Supporting Information

A SAR Study of the Positive Allosteric Modulator LY2033298 at the M_4 Muscarinic Acetylcholine Receptor

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Experimental:

Chemistry. All solvents and chemicals were purchased from standard suppliers and were used without any further purification. ^{1}H NMR and ^{13}C NMR spectra were acquired at 400.13 and 100.62 MHz respectively, on a Bruker Advance III 400 MHz UltrashieldPlus NMR spectrometer using TOPSPIN 2.1 software. Chemical shifts (δ) for all ^{1}H spectra are reported in parts per million (ppm) using tetramethylsilane (TMS, 0 ppm) as the reference. The data for all spectra are reported in the following format: chemical shift (δ), (multiplicity, coupling constants *J* (Hz), integral), where the multiplicity is defined as: s = singlet, d= doublet, t= triplet, q=quartet, p = pentet and m = multiplet. For ^{13}C NMR spectra C= quaternary carbon, CH= methine carbon, CH_2= methylene carbon, and CH_3= methyl carbon.

The purity and retention time of final products was determined on an analytical reverse-phase HPLC system fitted with a Luna C8 (2) 100 Å column (50 × 4.60 mm, 5 μm) using a binary solvent system; solvent A: 0.1% TFA/H_2O; solvent B: 0.1% TFA/80%MeOH/20%H_2O. Gradient elution was achieved using 100% A for 10 minutes, 20% A and 80% B over 2 minutes and 100% A over 10 minutes at a flow rate of 1 mL/min monitored at 214 nm using a Waters 996 Photodiode Array detector.

Thin layer chromatography (TLC) was carried out routinely on silica gel 60F_{254} pre-coated plates (0.25 mm, Merck).Flash column chromatography was carried out using Merck Silica gel 60, 230-400 mesh ASTM. Melting points were determined using an electronic MP50 Melting Point System by Mettler Toledo analytical 2009 and are uncorrected.

General procedure for formation of the thienopyridine bicycle: To a solution of **2** (1 equiv.) in *N*,*N*-dimethylformamide (15 mL) was added potassium carbonate (1.5 equiv.) and the required alkyl iodide (1.1 equiv). After stirring at room temperature for 1-2 h, 1 M aqueous potassium hydroxide (1 equiv.) was added dropwise. Following an additional 15 mins, 10-15 mL of water was added to cause precipitation and the product filtered and dried over high vacuum.

Ethyl 3-amino-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (3a).[1] White solid (63 mg, 79%). ^{1}H NMR ($CDCl_3$): δ 1.38 (t, *J* 7.1 Hz, 3H), 2.68 (d, *J* 0.9 Hz, 3H), 3.98 (s, 3H), 4.33 (q, *J* 7.1 Hz, 2H), 6.11 (br s, 2H, NH_2), 6.43 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 14.7 (CH_3), 20.4 (CH_3), 54.0 (CH_3), 60.4 (CH_2), 95.2 (C), 110.0 (CH), 119.4 (C), 145.9 (C), 149.5 (C), 160.6 (C),164.6 (C), 165.9 (C).

Ethyl 3-amino-6-ethoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (3b). Fluffy white solid (521 mg, 94%). ^{1}H NMR ($CDCl_3$): δ 1.36-1.41 (q, *J* 7.1 Hz, 6H), 2.68 (d, *J* 0.9 Hz, 3H), 4.33 (q, *J* 7.1 Hz, 2H), 4.22 (q, *J* 7.1 Hz, 2H), 6.12 (br s, 2H, NH_2), 6.42 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 14.7 (CH_3), 14.7 (CH_3), 20.4 (CH_3), 60.4 (CH_2), 62.6 (CH_2), 95.1 (C), 110.2 (CH), 119.3 (C), 146.0 (C), 149.5 (C), 160.4 (C), 164.2 (C), 165.9 (C).

Ethyl 3-amino-6-butoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (3c). White solid (535 mg, 88%). ^{1}H NMR ($CDCl_3$): δ 0.98 (t, *J* 7.4 Hz, 3H), 1.37 (t, *J* 7.1 Hz, 3H) 1.47 (m, 2H), 1.76 (m, 2H), 2.68 (s, 3H), 4.30-4.37 (m, 4H), 6.12 (br s, 2H, NH_2), 6.43 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 14.0 (CH_3), 14.7 (CH_3), 19.4 (CH_2), 20.4 (CH_3), 31.1 (CH_2), 60.3 (CH_2), 66.6 (CH_2), 95.1 (C), 110.2 (CH), 119.3 (C), 145.9 (C), 149.5 (C), 160.4 (C), 164.2 (C), 165.9 (C).

Ethyl 3-amino-6-(hexyloxy)-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (3d). White solid (462 mg, 71%). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, *J* 7.0 Hz, 3H), 1.32-1.36 (m, 4H), 1.37 (t, *J* 7.0 Hz, 3H), 1.41-1.48 (m, 2H), 1.76 (m, 2H), 2.67 (d, *J* 0.7 Hz, 3H), 4.30-4.36 (m, 4H), 6.11 (br s, 2H, NH_2), 6.42 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 14.2 (CH_3), 14.7 (CH_3), 20.4 (CH_3), 22.7 (CH_2), 25.9 (CH_2), 29.1 (CH_2), 31.7 (CH_2), 60.3 (CH_2), 66.8 (CH_2), 95.1 (C), 110.2 (CH), 119.3 (C), 145.8 (C), 149.6 (C), 160.7 (C), 164.6 (C), 165.9 (C).

General procedure for phthalimide protection: To a solution of **3a-d** (1 equiv.) in acetic acid (15 mL), was added phthalic anhydride (2 equiv.), and the mixture was heated at reflux for 1.5 days. The reaction was cooled to room temperature, then placed in an ice bath to initiate precipitation of the final product as a white solid.

Ethyl 3-(1,3-dioxoisoindolin-2-yl)-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (4a). White solid (53 mg, 55%). ^{1}H NMR ($CDCl_3$): δ 1.11 (t, *J* 7.1 Hz, 3H), 2.37 (s, 3H), 4.02 (s, 3H), 4.20 (q, *J* 7.1 Hz, 2H), 6.59 (s, 1H), 7.83 (m, 2H), 8.00 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 18.5 (CH_3), 54.2 (CH_3), 61.7 (CH_2), 112.2 (CH), 124.2 (CH), 124.7 (C), 125.4 (C), 127.6 (C), 132.4 (C), 134.7 (CH), 145.9 (C), 159.1 (C), 161.1 (C), 164.5 (C), 167.6 (C).

