***** *SUPPORTING INFORMATION FOR* *****

Selective isoxazolopyrimidine PAT1 (SLC26A6) inhibitors for therapy of intestinal disorders

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METHODS

Compounds

SLC26A6 transport assays were done on 377 commercially available (ChemDiv) structural analogs of original isoxazolopyrimidine compound ($PAT1_{inh}$-A0001). Commercial analogs were selected by holding the core scaffold constant and allowing variation of substituents on attached ring systems, typically substituted phenyl groups. For the initial screen, the compounds were used as supplied, and the best inhibitors were resynthesized in-house.

Cell culture and transport assays

Fischer rat thyroid (FRT) cells stably expressing murine slc26a6 and a halide sensing (with mutations H148Q/I152L/F46L) yellow fluorescent protein (FRT-YFP-slc26a3 cells) were generated and cultured as described.[1] For assay of iodide-chloride exchange, cells cultured on 96-well plates were incubated in phosphate buffered saline (PBS). For assay of slc26a6 function, baseline cellular fluorescence was measured for 2 seconds, after which 100 µl NaI-substituted PBS (140 mM NaCl replaced by 140 mM NaI) was added by syringe pump to drive Cl^-/I^- exchange. The initial rate of Cl^-/I^- exchange was determined by single exponential regression of the fluorescence quenching, as described.[1] All assay plates contained wells with negative (1% DMSO) and positive (500 µM niflumic acid) controls which were used to quantify percent inhibition. IC_{50} values were determined from assays done at different inhibitor concentrations using a single-site inhibition model. Selectivity studies were done using YFP-based assays of SLC26A4, SLC26A3, SLC26A9, CFTR and TMEM16A, as described.[1]

Cellular toxicity

FRT cells were plated in black-walled, clear-bottom tissue culture plates at a density of 20,000 cells/well. After 24 h, cells were incubated with 10 μM $PAT1_{inh}$-A0030, 0.1 % DMSO (vehicle control) or 20 % DMSO (positive control) for 24 hours for assay of cell viability using Alamar Blue (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Animals

Wild type CD1 mice (male and female, age 10-16 weeks) and CF mice (homozygous CFTR F508del, male and female, age 10-16 weeks) were bred in the UCSF Laboratory Animal Resource Center. All protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Closed-loop studies of intestinal fluid absorption

Mice were given free access to 5% dextrose in water but not solid food for 24 hours before experiments. Closed ileal loops were prepared and isolated as described.[1] Mice were anesthetized with isoflurane and body temperature was maintained during surgery at 36-38 °C using a heating pad. A small abdominal incision was made to expose small intestine for isolation of 2-3 closed ileal loops (length

2–3 cm) with sutures. Loops were injected with 100 µL phosphate-buffered saline (PBS, pH: 7.4, in mM: 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 1.8 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$) containing 30 µM $PAT1_{inh}$-A0030 or 10 µM tenapanor (MedChemExpress, Monmouth Junction, NJ) (or vehicle, 0.1% DMSO in PBS). The abdominal incision was closed with sutures and mice were allowed to recover from anesthesia. Intestinal loops were removed at 0 and 30 min (in separate mice) and loop length and weight were measured to quantify fluid absorption.

Statistics

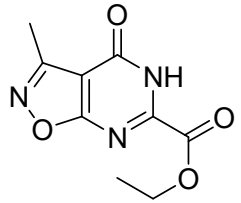
One-way ANOVA and post hoc Newman-Keuls multiple comparisons test was used for statistical analysis and $P < 0.05$ was considered statistically significant.

Chemistry: General

Unless otherwise indicated, all reaction solvents were anhydrous and obtained as such from commercial sources. Chemical and solvents were commercially available, and no further purification was required. Low resolution ESI-LCMS was carried out with an Agilent 1100 HPLC coupled to an Agilent 1956B MSD. RP-HPLC runs typically employed gradients of two solvents: [A]= H_2O (0.05% TFA) and [B] CH_3CN (0.05% TFA); RP-LCMS used the same solvent system using the modifier formic acid (88% aq). The standard HPLC and LCMS gradients proceeded with [A:B]= 95:5 to [A:B]= 5:95 over 15 minutes. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz instrument. ^{1}H NMR chemical shifts are relative to TMS (δ = 0.00 ppm), $CDCl_3$ (δ 7.26). ^{13}C NMR chemical shifts are relative to $CDCl_3$ (δ 77.2). When necessary, automated chromatographic purification was performed with a Biotage Isolera One instrument, using gradients of hexane:ethyl acetate, disposable 10g silica columns, and UV detection at 254 nm. The purity of assayed compounds was >95% based on HPLC-LCMS analysis at 254 nm, and absence of impurities was confirmed by examination at least one other wavelength (320 nm) as well as careful inspection of ^{1}H and ^{13}C-NMR spectra.

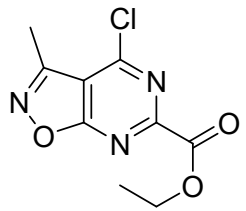
General procedure 1: reaction of chloroimine intermediate (4) with substituted benzylamines to generate final amidine products (5a-5c). Chloroimine (**4**) was dissolved in anhydrous acetonitrile (0.3M) and treated with substituted benzylamine (1.1 eq) and DIPEA (10 eq). The mixtures were heated at 110 ^{o}C for 1-2h. LCMS showed consumption of starting material and formation of

product, with no evidence of the aminolysis of the ethyl ester present in the scaffold. Reaction mixtures were taken up into brine and extracted twice with dichloromethane (2 x 10mL). Organic layers were dried over ~~Na_2SO_4~~, and concentrated *in vacuo*. Products were purified by automatic chromatography using a Biotage Isolera One instrument, with 10g columns, and typically isocratic 2:1 hexane:ethyl acetate. Final product yields were typically 40-56%.



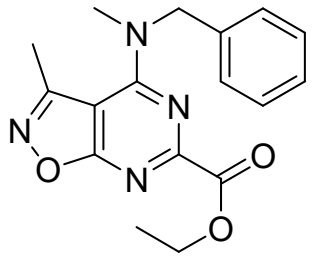
3

3-Methyl-4-oxo-4,5-dihydro-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (3). To a 20mL vial with a magnetic stir bar was added ethyl triethoxyacetate (4872 mg, 22.1 mmol), 5-amino-isoxazole-4-carboxamide (520 mg, 3.68 mmol), followed by acetic anhydride (2.44 mL, 7 mol eq). The vial was sealed and heated to 120 ^{o}C for 3h. The vial was cooled to RT and stored in a freezer, causing solid to form. The next day, the reaction mixture was added to a 25mL Erlenmeyer flask, and washed in with diethyl ether (20mL), causing additional precipitation. The product was isolated by vacuum filtration, yielding an off-white solid (295mg, 33% yield, mp 220-221 ^{o}C). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.35 (t, 3H, J = 7 Hz), 2.51 (s, 3H), 4.40 (q, 2H, J = 7 Hz), 13.5 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 11.53, 14.24, 63.71, 103.57, 150.20, 158.03, 159.44, 174.9. ESI-LCMS (low resolution) m/z calculated for $C_9H_9N_3O_4$ [M+H] 224.1, found [M+H] 224.1.



