**Novel Autotaxin Inhibitors for the Treatment of Osteoarthritis Pain: Lead Optimization via Structure-Based Drug Design**

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**EXPERIMENTAL SECTION**

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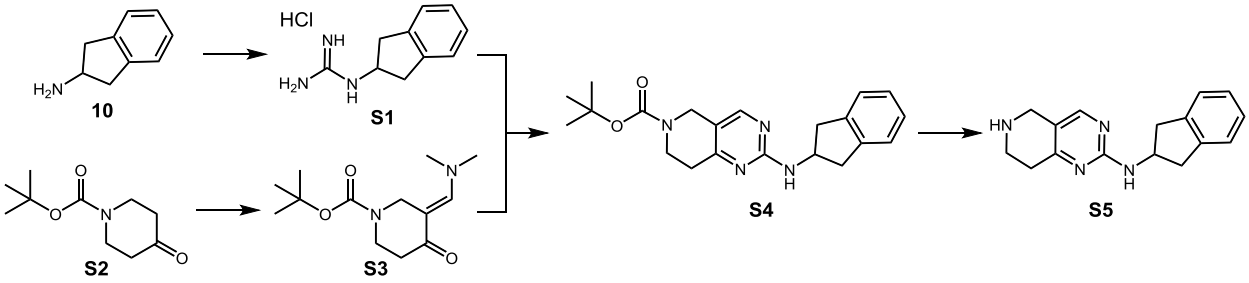
**General Methods**

All reagents and anhydrous solvents were obtained from commercial sources and used without further purification unless noted otherwise. $^{1}$H NMR spectra were recorded on Bruker 400 MHz or Varian 300 or 400 MHz spectrometers. $^{1}$H NMR chemical shifts are reported in ppm with the solvent as the internal standard (DMSO-*d*5 2.49 ppm, $CHCl_3$ 7.26 ppm, $CD_2HOD$ 3.31 ppm). $^{13}$C NMR chemical shifts are reported in ppm with the solvent as the internal standard (DMSO-*d*6 39.52 ppm or $CDCl_3$ 77.23 ppm). Compounds were analyzed for purity by $^{1}$H NMR, HPLC and HPLC-MS, and unless otherwise stated, purities of synthesized compounds were all found to be >95% by $^{1}$H NMR integration or the following HPLC methods: Agilent 1100 HPLC with VWD detectors, 2x50 mm Xbridge C18 4.6 mm × 100 mm × 3.5 μ m; Eluent: 0.1% formic acid in a gradient of 5% to 100% CH3CN in water over 1.75 min or 10mM $NH_4HCO_3$ in water with a gradient of 5% to 95% $CH_3CN$ in water over 1.75 min; Flow rate 1.2 mL/min; Column temp. 50 °C; λ 300 nm and 214 nm.

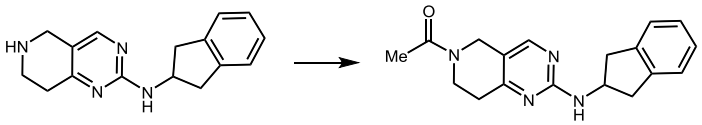



**1-(2,3-dihydro-1H-inden-2-yl)guanidine hydrochloride (S1)** A solution containing 2,3-dihydro-1H-inden-2-amine (**10**) (197 g, 1.16 mol, 1.08 equiv), 1*H*-pyrazole-1-carboximidamide hydrochloride (158 g, 1.08 mol, 1.00 equiv), diisopropylethylamine (400g, 540 mL, 3.09 mol, 2.87 equiv), and acetonitrile (2 L) was stirred at 62 °C for 2 hours, during which time a solid precipitated. The reaction mixture was cooled to 25 °C. The precipitate was filtered and washed with 300 mL acetonitrile and 300 mL methyl tert-butyl ether. The product was dried in the air at 25 °C for 1 h to give the title compound (200 g, 87%) as a white solid. $^{1}$H NMR (400 MHz, DMSO): δ 8.39 (d, *J* = 7.2 Hz, 1H), 8.00-7.90 (m, 8H), 4.40 (sex, *J* = 7.2 Hz, 1H), 3.28 (dd, *J* = 16, 7.6 Hz, 2H) 2.82 (dd, *J* = 16.0, 6.0 Hz, 2H). LC/MS ($ESI^+$): (*m/z*) 176 ($C_{10}H_{14}N_3$ = $(M\text{-}Cl)^+$).