Ethyl 3-(1,3-dioxoisoindolin-2-yl)-6-ethoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (4b). White solid (477 mg, 65%). ^{1}H NMR ($CDCl_3$): δ 1.11 (t, *J* 7.1 Hz, 3H), 1.42 (t, *J* 7.1 Hz, 3H), 2.36 (d, *J* 0.8 Hz, 3H), 4.20 (q, *J* 7.1 Hz, 2H), 4.47 (q, *J* 7.1 Hz, 2H), 6.57 (d, *J* 0.9 Hz, 1H), 7.84 (m, 2H), 8.00 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 14.6 (CH_3), 18.5 (CH_3), 61.7 (CH_2), 62.7 (CH_2), 112.4 (CH), 124.2 (CH), 124.6 (C), 125.2 (C), 127.7 (C), 132.5 (C), 134.7 (CH), 145.8 (C), 159.3 (C), 161.2 (C), 164.2 (C), 167.6 (C).

Ethyl 6-butoxy-3-(1,3-dioxoisoindolin-2-yl)-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (4c). White solid (613 mg, 86%). ^{1}H NMR ($CDCl_3$): δ 0.99 (t, *J* 7.4 Hz, 3H), 1.11 (t, *J* 7.1 Hz, 3H), 1.49 (m, 2H), 1.78 (m, 2H), 2.36 (d, *J* 0.9 Hz, 3H), 4.20 (q, *J* 7.1 Hz, 2H), 4.41 (t, *J* 6.6 Hz, 2H), 6.57 (m, 1H), 7.84 (m, 2H), 8.00 (m, 2H). ^{13}C NMR (DMSO- d_6): δ 13.5 (CH_3), 13.7 (CH_3), 17.6 (CH_3), 18.7 (CH_2), 30.4 (CH_2), 61.8 (CH_2), 66.3 (CH_2), 112.4 (CH), 124.0 (C), 124.1 (C), 124.2 (CH), 127.9 (C), 131.4 (C), 135.6 (CH), 146.5 (C), 157.7 (C), 160.3 (C), 164.0 (C), 167.1 (C).

Ethyl 3-(1,3-dioxoisoindolin-2-yl)-6-(hexyloxy)-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (4d). White solid (266 mg, 48%). ^{1}H NMR ($CDCl_3$): δ 0.91 (m, 3H), 1.11 (t, *J* 7.1 Hz, 3H), 1.36 (m, 4H), 1.46 (m, 2H), 1.79 (m, 2H), 2.36 (d, *J* 0.9 Hz, 3H), 4.20 (q, *J* 7.1 Hz, 2H), 4.40 (t, *J* 6.7 Hz, 2H), 6.57 (m, 1H), 7.83 (m, 2H), 8.00 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 14.2 (CH_3), 18.5 (CH_3), 22.8 (CH_2), 25.8 (CH_2), 29.0 (CH_2), 31.7 (CH_2), 61.7 (CH_2), 67 (CH_2), 112.4 (CH), 124.2 (CH), 124.6 (C), 125.2 (C), 127.7 (C), 132.5 (C), 134.7 (CH), 145.8 (C), 159.3 (C), 161.2 (C), 164.4 (C), 167.6 (C).

General procedure for halogenation at the 5′ position. Intermediate **4a-d** (1equiv.) was added to acetonitrile (15-25 mL) and warmed until completely dissolved. The required *N*-halogensuccinimide (2 equiv.) and 2-3 drops of concentrated HCl were added and the reaction mixture heated at reflux for 1.5–4 h under N_2. The reaction mixture was then diluted with chloroform (50 mL) and washed with water (3 × 20 mL), dried over anhydrous sodium sulfate, filtered and evaporated to dryness.

Ethyl-5-chloro-3-(1,3-dioxoisoindolin-2-yl)-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (5a). White solid (4.28 g, 86%). ^{1}H NMR ($CDCl_3$): δ 1.12 (t, *J* 7.1 Hz, 3H), 2.49 (s, 3H), 4.13 (s, 3H), 4.21 (q, *J* 7.1 Hz, 2H), 7.85 (m, 2H), 8.01 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 14.7 (CH_3), 55.4 (CH_3), 61.9 (CH_2), 118.8 (C), 124.3 (CH), 125.1 (C), 127.0 (C), 127.4 (C), 132.4 (C), 134.9 (CH), 143.0 (C), 155.9 (C), 159.5 (C), 160.9 (C), 167.6 (C).

Ethyl 5-chloro-3-(1,3-dioxoisoindolin-2-yl)-6-ethoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (5b).

White solid (386 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 1.12 (t, *J* 7.1 Hz, 3H), 1.49 (t, *J* 7.1 Hz, 3H), 2.48 (s, 3H), 4.21 (q, *J* 7.1 Hz, 2H), 4.57 (q, *J* 7.1 Hz, 2H), 7.85 (m, 2H), 8.01 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 14.6 (CH_3), 14.8 (CH_3), 61.9 (CH_2), 64.2 (CH_2), 118.9 (C), 124.3 (CH), 124.9 (C), 126.7 (C), 127.4 (C), 132.4 (C), 134.9 (CH), 142.8 (C), 156.0 (C), 159.2 (C), 160.9 (C), 167.6 (C).

Ethyl 6-butoxy-5-chloro-3-(1,3-dioxoisoindolin-2-yl)-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (5c). Yellow foam (650 mg, 99%). ^{1}H NMR ($CDCl_3$): δ 1.01 (t, *J* 7.4 Hz, 3H), 1.12 (t, *J* 7.1 Hz, 3H), 1.53 (m, 2H), 1.85 (m, 2H), 2.48 (s, 3H), 4.21 (q, *J* 7.1 Hz, 2H), 4.50 (t, *J* 6.6 Hz, 2H), 7.85 (m, 2H), 8.01 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 14.0 (CH_3), 14.7 (CH_3), 19.4 (CH_2), 30.9 (CH_2), 61.9 (CH_2), 68.1 (CH_2), 119.0 (C), 124.3 (CH), 124.8 (C), 126.7 (C), 127.4 (C), 132.4 (C), 134.9 (CH), 142.8 (C), 156.0 (C), 159.3 (C), 160.9 (C), 167.6 (C).

Ethyl 5-chloro-3-(1,3-dioxoisoindolin-2-yl)-6-(hexyloxy)-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (5d). Colourless oil (274 mg, 99%). ^{1}H NMR ($CDCl_3$): δ 0.91 (m, 3H), 1.12 (t, *J* 7.1 Hz, 3H), 1.37 (m, 4H), 1.50 (m, 2H), 1.86 (m, 2H), 2.49 (s, 3H), 4.21 (q, *J* 7.1 Hz, 2H), 4.49 (t, *J* 6.7 Hz, 2H), 7.85 (m, 2H), 8.01 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 14.1 (CH_3), 14.7 (CH_3), 22.7 (CH_2), 25.8 (CH_2), 28.8 (CH_2), 31.6 (CH_2), 61.9 (CH_2), 68.3 (CH_2), 119.0 (C), 124.3 (CH), 124.8 (C), 126.7 (C), 127.4 (C), 132.4 (C), 134.8 (CH), 142.8 (C), 156.0 (C), 159.3 (C), 160.9 (C), 167.5 (C).