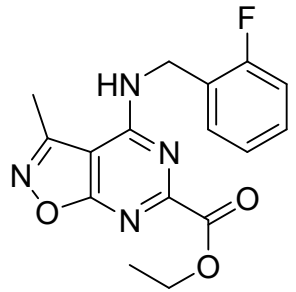
4

4-Chloro-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (4). To a 20mL vial without stirbar was added 3-Methyl-4-oxo-4,5-dihydro-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**3**) (40mg, 0.179mmol) and phosphorous oxychloride (0.5mL, 5.37 mmol, 30 mol eq). The reaction was heated to 100 ^{0}C for 1h. LCMS showed quantitative conversion from SM to product. To workup the product, it was diluted into DCM (10mL), transferred into a separatory funnel, and washed with Na_2CO_3 (10% aq) (10mL). The organic layer was collected, and the aqueous layer was back-extracted with additional DCM (5mL). The combined organic material was dried over Na_2SO_4 (anh) and concentrated *in vacuo*. After hi-vacuum, the product was isolated in quantitative yield (43mg). While there was a slight acidic odor, we found the material could be used in the next step without additional characterization or purification.



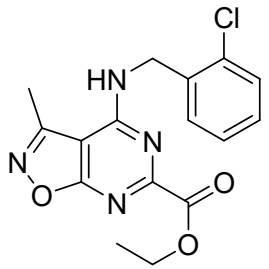
5a

4-(Benzyl-methyl-amino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (5a). Using general procedure 1, 4-Chloro-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**4**) (54 mg, 0.224 mmol) was reacted with N-methylbenzylamine (0.030 ml, 0.235 mmol) and DIPEA (0.410 ml, 2.35 mmol) to form the title molecule as an off white colorless solid (40 mg, 56% yield, mp 80-81 ^{o}C). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.47 (t, 3H, J = 7 Hz), 2.59 (s, 3H), 3.36 (s, 3H), 4.51 (q, 2H, J = 7 Hz), 5.09 (s, 2H), 7.36-7.39 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.21, 16.25, 38.61, 55.02, 62.52, 96.81, 127.88, 128.08, 128.99, 135.87, 152.88, 154.72, 159.98, 163.57, 177.33. ESI-LCMS (low resolution) m/z calculated for $C_{17}H_{18}N_4O_3$ [M+H] 327.1, found [M+H] 327.1.



5b

4-(2-Fluoro-benzylamino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (5b). Using general procedure 1, 4-Chloro-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**4**) (54 mg, 0.224 mmol) was reacted with 2-fluorobenzylamine (0.027 ml, 0.235 mmol) and DIPEA (0.410 ml, 2.35 mmol) to form the title molecule as an off white colorless solid (34 mg, 40% yield, mp 179-180 ^{o}C). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.48 (t, 3H, *J* = 7 Hz), 2.65 (s, 3H), 4.50 (q, 2H, *J* = 7 Hz), 4.97 (d, 2H, *J* = 6 Hz), 6.06 (bs, 1H), 7.06 (t, 1H, *J* = 8 Hz), 7.12 (t, 1H, *J* = 8 Hz), 7.30 (m, 1H), 7.58 (t, 1H, *J* = 8 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 12.58, 14.20, 39.64, 62.62, 97.36, 115.4 (d, *J* = 21 Hz), 124.2 (d, *J* = 14 Hz), 124.4 (d, *J* = 3 Hz), 129.90 (d, *J* = 9 Hz), 131.31 (d, *J* = 4 Hz), 153.2, 156.6, 158.5, 161.3 (d, *J* = 245 Hz), 163.4, 176.03. ESI-LCMS (low resolution) m/z calculated for $C_{16}H_{15}FN_4O_3$ [M+H] 331.1, found [M+H] 331.1.



5c

4-(2-Chloro-benzylamino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (5c). Using general procedure 1, 4-Chloro-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**4**) (54 mg, 0.224 mmol) was reacted with 2-chlorobenzylamine (0.028 ml, 0.235 mmol) and DIPEA (0.410 ml, 2.35 mmol) to form the title molecule as an off white colorless solid

(37 mg, 49% yield, mp 155-156 °C). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.49 (t, 3H, *J* = 7 Hz), 2.65 (s, 3H), 4.52 (q, 2H, *J* = 7 Hz), 5.01 (d, 2H, *J* = 6 Hz), 6.11 (bs, 1H), 7.26-7.27 (m, 2H), 7.39 (d, 1H, *J* = 5 Hz), 7.67 (d, 1H, *J* = 5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 12.58, 14.23, 43.44, 62.62, 97.47, 127.24, 129.64, 131.88, 133.86, 134.63, 153.14, 156.61, 158.39, 163.40, 176.06. ESI-LCMS (low resolution) m/z calculated for $C_{16}H_{15}ClN_4O_3$ [M+H] 347.1, found [M+H] 347.1.