**Tert-butyl 2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate (S4)** A solution of 1,1-dimethoxy-N-N-dimethyl-methanamine (224 g, 1.88 mol, 2.15 equiv) and tert-butyl 4-oxopiperidine-1-carboxylate (**S2**) in dimethylformamide (1.2 L) was stirred at 109 °C under nitrogen for four hours to give intermediate tert-butyl (E)-3-((dimethylamino)methylene)-4-oxopiperidine-1-carboxylate (**S3**) which was not isolated. The mixture was cooled to 25 °C, then ethanol (700 mL) was added followed by 1-(2,3-dihydro-1H-inden-2-yl)guanidine hydrochloride (**S1**) (185 g, 873 mmol, 1.00 equiv) and potassium carbonate (475 g, 3.44 mol) to give a white suspension. The

suspension was stirred at 80-90 °C for 24 h, then cooled to 25 °C and poured into 5 L of ice/water to give a yellow suspension. The suspension was extracted with ethyl acetate (3 x 3 L). The combined organic extracts were washed with 10% lithium chloride solution (3 L), water (3 L), and saturated sodium chloride solution (3 L). The solution was then dried over anhydrous sodium sulfate, filtered and concentrated to give about 300 mL of a red solution. The solution was filtered through a silica gel plug (10 cm height, 5 cm diameter), then concentrated to dryness to give the title compound as an impure red gel (320 g, 100%) which was used without further purification. LC/MS ($ESI^+$): (*m/z*) 367 ($C_{21}H_{27}N_4O_2$ = $(M+1)^+$).

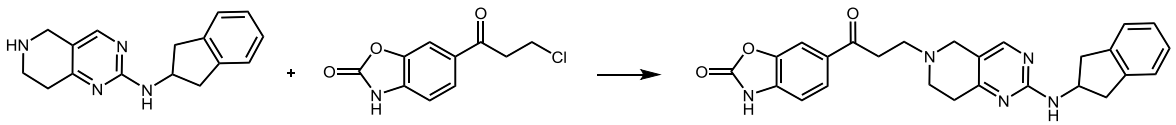
**N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (S5)** To a solution of tert-butyl 2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate (**S4**) (319 g, 871 mmol, 1.00 equiv) in tetrahydrofuran (1.5 L) was added portionwise hydrochloric acid (900 mL, 5 M in water, 4.5 mol, 5.17 equiv) then stirred at 50 °C for one hour. The reaction mixture was cooled to 25 °C, then methyl tert-butyl ether (3 L) and water (1 L) was added. The solution was allowed to stand at 20 °C for 16 hours. The two phases were separated, then the aqueous phase was extracted with dichloromethane (2 L). The organic extracts were discarded and then the aqueous phase pH was adjusted to 10 using 4 M sodium hydroxide. The resulting solution was extracted with ethyl acetate (3 x 3 L). The combined organic extracts were then washed with saturated sodium chloride (2 L), dried over anhydrous sodium sulfate, filtered, and concentrated to give a red gel. The gel was redissolved in ethyl acetate (300 mL) and petroleum ether (200 mL) at 50 °C and then allowed to precipitate over 24 hours. The precipitate was filtered and dried to give the title compound (85 g, 37%). $^1H$ NMR (400 MHz, DMSO): δ 7.98 (s, 1H) 7.23-7.08 (m, 5H), 4.58 (sex, J = 7.2 Hz, 1H), 3.66 (s, 2H), 3.21 (dd, *J* = 15.6, 7.2 Hz, 2H), 2.95 (t, *J* = 6.0 Hz, 2H), 2.84 (dd, *J* = 15.6, 7.2 Hz, 2H), 2.70 (bs, 1H), 2.54 (t, *J* = 6.0 Hz), 2H). LC/MS ($ESI^+$): (*m/z*) 267 ($C_{16}H_{19}N_4$ = $(M+1)^+$).




**S5** **1**

**1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)ethan-1-one (1)** To a solution containing N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (900 mg, 3.38 mmol, 1.00 equiv), DMAP (20 mg, 0.17 mmol, 0.05 equiv), dichloromethane (17 mL) and diisopropylethylamine (1.33 mL, 7.60 mmol, 2.25 equiv) was added acetyl chloride (0.26 mL,