Ethyl 5-bromo-3-(1,3-dioxoisoindolin-2-yl)-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (5e). White solid (289 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 1.12 (t, *J* 7.1 Hz, 3H), 2.53 (s, 3H), 4.12 (s, 3H), 4.21 (q, *J* 7.1 Hz, 2H), 7.85 (m, 2H), 8.01 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 17.9 (CH_3), 55.6 (CH_2), 61.9 (CH_3), 110.5 (C), 124.3 (CH), 125.3 (C), 126.9 (C), 127.3 (C), 132.4 (C), 134.9 (CH), 145.3 (C), 156.9 (C), 160.1 (C), 160.9 (C), 167.5 (C).

Ethyl 3-(1,3-dioxoisoindolin-2-yl)-5-iodo-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (5f). Pale yellow solid (1.11 g, 84%). ^{1}H NMR ($CDCl_3$): δ 1.12 (t, *J* 7.1 Hz, 3H), 2.59 (s, 3H), 4.10 (s, 3H), 4.21 (q, *J* 7.1 Hz, 2H), 7.85 (m, 2H), 8.01 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 14.0 (CH_3), 24.0 (CH_3), 55.9 (CH_3), 61.9 (CH_2), 88.9 (C), 124.3 (CH), 125.1 (C), 126.5 (C), 127.1 (C), 132.4 (C), 134.9 (CH), 149.5 (C), 158.4 (C), 160.9 (C), 161.7 (C), 167.5 (C).

General procedure for phthalamide deprotection. To a solution of intermediate **5a-f** (1equiv.) in ethanol (15-20 mL) was added dropwise hydrazine monohydrate (4 equiv.), and the mixture was heated at reflux for 1h. After this time, the reaction mixture was cooled to room temperature and the white precipitate filtered and washed with chloroform (10 mL). The filtrate was collected and diluted with a further 20 mL of chloroform and washed with water (3 × 20 mL), dried over anhydrous sodium sulfate, filtered and evaporated to dryness.

Ethyl 3-amino-5-chloro-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (6a). Pale yellow solid (85 mg, 84%). ^{1}H NMR ($CDCl_3$): δ 1.38 (t, *J* 7.1 Hz, 3H), 2.83 (s, 3H), 4.08 (s, 3H), 4.33 (q, *J* 7.1 Hz, 2H), 6.14 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 14.6 (CH_3), 16.4 (CH_3), 55.2 (CH_3), 60.6 (CH_2), 116.3 (C), 120.1 (C), 142.9 (C), 149.1 (C), 157.3 (C), 159.5 (C), 165.7 (C), 168.4 (C).

Ethyl 3-amino-5-chloro-6-ethoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (6b). Yellow solid (234 mg, 89%). ^{1}H NMR ($CDCl_3$): δ 1.38 (t, *J* 7.1 Hz, 3H), 1.46 (2.83 t, *J* 7.1 Hz, 3H), 2.82 (s, 3H), 4.33 (q, *J* 7.1 Hz, 2H), 4.51 (q, *J* 7.1 Hz, 2H), 6.14 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 14.6 (2 × CH_3), 16.4 (CH_3), 60.5 (CH_2), 63.9 (CH_2), 96.6 (C), 116.4 (C), 119.9 (C), 142.8 (C), 149.1 (C), 157.3 (C), 159.2 (C), 165.7 (C).

Ethyl 3-amino-6-butoxy-5-chloro-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (6c). Yellow solid (215 mg, 46%). ^{1}H NMR ($CDCl_3$): δ 0.99 (t, *J* 7.4 Hz, 3H), 1.37 (2.83 t, *J* 7.1 Hz, 3H), 1.51 (m, 2H), 1.81 (m, 2H), 2.82 (s, 3H), 4.33 (q, *J* 7.1 Hz, 2H), 4.44 (t, *J* 6.6 Hz, 2H), 6.13 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 14.0 (CH_3), 14.6 (CH_3), 16.4 (CH_3), 19.4 (CH_2), 31.0 (CH_2), 60.5 (CH_2), 67.8 (CH_2), 96.6 (C), 116.5 (C), 119.9 (C), 142.7 (C), 149.1 (C), 157.4 (C), 159.4 (C), 165.7 (C).

Ethyl 3-amino-5-chloro-6-(hexyloxy)-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (6d). Yellow solid (173 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 0.91 (m, 3H), 1.35 (m, 4H), 1.37 (t, *J* 7.1 Hz, 3H), 1.48 (m, 2H), 1.83 (m, 2H), 2.81 (s, 3H), 4.33 (q, *J* 7.1 Hz, 2H), 4.43 (t, *J* 6.7 Hz, 2H), 6.13 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 14.2 (CH_3), 14.6 (CH_3), 16.4 (CH_3), 22.7 (CH_2), 25.8 (CH_2), 28.9 (CH_2), 31.7 (CH_2), 60.5 (CH_2), 68.1 (CH_2), 96.6 (C), 116.5 (C), 119.9 (C), 142.7 (C), 149.1 (C), 157.4 (C), 159.4 (C), 165.7 (C).

Ethyl 3-amino-5-bromo-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (6e). Fluffy beige solid (123 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 1.38 (t, *J* 7.1 Hz, 3H), 2.87 (s, 3H), 4.74 (s, 3H), 4.33 (q, *J* 7.1 Hz, 2H), 6.07 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 14.6 (CH_3), 19.8 (CH_3), 55.4 (CH_3), 60.6 (CH_2), 96.7 (C), 107.8 (C), 120.5 (C), 145.2 (C), 149.0 (C), 158.3 (C), 160.1 (C), 165.7 (C).

Ethyl 3-amino-5-iodo-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (6f). Pale yellow solid (336 mg, 89%). ^{1}H NMR ($CDCl_3$): δ 1.38 (t, *J* 7.1 Hz, 3H), 2.92 (s, 3H), 4.04 (s, 3H), 4.33 (q, *J* 7.1 Hz, 2H), 5.93 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 14.6 (CH_3), 25.9 (CH_3), 55.7 (CH_3), 60.6 (CH_2), 85.8 (C), 96.3 (C), 120.4 (C), 148.8 (C), 149.4 (C), 159.8 (C), 161.9 (C), 165.7 (C).

General procedure for ester hydrolysis. Compounds **6a-f** (1 equiv) was added to ethanol (10 mL) and 2 M NaOH (10 mL) and heated at reflux for 1.5 h. The reaction mixture was then cooled to room temperature and an excess of 2 M HCl was added causing precipitation of the product. The product was then filtered and washed with a small amount of cold water.