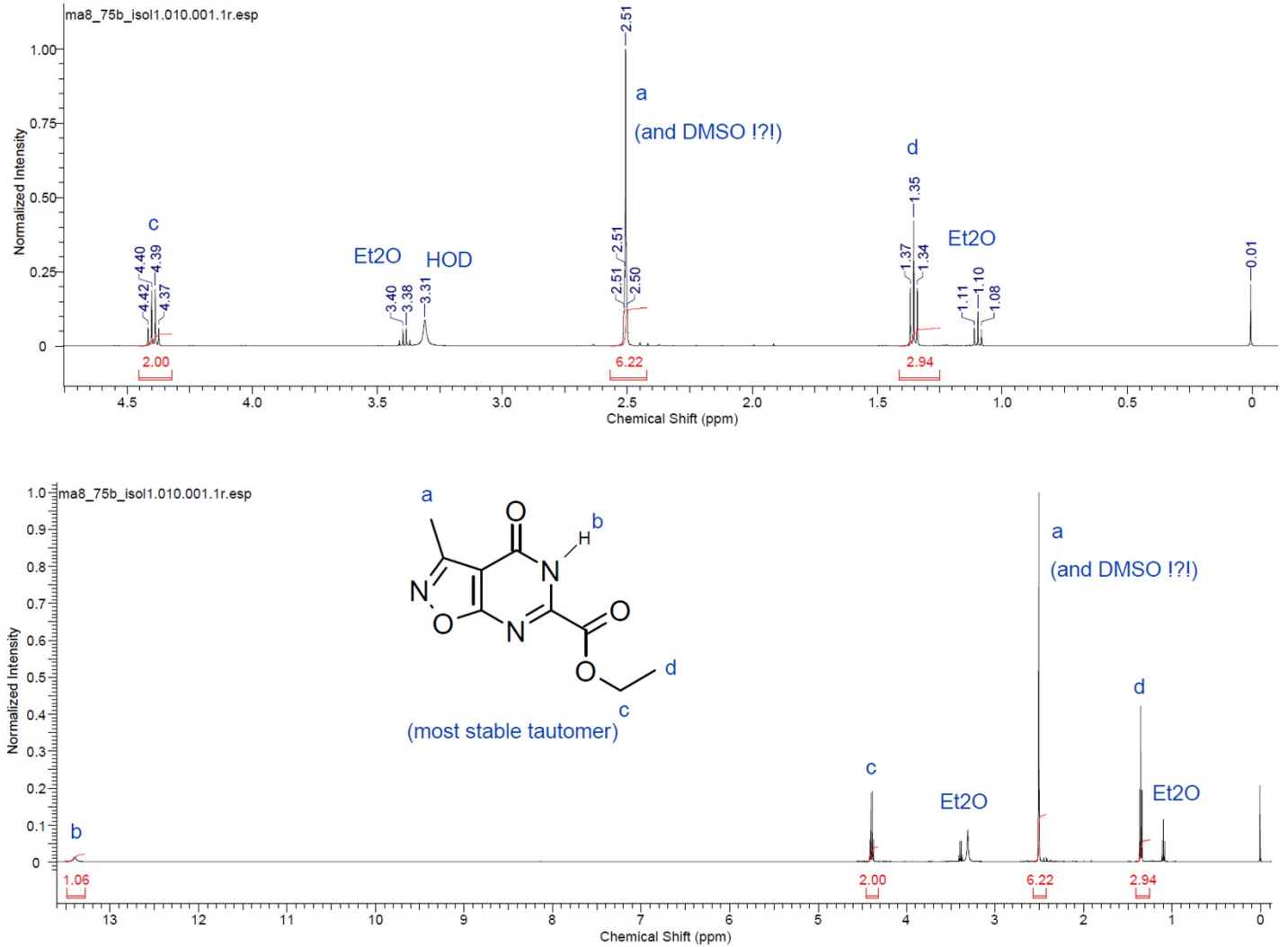


Figure S1. ^{1}H-NMR of 3-Methyl-4-oxo-4,5-dihydro-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**3**) (500 MHz, DMSO-*d6*).

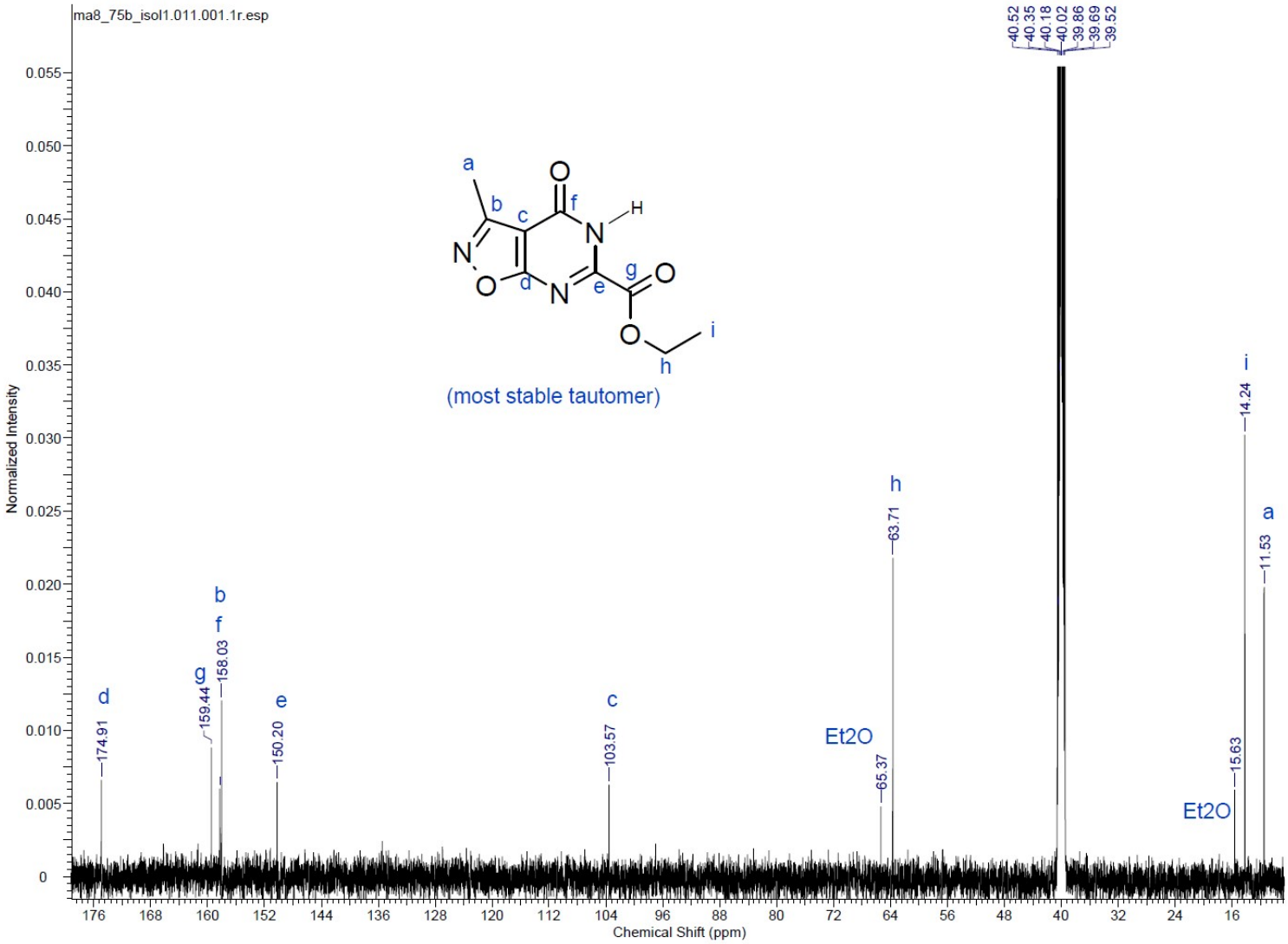


Figure S2. ^{13}C-NMR of 3-Methyl-4-oxo-4,5-dihydro-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**3**) (125 MHz, DMSO-*d6*).

