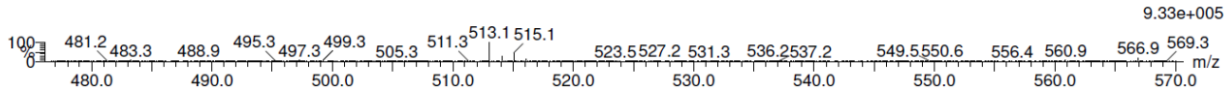
Elemental Composition Report

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
72 formula(e) evaluated with 2 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-250 N: 4-4 O: 0-20 32S: 2-2 35Cl: 1-1

RAM-05OCT21-478-II-CC 178 (3.028) AM2 (Ar,25000.0,0.00,0.00); ABS
TOF MS ES+



Minimum:				-1.5				
Maximum:		5.0	3.0	100.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
513.0608	513.0611	-0.3	-0.6	20.5	388.4	0.778	45.93	C27 H18 N4 O 32S2 35Cl
	513.0575	3.3	6.4	-1.5	388.3	0.615	54.07	C9 H26 N4 O14 32S2 35Cl