3-Amino-5-chloro-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylic acid (7a). Beige-yellow solid (179 mg, 84%). ^{1}H NMR (DMSO- d_6): δ 2.77 (s, 3H), 3.96 (s, 3H), 6.41 (br s, 2H, NH_2). LCMS (ESI) *m/z*: 273.0 $[M+H]^+$ (90%), 275.0 (30%).

3-Amino-5-chloro-6-ethoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylic acid (7b). Beige-yellow solid (193 mg, 91%). ^{1}H NMR (DMSO- d_6): δ 1.37 (t, *J* 7.1 Hz, 3H), 2.79 (s, 3H), 4.43 (q, *J* 7.0 Hz, 2H), 6.76 (br s, 2H, NH_2). ^{13}C NMR (DMSO- d_6): δ 14.3 (CH_3), 16.3 (CH_3), 63.4 (CH_2), 94.9 (C), 114.9 (C), 120.1 (C), 144.4 (C), 149.6 (C), 156.0 (C), 158.3 (C), 166.2 (C).

3-Amino-6-butoxy-5-chloro-4-methylthieno[2,3-*b*]pyridine-2-carboxylic acid (7c). Yellow solid (91 mg, 49%). ^{1}H NMR (DMSO- d_6): δ 0.94 (t, *J* 7.4 Hz, 3H), 1.45 (m, 2H), 1.73 (m, 2H) 2.79 (s, 3H), 4.38 (t, *J* 6.5 Hz, 2H), 6.75 (br s, 2H, NH_2). ^{13}C NMR (DMSO- d_6): δ 13.7 (CH_3), 16.3 (CH_3), 18.7 (CH_2), 30.3 (CH_2), 67.2 (CH_2), 94.9 (C), 115.0 (C), 120.1 (C), 144.4 (C), 149.6 (C), 156.0 (C), 158.5 (C), 166.2 (C).

3-Amino-5-chloro-6-(hexyloxy)-4-methylthieno[2,3-*b*]pyridine-2-carboxylic acid (7d). Beige solid (113 mg, 81%). ^{1}H NMR (DMSO- d_6): δ 0.87 (m, 3H), 1.32 (m, 4H), 1.43 (m, 2H), 1.74 (m, 2H) 2.79 (s, 3H), 4.36 (t, *J* 6.5 Hz, 2H), 6.75 (br s, 2H, NH_2). ^{13}C NMR (DMSO- d_6): δ 13.9 (CH_3), 16.3 (CH_3), 22.0 (CH_2), 25.1 (CH_2), 28.2 (CH_2), 30.7 (CH_2), 67.4 (CH_2), 95.1 (C), 115.0 (C), 120.2 (C), 144.4 (C), 149.5 (C), 155.9 (C), 158.4 (C), 166.3 (C).

3-Amino-5-bromo-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylic acid (7e). Beige solid (91 mg, 96%). ^{1}H NMR (DMSO- d_6): δ 2.82 (s, 3H), 3.95 (s, 3H), 6.41 (br s, 2H, NH_2). ^{13}C NMR (DMSO- d_6): δ 19.0 (CH_3), 54.6 (CH_3), 96.4 (C), 105.5 (C), 123.1 (C), 140.4 (C), 145.4 (C), 154.9 (C), 157.5 (C), 168.1 (C).

3-Amino-5-iodo-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylic acid (7f). White solid (290 mg, 95%). ^{1}H NMR (DMSO- d_6): δ 2.88 (s, 3H), 3.93 (s, 3H), 6.48 (br s, 2H, NH_2). ^{13}C NMR (DMSO- d_6): δ 25.1 (CH_3), 55.1 (CH_3), 55.2 (C), 55.7 (C), 85.3 (C), 96.1 (C), 122.3 (C), 140.3 (C), 148.9 (C), 149.7 (C).

General procedure for BOP coupling. Carboxylic acids **7a-f** (1 equiv) were added to *N,N*-dimethylformamide (10 mL), followed by *N,N*-diisopropylethylamine (1.05 equiv.) and BOP (1.05 equiv.) under N_2 at room temperature and stirred for 5-10 mins. Cyclopropylamine or

the required *N*-alkylamine (1.1 equiv.) was subsequently added and the reaction mixture stirred at room temperature for 2-3 h. The solvent was then removed in vacuo and the resulting residue partitioned between dichloromethane (30 mL) and saturated sodium bicarbonate (50 mL). The organic layer was removed and the aqueous phase was further extracted with 3 × 20 mL portions of dichloromethane. The organic fractions were combined, washed with water (50 mL), brine (50 mL), dried over anhydrous sodium sulfate, filtered and concentrated to yield the crude product as an oily residue. To remove excess HMPA, crude products are dissolved in ethyl acetate and washed with 3 × 50 mL portions of 2 M brine. Purification of the product was performed by column chromatography and /or recrystalisation.

3-Amino-5-chloro-*N*-cyclopropyl-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (8a).[2] White crystalline solid (117 mg, 57%). mp: 175.5-176.7 °C (DCM/hexane). ^{1}H NMR ($CDCl_3$): δ 0.63 (m, 2H), 0.86 (m, 2H), 2.82 (m, 1H), 2.83 (s, 3H), 4.07 (s, 3H), 5.55 (br s, 1H, NH), 6.36 (br s, 2H , NH_2). ^{13}C NMR ($CDCl_3$): δ 7.1 (CH_2), 16.4 (CH_3), 23.0 (CH), 55.2 (CH_3), 97.6 (C), 116.6 (C), 121.1 (C), 143.1 (C), 147.7 (C), 154.5 (C), 159.3 (C), 167.3 (C). HPLC purity (λ= 214 nm): 98% t_R 9.95 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 312.0574 calcd for $C_{13}H_{14}ClN_3O_2S$; found $[M+H]^+$ 312.0569.

3-Amino-5-chloro-*N*-cyclopropyl-6-ethoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (8b). Yellow solid (21 mg, 15%). mp: 211.2- 212.7 °C. ^{1}H NMR ($CDCl_3$): δ 0.63 (m, 2H), 0.85 (m, 2H), 1.45 (t, *J* 7.1 Hz, 3H), 2.82 (m, 1H), 2.82 (s, 3H), 4.49 (q, *J* 7.1 Hz, 2H), 5.54 (br s, 1H, NH), 6.35 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 7.0 (CH_2), 14.8 (CH_3), 16.4 (CH_3), 23.0 (CH), 63.8 (CH_2), 97.5 (C), 116.7 (C), 120.9 (C), 143.0 (C), 147.8 (C), 154.6 (C), 159.1 (C), 167.4 (C). HPLC purity (λ= 214 nm): 98% t_R 10.38 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 326.0730 calcd for $C_{14}H_{16}ClN_3O_2S$; found $[M+H]^+$ 326.0738.