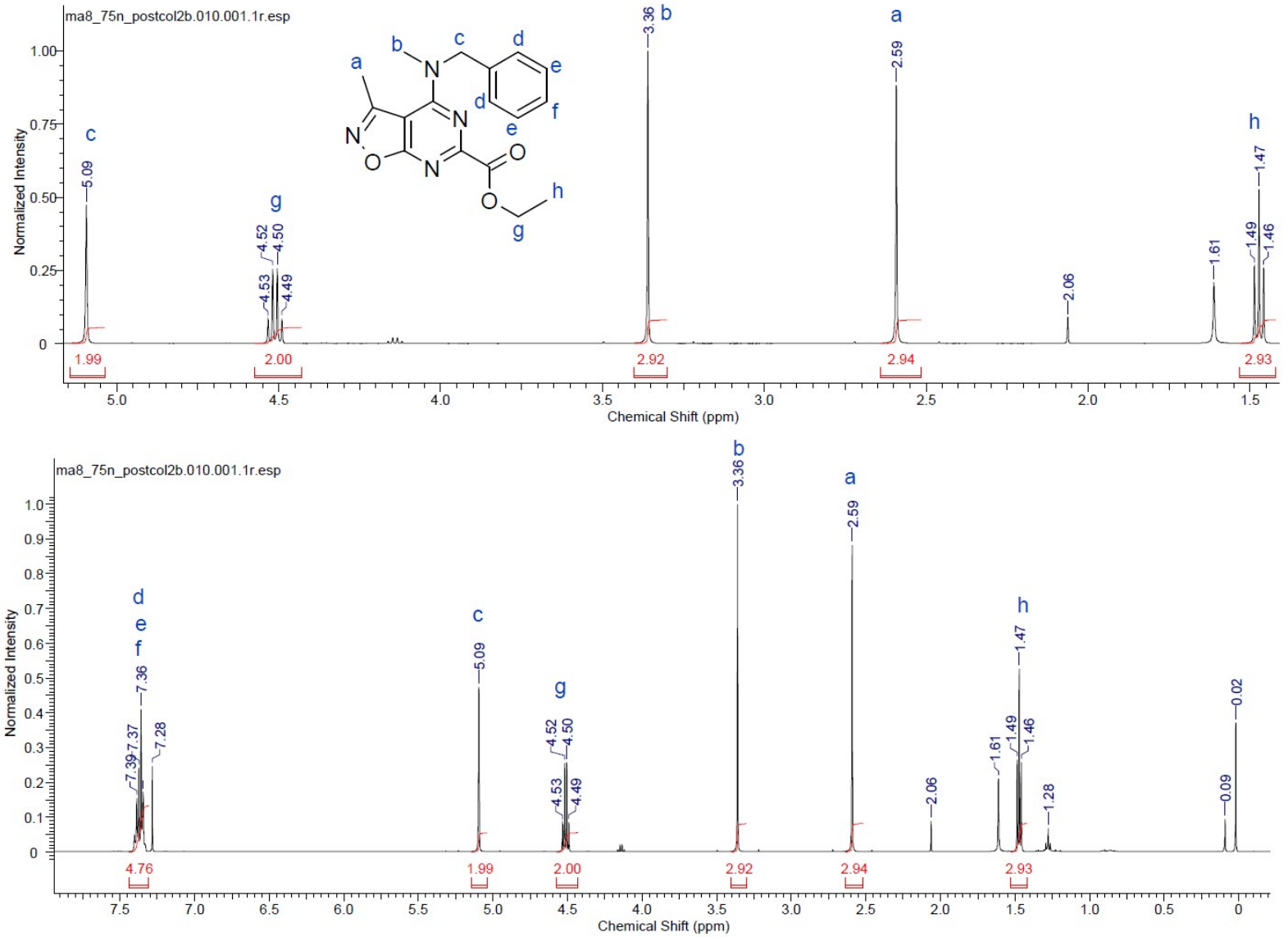


Figure S3. ^{1}H-NMR of 4-(Benzyl-methyl-amino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester **5a** (500 MHz, $CDCl_3$).