3.72 mmol, 1.1 equiv) and stirred at rt for 16 hours. The reaction mixture was diluted with 50 mL DCM, then washed with saturated sodium bicarbonate solution (10 mL) and saturated sodium chloride solution (10 mL). The solution was dried over anhydrous sodium sulfate, filtered, and concentrated. The crude material was purified via normal phase chromatography (gradient elution: 0-5% methanol in dichloromethane) to give the title compound (827 mg, 2.68 mmol, 79%) as a colorless solid. $^{1}$H NMR (400 MHz, DMSO): δ 60:40 mixture of rotamers, * indicates minor rotamer 8.16 (s, 0.6H), *8.15 (s, 0.4H), *7.33 (d, *J* = 7.2 Hz, 0.4H), 7.31 (d, *J* = 7.2 Hz, 0.6H), 7.25-7.11 (m, 4H), 4.60 (sex, *J* = 7.2 Hz, 1 H), *4.50 (s, 0.8H), 4.45 (s, 1.2H), 3.72-3.66 (m, 2H), 3.22 (dd, *J* = 15.6, 7.6 Hz, 2H), 2.86 (dd, *J* = 16.0, 7.2 Hz), 2.74 (t, *J* = 5.2 Hz, 1.2H), *2.61 (t, *J* = 5.2 Hz, 0.8H), 2.09 (s, 1.8H), *2.08 (s, 1.2H). $^{13}$C NMR (400 MHz, DMSO): δ * indicates minor rotamer 169.2, 163.8, *161.4, 161.3, 156.3, 141.9, 126.7, 124.9, 115.5, *115.3, 52.6, 44.4, 43.08, 38.5, 32.1, *31.4, *22.2, 21.7. LC/MS (ESI$^{+}$): (*m/z*) 309 ($C_{18}H_{21}N_4O$ = (M+1)$^{+}$).

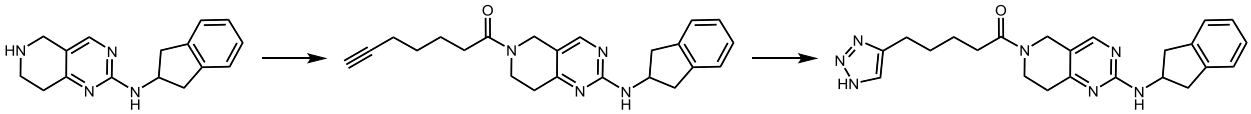



**S5** **S6** **2**

**6-(3-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)propanoyl)benzo[d]oxazol-2(3H)-one (2)** To a flask containing N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (327 mg, 1.23 mmol, 1.03 equiv) was added dichloromethane (4 mL), diisopropylethylamine (0.26 mL, 1.47 mmol, 1.2 equiv) and 6-(3-chloropropanoyl)benzo[d]oxazol-2(3H)-one (**S6**)[1] (270 mg, 1.19 mmol, 1.00 equiv). The reaction mixture was stirred at 40 °C for 10 minutes. The resulting solution was purified directly by silica gel chromatography (gradient elution: 0-20% methanol in dichloromethane) to give the title compound (487 mg, 1.07 mmol, 87%) as a white solid. $^{1}$H NMR (400 MHz, DMSO): δ 12.0 (bs, 1H), 7.99 (s, 1H), 7.86-7.82 (m, 2H), 7.18-7.12 (m, 4H), 7.11-7.07 (m, 2H), 4.54 (sex, *J* = 7.6 Hz, 1H), 3.43 (bs, 2H), 3.27 (t, *J*= 7.0 Hz, 2H), 3.21 (dd, *J* = 16.0, 7.7 Hz, 2H), 2.86 (t, *J*= 6.8 Hz, 2H), 2.84 (dd, *J* = 16.0, 7.3 Hz, 2H), 2.76, (t, *J*= 5.6 Hz, 2H), 2.64 (t, *J* = 5.6 Hz). $^{13}$C NMR (400 MHz, DMSO): δ 198.0, 163.9, 161.5, 155.9, 155.2, 143.8, 142.1,

[1] St-Coeur, P. D., Ferguson, D., Morin, P., Touaibia, M.; PF-8380 and closely related analogs: synthesis and structure-activity relationship towards autotaxin inhibition and glioma cell viability. *Arch. Pharm. Chem. Life Sci.* **2013**, *346*, 91-97.

135.8, 131.4, 126.7, 125.6, 124.9, 116.7, 109.9, 109.4, 53.1, 52.5, 51.9, 50.3, 49.1, 36.4, 32.0. LC/MS ($ESI^+$): (*m/z*) 456 ($C_{26}H_{26}N_5O_3$ = [M+1]+).