3-Amino-6-butoxy-5-chloro-*N*-cyclopropyl-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (8c). Yellow solid (61 mg, 65%). mp: 131.9- 134.2 °C. ^{1}H NMR ($CDCl_3$): δ 0.63 (m, 2H), 0.85 (m, 2H), 0.99 (t, *J* 7.4 Hz, 3H), 1.51 (m, 2H), 1.81 (m, 2H), 2.82 (m, 1H), 2.82 (s, 3H), 4.41 (t, *J* 6.6 Hz, 2H), 5.64 (br s, 1H, NH), 6.45 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 7.0 (CH_2), 14.0 (CH_3), 16.4 (CH_3), 19.4 (CH_2), 23.0 (CH), 31.0 (CH_2), 67.7 (CH_2), 97.4 (C), 116.8 (C), 120.8 (C), 142.9 (C), 147.8 (C), 154.6 (C), 159.2 (C), 167.4 (C). HPLC purity (λ= 214 nm): 95% t_R 11.63 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 354.1043 calcd for $C_{16}H_{20}ClN_3O_2S$; found $[M+H]^+$ 354.1029.

3-Amino-5-chloro-*N*-cyclopropyl-6-(hexyloxy)-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (8d). Yellow solid (47 mg, 42%). mp: 126.8- 128.3 °C. ^{1}H NMR ($CDCl_3$): δ

0.63 (m, 2H), 0.84 (m, 2H), 0.89-0.94 (m, 3H), 1.33-1.37 (m, 4H), 1.46 (m, 2H), 1.82 (m, 2H), 2.80 (s, 3H), 2.81 (m, 1H), 4.39 (t, *J* 6.7 Hz, 2H), 5.56 (br s, 1H, NH), 6.43 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 7.0 (CH_2), 14.1 (CH_3), 16.3 (CH_3), 22.7 (CH_2), 23.0 (CH), 25.8 (CH_2), 28.8 (CH_2), 31.6 (CH_2), 68.0 (CH_2), 97.4 (C), 116.8 (C), 120.7 (C), 142.9 (C), 147.8 (C), 154.5 (C), 159.2 (C), 167.4 (C). HPLC purity (λ= 214 nm): 96% t_R 12.72 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 382.1356 calcd for $C_{18}H_{24}ClN_3O_2S$; found $[M+H]^+$ 382.1359.

3-Amino-5-bromo-*N*-cyclopropyl-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (8e). Yellow solid (67 mg, 70%). mp: 199.1- 201.5 °C. ^1H NMR ($CDCl_3$): 0.64 (m, 2H), 0.85 (m, 2H), 2.82 (m, 1H), 2.88 (s, 3H), 4.06 (s, 3H), 5.57 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 7.1 (CH_2), 19.7 (CH_3), 23.0 (CH), 55.7 (CH_3), 97.5 (C), 108.3 (C), 121.4 (C), 145.4 (C), 147.7 (C), 155.6 (C), 160.0 (C), 167.4 (C). HPLC purity (λ= 214 nm): 98% t_R 11.23 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 356.0068 calcd for $C_{13}H_{14}BrN_3O_2S$; found $[M+H]^+$ 356.0057.

3-Amino-*N*-cyclopropyl-5-iodo-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (8f). Beige solid (158 mg, 72%). mp: 226.1- 227.6 °C. ^1H NMR ($CDCl_3$): 0.64 (m, 2H), 0.86 (m, 2H), 2.82 (m, 1H), 2.94 (s, 3H), 4.04 (s, 3H), 5.56 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 7.1 (CH_2), 23.0 (CH), 25.9 (CH_3), 55.7 (CH_3), 86.3 (C), 97.2 (C), 121.2 (C), 147.4 (C), 149.5 (C), 157.1 (C), 161.7 (C), 167.3 (C). HPLC purity (λ= 214 nm): 98% t_R 9.76 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 403.9930 calcd for $C_{13}H_{14}IN_3O_2S$; found $[M+H]^+$ 403.9923.

3-Amino-5-chloro-*N*-ethyl-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (9a). Yellow solid (52 mg, 56%). mp: 180.9- 182.4 °C. ^1H NMR ($CDCl_3$): δ 1.25 (t, *J* 7.2 Hz, 3H), 2.80 (s, 3H), 3.45 (m, 2H), 4.06 (s, 3H), 5.40 (t, *J* 5.7 Hz, 1H, NH), 6.28 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 15.2 (CH_3), 16.3 (CH_3), 34.7 (CH_2), 55.1 (CH_3), 98.3 (C), 116.5 (C), 121.2 (C), 143.0 (C), 147.3 (C), 154.4 (C), 159.2 (C), 165.7 (C). HPLC purity (λ= 214 nm): 99% t_R 9.67 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 300.0574 calcd for $C_{12}H_{14}ClN_3O_2S$; found $[M+H]^+$ 300.0572.

3-Amino-*N*-butyl-5-chloro-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (9b). Beige solid (51 mg, 66%). mp: 138.7- 139.9 °C. ^1H NMR ($CDCl_3$): δ 0.96 (t, *J* 7.3 Hz, 3H), 1.42 (m, 2H), 1.59 (m, 2H) 2.80 (s, 3H), 3.41 (m, 2H), 4.06 (s, 3H), 5.41 (t, *J* 5.7 Hz, 1H, NH), 6.28 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 16.3 (CH_3), 20.7 (CH_2), 32.0 (CH_2), 39.6 (CH_2), 55.1 (CH_3), 98.3 (C), 116.5 (C), 121.2 (C), 143.0 (C), 147.3 (C), 154.4 (C), 159.1 (C), 165.7 (C). HPLC purity (λ= 214 nm): 99% t_R 10.73 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 328.0887 calcd for $C_{14}H_{18}ClN_3O_2S$; found $[M+H]^+$ 328.0889.

3-Amino-5-chloro-*N*-hexyl-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (9c). Yellow solid (54 mg, 64%). mp: 109.5- 110.8 °C. ^{1}H NMR ($CDCl_3$): δ 0.90 (t, *J* 7.3 Hz, 3H), 1.29- 1.39 (m, 6H), 1.60 (m, 2H) 2.80 (s, 3H), 3.38 (m, 2H), 4.05 (s, 3H), 5.43 (t, *J* 5.7 Hz, 1H, NH), 6.26 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$): δ 14.1 (CH_3), 16.3 (CH_3), 22.7 (CH_2), 26.8 (CH_2), 29.9 (CH_2), 31.6 (CH_2), 39.8 (CH_2), 55.1 (CH_3), 98.3 (C), 116.5 (C), 121.1 (C), 143.0 (C), 147.2 (C), 154.3 (C), 159.1 (C), 165.7 (C). HPLC purity (λ= 214 nm): 98% t_R 11.77 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 356.1200 calcd for $C_{16}H_{22}ClN_3O_2S$; found $[M+H]^+$ 356.1205.