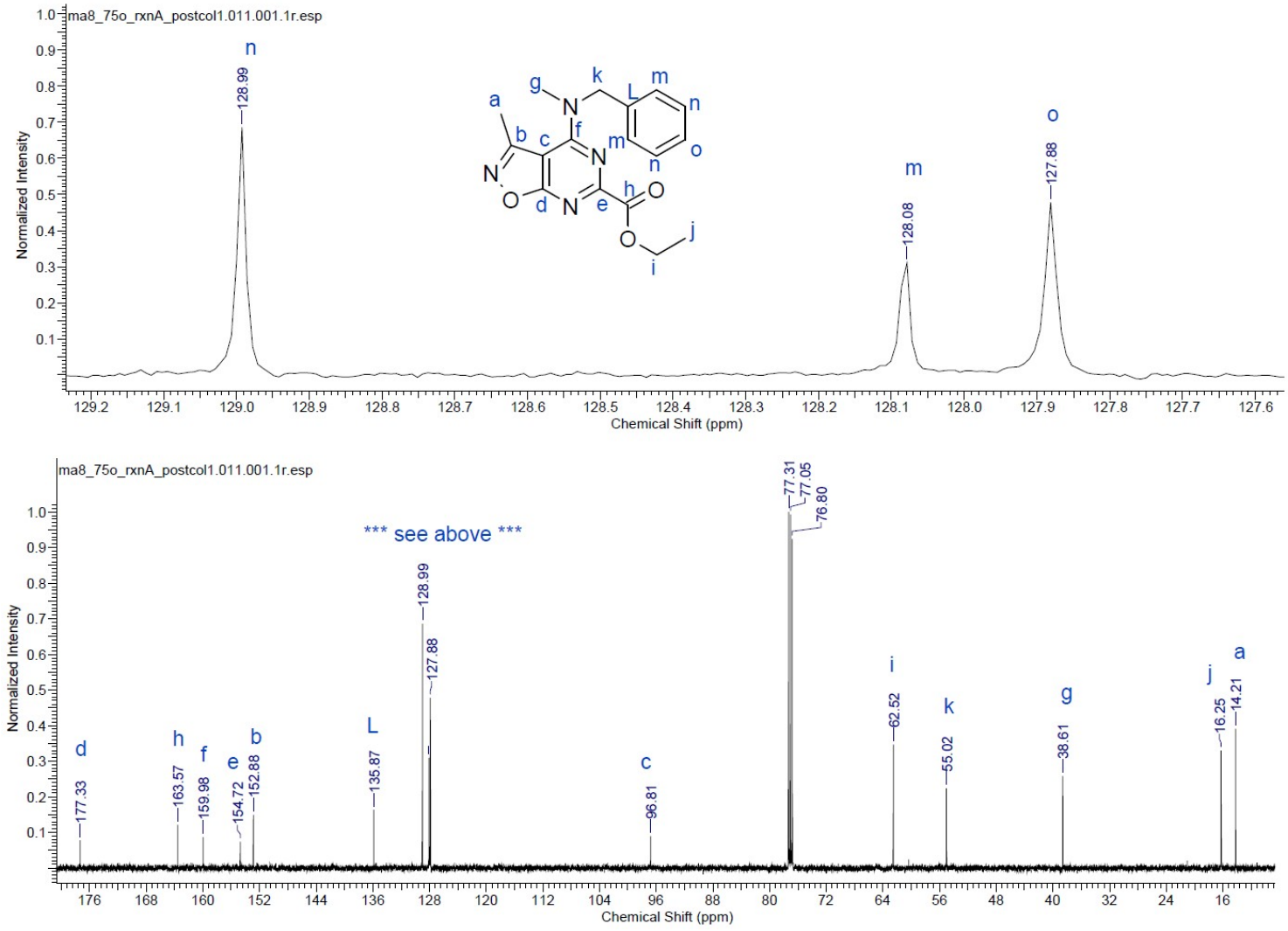


Figure S4. ^{13}C-NMR of 4-(Benzyl-methyl-amino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester **5a** (125MHz, $CDCl_3$).

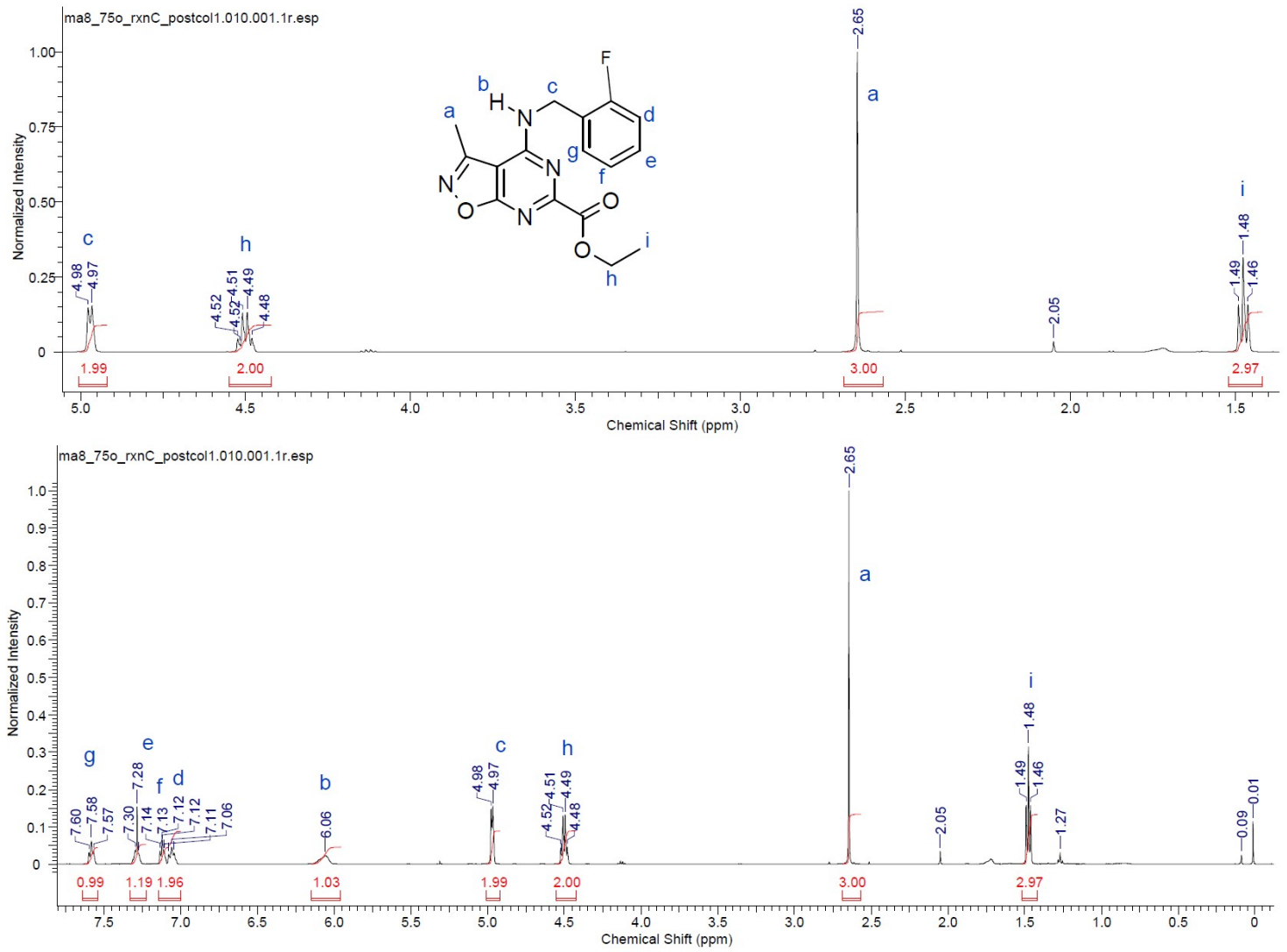


Figure S5. ^{1}H-NMR of 4-(2-Fluoro-benzylamino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**5b**) (500 MHz, $CDCl_3$).