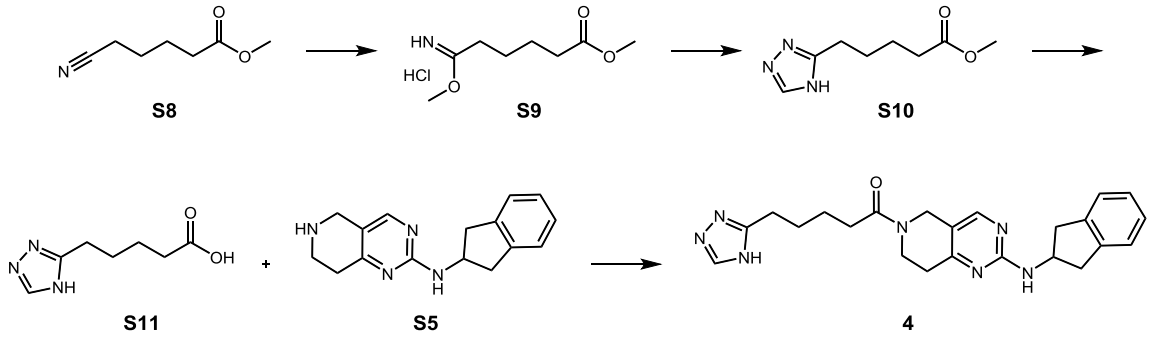


S5 S7 3

**1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)hept-6-yn-1-one (S7)** To a flask containing N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (300 mg, 1.13 mmol, 1.00 equiv) was added 6-heptynoic acid (156 mg, 1.24 mmol, 1.1 equiv), dichloromethane (2 mL), and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide Hydrochloride (320 mg, 1.69 mmol, 1.5 equiv) then stirred at 25 °C for 3 hours. The resulting solution was purified directly by silica gel chromatography (gradient elution: 0-100% ethyl acetate in hexanes) to give the title compound (420 mg, 1.12 mmol, 99%) as a colorless gum. $^1$H NMR (400 MHz, DMSO): ~60:40 mixture of rotamers * indicates minor rotamer δ 8.12 (s, 0.6H), *8.10 (s, 0.4H), 7.29 (d, *J* = 7.2 Hz, 0.6H), *7.27 (d, *J* = 7.2 Hz, 0.4H), 7.18-7.08 (m, 4H), 4.55 (sex, *J*= 7.2 Hz, 1H), *4.47 (s, 0.8H), 4.42 (s, 1.2H), 3.70-3.64 (m, 2H), 3.17 (dd, *J* = 16.0, 7.2 Hz, 2H), 2.82 (dd, *J* = 16.0, 7.2 Hz, 2H), 2.73 (t, *J* = 2.4 Hz, 0.6H), *2.72 (t, *J* = 2.4 Hz, 0.4H), 2.68 (t, *J* = 6.0 Hz, 1.2H), *2.56 (t, *J* = 6.0 Hz, 0.8H), 2.41-2.34 (m, 2H), 2.17-2.11 (m, 2H), 1.60-1.50 (m, 2H), 1.49-1.39 (m, 2H). LC/MS ($ESI^+$): (*m/z*) 375 ($C_{23}H_{27}N_4O$ = $(M+1)^+$).

**1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-5-(1H-1,2,3-triazol-4-yl)pentan-1-one (3)** To a flask containing 1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)hept-6-yn-1-one (**S7**) (230 mg, 0.61 mmol, 1.00 equiv) was added dimethylformamide (6 mL), water (3 mL), Copper(II) sulfate pentahydrate (31 mg, 0.12 mmol, 0.2 equiv) and sodium ascorbate (243 mg, 1.23 mmol, 2.00 equiv). The flask was then evacuated and backfilled with nitrogen x 2 then azidotrimethylsilane (655 uL, 4.91 mmol, 8 equiv) was added and the reaction mixture heated to 90 °C for 2 hours. The reaction mixture was diluted with water (250 mL) and ethyl acetate (50 mL). The organic layer was washed with brine (30 mL), dried over magnesium sulfate, and concentrated. The crude material was purified by silica gel chromatography (gradient elution: 0-8% methanol in ethyl acetate) to give the title compound (87 mg, 0.208 mmol, 34%) as a colorless foam. $^1$H NMR (400 MHz, DMSO): ~60:40 mixture of rotamers * indicates minor rotamer (triazole tautomers also present) δ 8.10 (bs, 0.6H), *8.09 (bs, 0.4H), *7.78 (bs, 0.2H), 7.52 (bs, 0.4H), 7.44 (bs, 0.4H), 7.31-7.26 (m, 1H), 7.18-7.08 (m, 4H), 4.55 (sex, *J* = 7.6 Hz, 1H), *4.46 (bs, 0.8H), 4.42 (bs, 1.2H), 3.70-3.64 (m, 2H),

3.18 (dd, *J* = 16.0, 7.6 Hz, 2H), 2.82 (dd, *J* = 16.0, 7.6 Hz, 2H), 2.70-2.55 (m, 4H), 2.42-2.35 (m, 2H), 1.65-1.45 (m, 4H). LC/MS (ESI$^+$): (*m/z*) 418 ($C_{23}H_{28}N_7O$ = $(M+1)^+$).