General procedure for amine substituted derivatives. Compound **8a** (1 equiv) was dissolved in dry DCM (3 mL) under N_2. Triethylamine (2 equiv) was added and the reaction mixture cooled to 0 ^{0}C. The required acyl chloride (1.05 equiv) was added dropwise and the reaction heated at reflux for 2 d during which an additional (1-2 equiv) of the acyl chloride was added. Upon cooling to room temperature formed a white precipitate that was filtered and washed with a small amount of DCM.

3-Acetamido-5-chloro-*N*-cyclopropyl-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (10a). White solid (29 mg, 48%). mp: 289.9- 291.2 °C ^{1}H NMR (d_6-DMSO): δ 0.54 (m, 2H), 0.73 (m, 2H), 2.08 (s, 3H), 2.61 (s, 3H), 2.81 (m, 1H), 4.02 (s, 3H), 8.02 (d, *J* 3.7 Hz, 1H, NH), 9.78 (s, 1H, NH). ^{13}C NMR (d_6-DMSO): δ 6.09 (CH_2), 14.6 (CH_3), 22.8 (CH_3), 23.0 (CH), 54.9 (CH_3), 116.6 (C), 124.4 (C), 128.6 (C), 129.4 (C), 143.6 (C), 153.1 (C), 157.7 (C), 161.8 (C), 170.5 (C). HPLC purity (λ= 214 nm): 96% t_R 8.32 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 354.0679 calcd for $C_{15}H_{16}ClN_3O_3S$; found $[M+H]^+$ 354.0672.

3-Butyramido-5-chloro-*N*-cyclopropyl-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (10b). White solid (31 mg, 48%). mp: 282.0- 283.5 °C. ^{1}H NMR (d_6-DMSO): δ 0.54 (m, 2H), 0.73 (m, 2H), 0.95 (t, *J* 7.4 Hz, 3H), 1.63 (m, 2H), 2.34 (t, *J* 7.3 Hz, 2H), 2.60 (s, 3H), 2.81 (m, 1H), 4.02 (s, 3H), 8.04 (d, *J* 3.7 Hz, 1H, NH), 9.74 (s, 1H, NH). ^{13}C NMR (d_6-DMSO): δ 6.01 (CH_2), 13.8 (CH_3), 14.7 (CH_3), 18.2 (CH_2), 23.0 (CH), 37.4 (CH_2), 55.0 (CH_3), 116.6 (C), 124.5 (C), 128.5 (C), 129.6 (C), 134.9 (C), 143.6 (C), 153.1 (C), 157.8 (C), 173.2 (C). HPLC purity (λ= 214 nm): 96% t_R 7.27 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 382.0992 calcd for $C_{17}H_{20}ClN_3O_3S$; found $[M+H]^+$ 382.0996.

5-Chloro-*N*-cyclopropyl-3-hexanamido-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (10c). White solid (29 mg, 45%). mp: 256.4- 257.9 °C ^{1}H NMR (d_6-DMSO): δ 0.52 (m, 2H), 0.73 (m, 2H), 0.90 (m, 3H), 1.30-1.33 (m, 4H), 1.60 (m, 2H), 2.36 (t, *J* 7.3 Hz, 2H), 2.60 (s, 3H), 2.79 (m, 1H), 4.02 (s, 3H), 8.01 (d, *J* 3.7 Hz, 1H, NH), 9.73 (s, 1H, NH).

^{13}C NMR (d_6-DMSO): δ 6.00 (CH_2), 13.8 (CH_3), 14.7 (CH_3), 21.9 (CH_2), 23.0 (CH), 24.3 (CH_2), 30.9 (CH_2), 35.4 (CH_2), 55.0 (CH_3), 116.6 (C), 124.5 (C), 128.5 (C), 129.6 (C), 143.6 (C), 153.1 (C), 157.7 (C), 161.9 (C), 173.4 (C). HPLC purity (λ= 214 nm): 96% t_R 10.73 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 410.1305 calcd for $C_{19}H_{24}ClN_3O_3S$; found $[M+H]^+$ 410.1299.

Synthesis of compound 12.

3-Amino-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxylic acid (11). **3a** was dissolved in ethanol (30 mL) followed by the addition of 1 M sodium hydroxide solution (2.08 mL) where a white precipitate formed. The reaction mixture was refluxed for 2 h. The reaction mixture was cooled to room temperature where a white precipitate formed. The solid was filtered and dissolved in a minimum amount of water. 1 M aqueous hydrogen chloride was added dropwise where a white precipitate formed. The solid was filtered, washed with water and dried under vacuum to give the title compound (515 mg, 58%). ^{1}H NMR (d_6-DMSO) δ 2.69 (s, 3H), 3.90 (s, 3H), 6.62 (d, *J* 0.9, 1H), 6.69 (br s, 2H), 11.50 (br s, 1H). ^{13}C NMR (d_6-DMSO) δ 19.8 (CH_3), 53.6 (CH_3), 109.3 (CH), 119.5 (C), 147.6 (C), 149.9 (C), 159.0 (C), 161.6 (C), 163.9 (C), 166.4 (C).

3-Amino-*N*-cyclopropyl-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide hydrochloride (12). Compound **11** (362 mg, 1.52 mmol) was dissolved in dry DMF (5 mL) under N_2 atmosphere. Diisopropylethylamine (520 μL, 3.04 mmol) was added to the solution followed by BOP (605 mg, 1.60 mmol). The mixture was stirred for 10 mins until completely dissolved. Cyclopropylamine (116 μL, 1.67 mmol) dissolved in dry DMF (1 mL) was slowly added to the mixture. The reaction mixture was stirred at RT under N_2 for 3 h. Note: Additional amine may be required to consume starting material. The reaction mixture was partitioned between ethyl acetate (30 mL) and water (30 mL). The aqueous layer was extracted with ethyl acetate (2 × 20 mL). The combined ethyl acetate layers were washed with brine (20 mL), dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. ^{1}H NMR ($CDCl_3$) δ 0.61-0.65 (m, 2H), 0.82-0.87 (m, 2H), 2.69 (d, *J* 0.9 Hz, 3H), 2.80-2.84 (m, 1H), 3.97 (s, 3H), 5.54 (br s, 1H), 6.34 (br s, 2H), 6.44 (d, *J* 0.9 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 6.9 (CH_3), 20.2 (CH_2), 22.8 (CH), 53.9 (CH_3), 110.2 (CH), 120.3 (C), 145.9 (C), 148.0 (C), 157.7 (C), 162.1 (C), 164.3 (C), 167.4 (C). The product was dissolved in ethyl acetate and converted to the HCl salt using ethereal hydrogen chloride to give the product as a white solid (185 mg, 39%). mp: 180- 181 °C. HPLC purity (λ= 254 nm): 99.7% t_R 8.26 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 278.0963 calcd for $C_{13}H_{15}N_3O_2S$; found $[M+H]^+$ 278.0958.