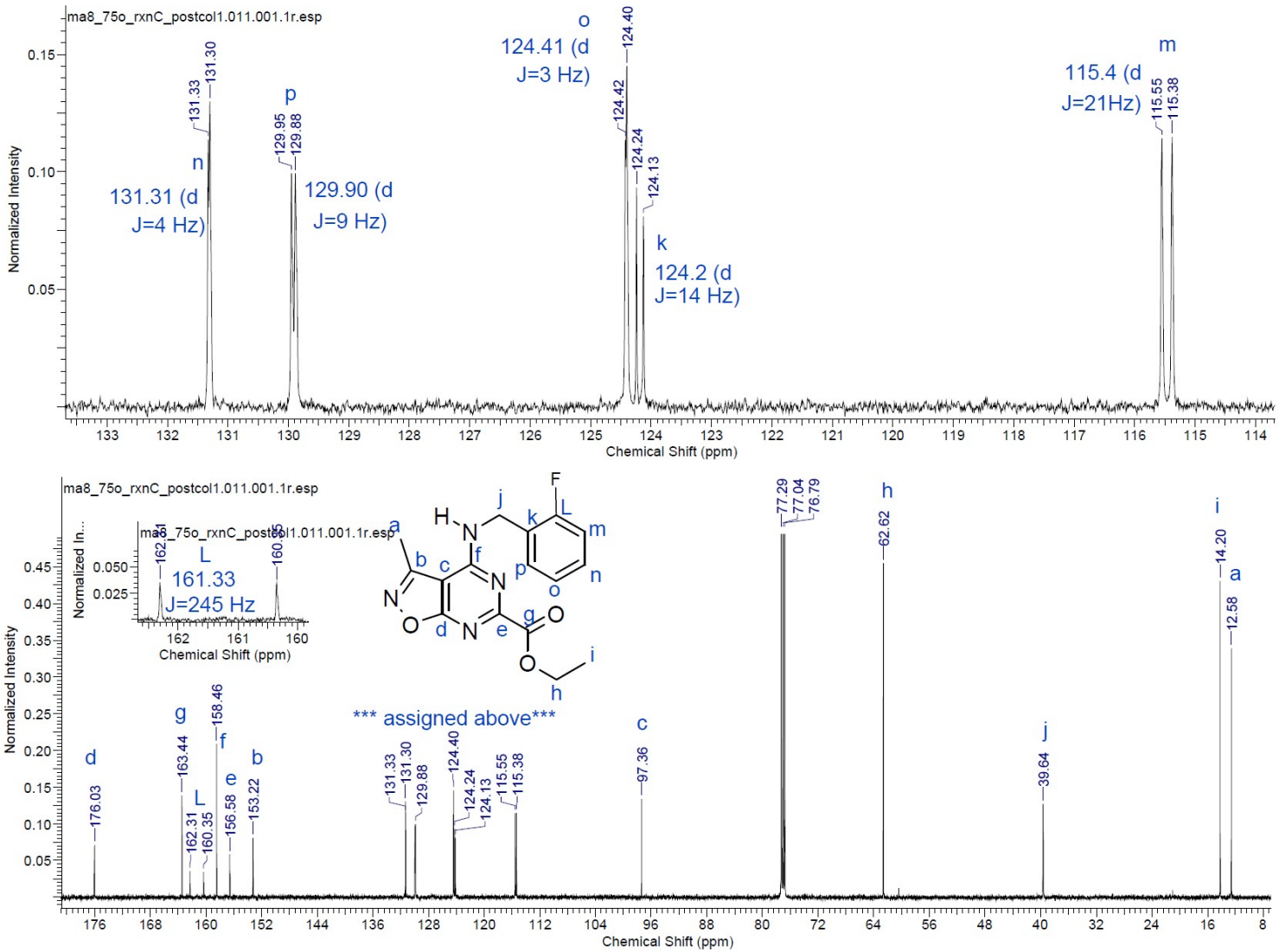


Figure S6. ^{13}C-NMR of 4-(2-Fluoro-benzylamino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**5b**) (125MHz, $CDCl_3$).

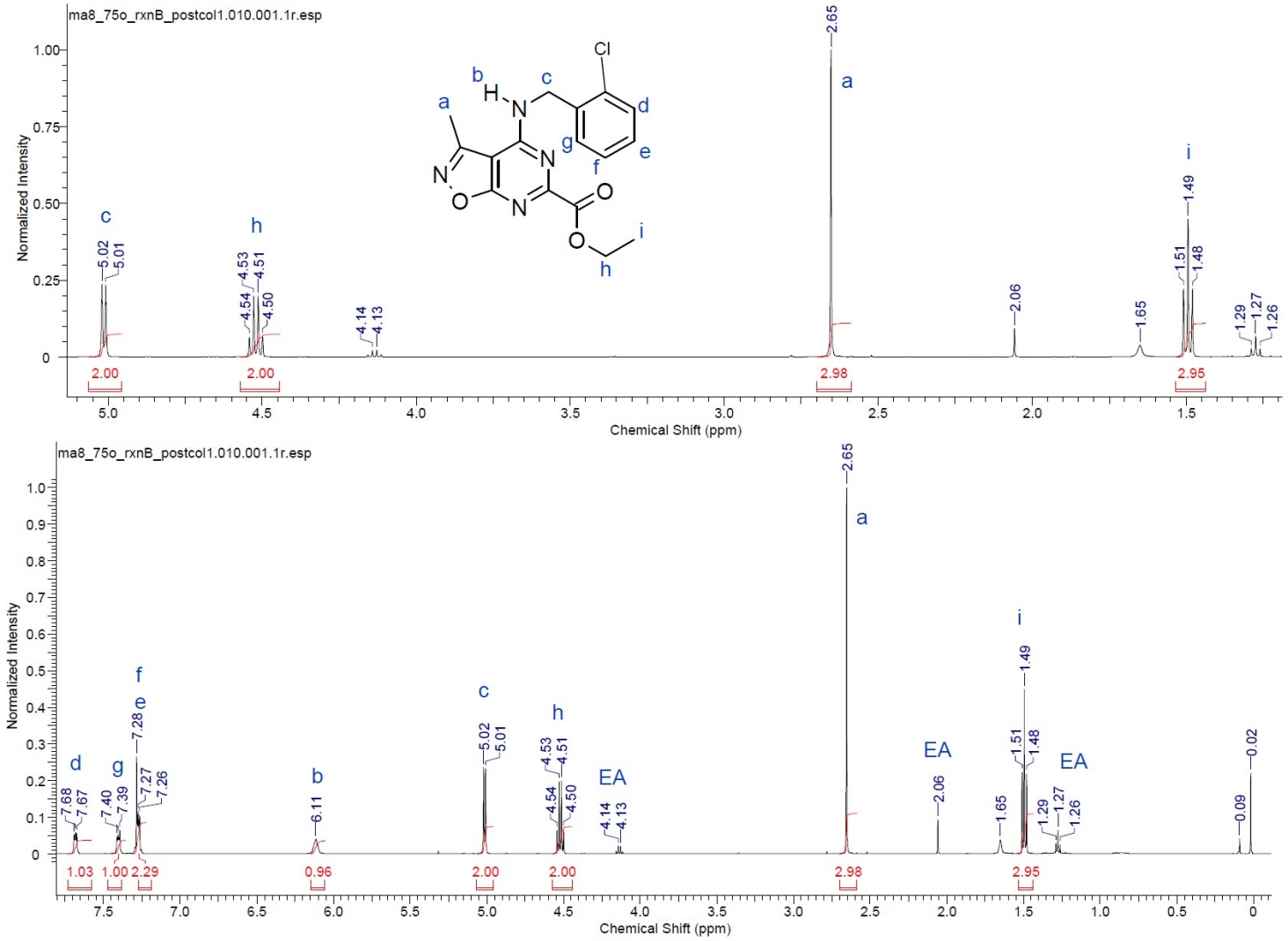


Figure S7. ^{1}H-NMR of 4-(2-Chloro-benzylamino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**5c**) (500 MHz, $CDCl_3$).