Synthesis of compound 18.

5-Chloro-4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (14).[3] A mixture of cyanopyridone (**13,** 500 mg, 3.37 mmol) and sulfuryl chloride (1.09 mL, 13.5 mmol) in dry dichloroethane (10 mL) was refluxed for 6 h. The reaction mixture was cooled to RT and the precipitate was filtered and washed with DCM (50 mL) to give the desired product without requiring further purification (257 mg, 42%). ^{1}H NMR (d_6-DMSO) δ 2.35 (s, 3H), 2.41 (s, 3H), 12.8 (br s, 1H). ^{13}C NMR (d_6-DMSO) δ 18.2 (CH_3), 20.0 (CH_3), 100.8 (C), 115.4 (C), 115.5 (C), 121.6 (C), 159.2 (C), 159.3 (C).

2,5-Dichloro-4,6-dimethylnicotinonitrile (15).[3] Compound **14** (502 mg, 2.80 mmol) and $POCl_3$ (2 mL) were combined in a microwave vessel. The mixture was heated to 100 °C for 1 h in a microwave. The resulting green solution was poured into ice (10 g). Following this, gas evolution occurred and a white precipitate formed. The solid was filtered and washed with ice water (3 × 15 mL). The solid was dried under vacuum, and the resulting white solid was used in subsequent reactions without further purification (487 mg, 88%). ^{1}H NMR (d_6-DMSO) δ 2.57 (s, 3H), 2.61 (s, 3H). ^{13}C NMR (d_6-DMSO) δ 19.7 (CH_3), 23.2 (CH_3), 109.3 (C), 114.0 (C), 130.3 (C), 148.2 (C), 152.3 (C), 160.1 (C).

5-Chloro-4,6-dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile (16).[4] Thiourea (318 mg, 4.17 mmol) was added to a stirred solution of **15** (419 mg, 2.09 mmol) in absolute ethanol (25 mL). The mixture was refluxed for 24 h. The mixture was cooled to RT following which the product recrystallised from solution to form yellow needles. The product was then filtered and washed with cold ethanol and dried under vacuum (121 mg, 29%). ^{1}H NMR (d_6-DMSO) δ 2.45 (s, 3H), 2.46 (s, 3H), 13.1 (br s, 1H). ^{13}C NMR (d_6-DMSO) δ 18.1 (CH_3), 20.0 (CH_3), 114.9 (C), 116.0 (C), 119.3 (C), 151.0 (C), 154.0 (C), 175.6 (C).

2-Chloro-*N*-cyclopropylacetamide (17).[5] *N*-Cyclopropylamine (8.25 mmol) and triethylamine (1.26 mL, 9.08 mmol) was dissolved in dichloromethane (5-10 mL) under nitrogen and cooled to -2 to -5 °C in an ice/acetone bath. Chloroacetyl chloride (656 µL, 8.25 mmol) dissolved in dichloromethane (3 mL) was added dropwise to the mixture and a vigorous reaction occurred with a precipitate forming. The mixture was allowed to stir for 30 min at 0 °C and was then returned to RT and stirred for a further 30 min. The precipitate was then filtered and washed with dichloromethane. The filtrate was washed with 2 M aqueous hydrochloric acid (2 × 5 mL) and brine (2 ×15 mL). The organic layer was then dried over anhydrous sodium sulfate, filtered and concentrated in vacuo which was then recrystallised from dichloromethane/hexane to afford the desired compound (1.52 g, 65%). mp: 83- 84 °C.

^{1}H NMR ($CDCl_3$) δ 0.56-0.60 (m, 2H), 0.81-0.86 (m, 2H), 2.72-2.79 (m, 1H), 4.03 (s, 2H), 6.67 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ 6.5 (CH_2), 22.8 (CH), 42.5 (CH_2), 167.3 (C).

3-Amino-5-chloro-*N*-cyclopropyl-4,6-dimethylthieno[2,3-*b*]pyridine-2-carboxamide hydrochloride (18). Sodium metal (40 mg, 1.55 mmol), was dissolved in methanol (15 mL) and stirred for 5 minutes. **16** (100 mg, 503 μmol) and **17** (74 mg, 554 μmol) were added to the sodium methoxide solution. The mixture was refluxed for 3 h, then cooled to RT. The solvent was removed by rotary evaporation and the product was purified by silica gel flash column chromatography eluting with a ratio of 2:1 ethyl acetate and hexane to afford the compound as the free base. ^{1}H NMR (d_6-DMSO) δ 0.56-0.62 (m, 2H), 0.62-0.69 (m, 2H), 2.62 (s, 3H), 2.74-2.81 (m, 1H), 2.82 (s, 3H), 6.86 (br s, 2H), 7.83 (d, *J* 3.7 Hz, 1H). ^{13}C NMR (d_6-DMSO) δ 5.7 (CH_2), 16.2 (CH_3), 22.8 (CH), 23.7 (CH_3), 99.6 (C), 124.4 (C), 127.5 (C), 141.8 (C), 147.1 (C), 155.4 (C), 155.8 (C), 166.3 (C). The compound was then converted to the HCl salt using ethereal hydrogen chloride to give the title compound as a bright yellow solid; (50 mg, 30%). mp: 214.5-215.8 °C. HPLC purity (λ= 254 nm): 98% t_R 9.21 min. HRMS (ESI)-TOF (*m/z)*: $[M+H]^+$ 296.0624 calcd for $C_{13}H_{14}ClN_3OS$; found $[M+H]^+$ 296.0616.

Synthesis of compound 21.

4-Mercapto-2,6-dimethylpyrimidine-5-carbonitrile (20).[6] Acetyl chloride (4.34 mL, 61.0 mmol) and ammonium thiocyanate (**19**, 5.08 g, 66.7 mmol) were suspended in dioxane (100 mL) and refluxed for 15 mins. 3-Aminocrotonotrile (10.0g, 122 mmol) was added to the mixture and refluxed for 4 h. The reaction mixture was poured into an ice water mixture (300 mL). No precipitate had formed and the aqueous layer was washed with ethyl acetate (200 mL). 6 M aqueous HCl solution was added to the aqueous layer to attain a pH of 2-3 and extracted with ethyl acetate (3 × 200 mL). The organic layer was washed with brine (200 mL), dried over anhydrous Na_2SO_4 , filtered and concentrated in vacuo to give the title compound without any further purification (2.29 g, 23%). ^{1}H NMR (d_6-DMSO) δ 2.44 (s, 6H), 3.34 (br s, 1H). ^{13}C NMR (d_6-DMSO) δ 21.5 (CH_3), 23.4 (CH_3), 110.4 (C), 115.7 (C), 136.4 (C), 161.4 (C), 169.1 (C).