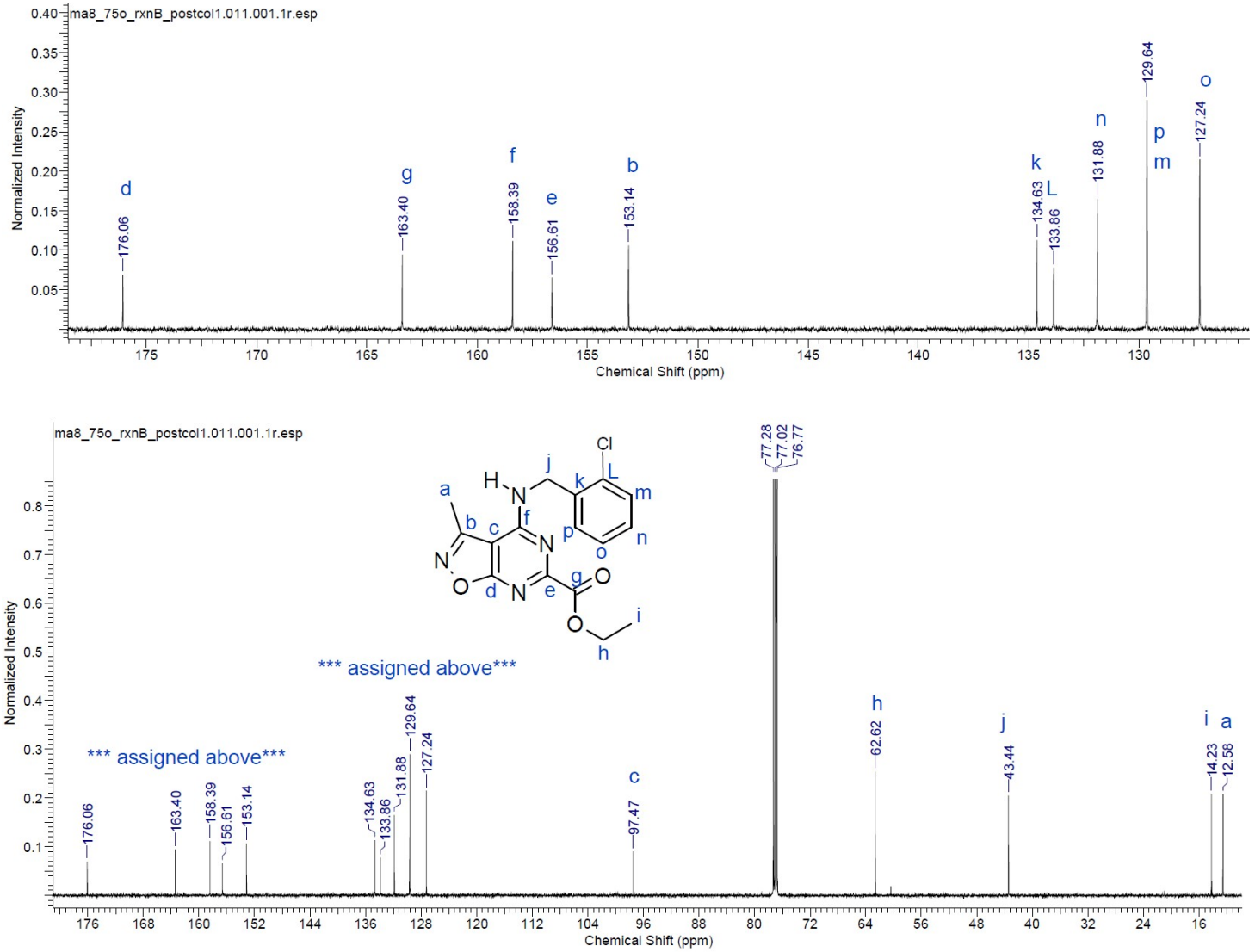


Figure S8. ^{13}C-NMR of 4-(2-Chloro-benzylamino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**5c**) (125MHz, $CDCl_3$).

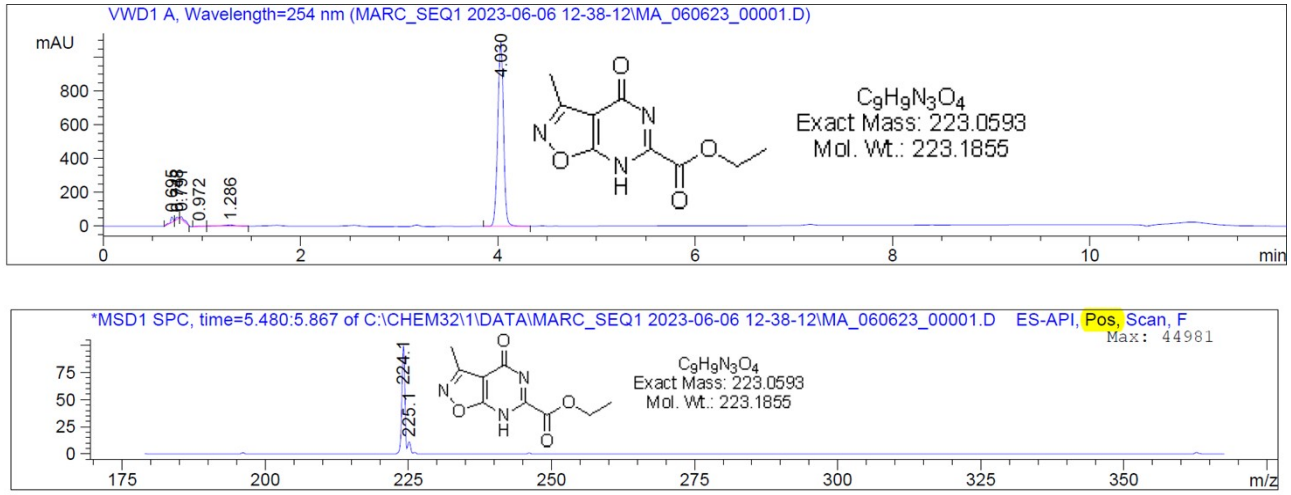


Figure S9. LC-ESI(+)-MS of 3-Methyl-4-oxo-4,5-dihydro-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**3**).