5-Amino-*N*-cyclopropyl-2,4-dimethylthieno[2,3-*d*]pyrimidine-6-carboxamide (21). Sodium metal (140 mg, 6.05 mmol) was dissolved in absolute ethanol (25 mL) and stirred for 5 mins. Compound **20** (207 mg, 1.21 mmol) was added to the solution followed by **17** (178 mg, 1.33 mmol). The mixture was refluxed for 4 h. The reaction mixture was cooled to RT and the solvent was removed by rotary evaporation. The product was purified by silica gel

flash column chromatography eluted with a gradient of ethyl acetate and petroleum benzene to give the product as a pale yellow solid as the free base (237 mg, 75%). mp: 194.9- 196.6 °C. ^{1}H NMR (d_6-DMSO) δ 0.55-0.60 (m, 2H), 0.62-0.69 (m, 2H), 2.62 (s, 3H), 2.74-2.81 (m, 1H), 2.84 (s, 3H), 6.98 (br s, 2H), 7.87 (d, *J* 3.5 Hz, 1H). ^{13}C NMR (d_6-DMSO) δ 5.8 (CH_2), 22.9 (CH), 23.3 (CH_3), 25.3 (CH_3), 96.8 (C), 120.7 (C), 145.5 (C), 163.1 (C), 164.4 (C), 165.7 (C), 166.1 (C). HPLC purity (λ= 254 nm): 99.5% t_R 5.93 min. HRMS (ESI)-TOF (*m/z*): $[M+H]^+$ 263.0967 calcd for $C_{12}H_{14}N_4OS$; found $[M+H]^+$ 263.0960.

Pharmacology.

ERK1/2 Phosphorylation Assay. FlpIn CHO cells stably expressing the M_4 receptor were seeded into 96-well plates at a density of 30 000 cells/ well. After 5h, cells were washed with phosphate-buffered saline (PBS) and incubated in serum-free DMEM overnight before assaying. Initially, time-course experiments were conducted at least twice for each ligand to determine the time required to maximally promote ERK1/2 phosphorylation via the dopamine $D_{2L}R$. Interaction studies were performed using varying concentrations of test ligand and increasing concentrations ACh at 37 °C with a stimulation time of 6 minutes. Stimulation of the cells was terminated by removing the media followed by the addition of 100 μL of SureFire lysis buffer (PerkinElmer) to each well. The plate was shaken for 5 min at rt before transferring 5 μL of the lysates to a white 384-well Proxiplate (PerkinElmer). Then, 8 μL of a 240:1440:7:7 mixture of Surefire activation buffer:Surefire reaction buffer:Alphascreen acceptor beads:Alphascreen donor beads was added to the samples and incubated in the dark at 37 °C for 1.5 h. Plates were read using a Fusion-TM plate reader.

[^{3}H]NMS binding assay. FlpIn CHO cells stably expressing the human M_4 receptor were grown and maintained in DMEM containing 10% fetal bovine serum (FBS), and 200 μg/mL of Hygromycin-B. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 and 95% O_2.Radioligand binding experiments were performed on whole cells, seeded at 25,000 cells/well and grown overnight at 37 °C. Cells were washed twice with 100 μL of binding buffer (10 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4). Assays were performed in a total volume of 100 μl with a 1/10 dilution of drug, for a duration of 4 hours at 4 °C. Assays were terminated by buffer removal followed by rapid washing, twice, with ice-cold 0.9% NaCl (100 μl). OptiPhaseSupermix scintillation cocktail (100 μl) was added, plates were sealed (TopSeal™), and radioactivity was measured in a MicroBeta^{2}LumiJETmicroplate counter. All inhibition binding experiments were performed

with 0.3 nM [^{3}H]NMS (K_D concentration) in presence of increasing concentrations of ACh with required analogues.

Data analysis

The data analysis and equations presented below are the same as previously published.[7]

Computerized nonlinear regression was performed using Prism 6 (GraphPad Software, San Diego, CA).

Functional experiments measuring the interactions between ACh and allosteric modulators were fitted to an operational model of allosterism and agonism (equation 1) to derive functional estimates of modulator affinity, cooperativity and efficacy. Note that this model assumes the orthosteric agonist is a full agonist both in the presence and absence of allosteric modulators (which was the case in our studies).

$$Response = Basal + \frac{(E_m - Basal)([A](K_B + \alpha\beta[B]) + (\tau_b[B][EC_{50}])}{[EC_{50}](K_B + [B]) + ([A](K_B + \alpha\beta[B]) + \tau_b[B][EC_{50}])} \quad (1)$$

where E_m is the maximum attainable system response for the pathway under investigation; [A] and [B] are the concentrations of orthosteric agonist and allosteric modulator/agonist, respectively; K_B is the dissociation constant of the allosteric modulator; EC_{50} is the concentration of orthosteric (full) agonist yielding 50% of the response between minimal and maximal receptor activation in the absence of allosteric ligand; n is a transducer slope factor linking occupancy to response; α and β are the cooperativity factors governing allosteric effects of the modulator on orthosteric agonist binding affinity and signalling efficacy, respectively; and τ_A and τ_B are operational measures of the ligands' respective signaling efficacies that incorporate receptor expression levels and efficiency of stimulus-response coupling. Statistical comparisons were performed with Prism using a one-way ANOVA followed by Newman-Keuls post test.

Competition binding curves between [^{3}H]NMS and ACh in the absence or presence of allosteric ligands, were fitted to the allosteric ternary complex model (equation 2):

$$Y = \frac{[A]}{[A] + \left(\frac{K_A K_B}{\alpha'[B] + K_B}\right)\left(1 + \frac{[I]}{K_I} + \frac{[B]}{K_B} + \frac{\alpha[I][B]}{K_I K_B}\right)} \quad (2)$$

where Y is percentage (vehicle control) binding, [A], [B], and [I] are the concentrations of [^{3}H]NMS, modulator and ACh, respectively, K_A and K_B are the equilibrium dissociation

constants of [^{3}H]NMS and modulator, respectively, K_B is the equilibrium dissociation constant of the modulator and $\alpha'$and α are the cooperativities (or analogues) between modulator and [^{3}H]NMS or ACh, respectively. Values of α (or α') > 1 denote positive cooperativity; values < 1 (but > 0) denote negative cooperativity, and values =1 denote neutral cooperativity.

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