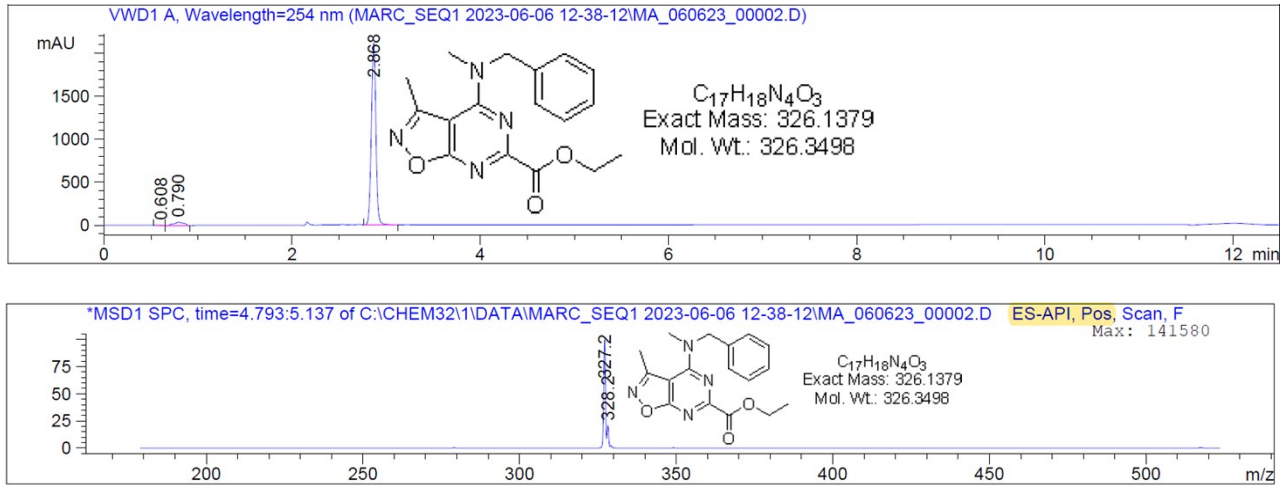


Figure S10. LC-ESI(+)-MS of 4-(Benzyl-methyl-amino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**5a**).

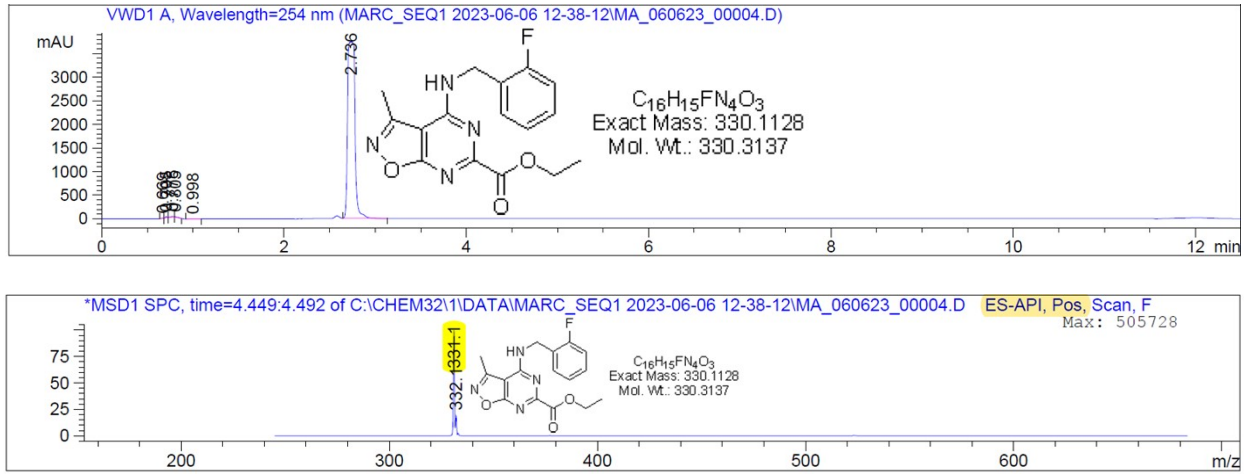


Figure S11. LC-ESI(+)-MS of 4-(2-Fluoro-benzylamino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**5b**).

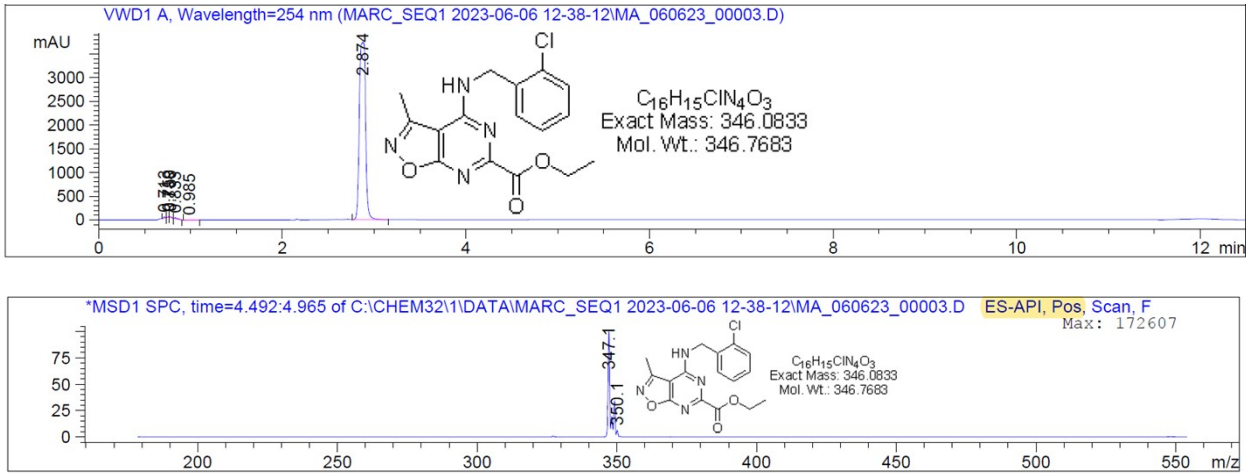


Figure S12. LC-ESI(+)-MS of 4-(2-Chloro-benzylamino)-3-methyl-isoxazolo[5,4-d]pyrimidine-6-carboxylic acid ethyl ester (**5c**).

Reference

1. O. Cil, P. M. Haggie, J. T. Tan, A. A. Rivera and A. S. Verkman, *JCI insight*, 2021, **6**.