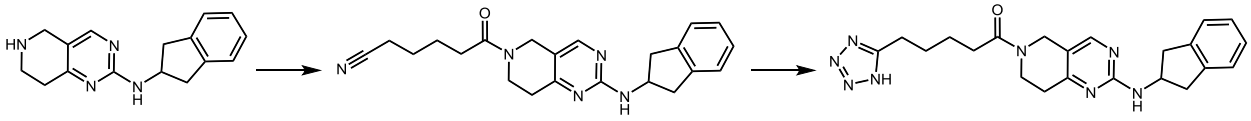

**Methyl 6-imino-6-methoxyhexanoate hydrochloride (S9)** Hydrogen chloride was bubbled through a flask at 0 °C containing methyl 5-cyanopentanoate (**S8**) (3.11 g, 22.02 mmol, 1.00 equiv), methanol (5 mL) and diethyl ether (10 mL) for 1 hour. The reaction mixture was warmed to 25 °C over one hour. To this mixture was added diethyl ether (50 mL) which caused the product to precipitate. The mixture was stirred vigorously for 15 minutes then filtered and rinsed with diethyl ether (20 mL) to give the title compound (3.82 g, 18.2 mmol, 83%) as a white solid. $^1$H NMR (400 MHz, DMSO): δ 4.03 (s, 3H), 3.56 (s, 3H), 2.59 (t, *J* = 7.6 Hz, *2*H), 2.31 (t, *J* = 7.6 Hz, *2*H), 1.47-1.62 (m, 4H).

**Methyl 5-(4H-1,2,4-triazol-3-yl)pentanoate (S10)** To a flask containing methyl 6-imino-6-methoxyhexanoate hydrochloride (**S9**) (542 mg, 2.58 mmol, 1.00 equiv) was added methanol (1 mL), triethylamine (360 μL, 2.58 mmol, 1.00 equiv), and formylhydrazine (155 mg, 2.58 mmol, 1.00 equiv) dissolved in methanol (3 mL). The resulting solution was stirred at 25 °C for 16 hours then to 70 °C for three hours. The reaction mixture was concentrated *in vacuo*, then diluted with ethyl acetate (30 mL). The precipitated triethylamine hydrochloride was filtered off. The supernatant was concentrated to give the title compound (498 mg, 2.72 mmol, 100%) as an impure colorless oil which was used without further purification. LC/MS (ESI$^+$): (*m/z*) 184 ($C_{18}H_{14}N_3O_2$ = $(M+1)^+$).

**5-(4H-1,2,4-triazol-3-yl)pentanoic acid (S11)** To a flask containing methyl 5-(4H-1,2,4-triazol-3-yl)pentanoate (**S10**) (498 mg, 2.72 mmol, 1.00 equiv) was added methanol (3 mL), tetrahydrofuran (3 mL), water (2 mL) and lithium hydroxide (261 mg, 10.9 mmol, 4.0 equiv). The solution was stirred at 25 °C for 90 minutes then cooled to 0 °C. To this solution was added hydrogen chloride solution (5M in water, 2.18 mL, 10.9 mmol, 4.0 equiv). The solution was concentrated on the rotovap then the resulting crude material was dissolved in dichloromethane (30 mL) and then dried over magnesium sulfate,

filtered, and concentrated to give the title compound (1.60 g) which formed an adduct with methanol. The semi pure material was dissolved in isopropyl alcohol and concentrated to give an isopropanol adduct (30 wt% **S11** by $^1$H NMR). $^1$H NMR (400 MHz, DMSO): δ 13.80 (bs, 1H), 11.86 (bs, 1H), 7.80 (bs, 1H), 2.66-2.55 (m, 2H), 2.17 (t, *J* = 7.2 Hz, 2H), 1.62 (p, *J* = 7.2 Hz, 2H), 1.42 (p, *J* = 7.2 Hz, 2H). LC/MS (ESI$^+$): (*m/z*) 170 ($C_7H_{12}N_3O_2$ = $(M+1)^+$).

**1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-5-(4H-1,2,4-triazol-3-yl)pentan-1-one (4)** To a flask containing 5-(4H-1,2,4-triazol-3-yl)pentanoic acid (**S11**) (0.4 g) and N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (190 mg, 0.71 mmol, 1.00 equiv) was added dimethylformamide (2 mL), dichloromethane (2 mL), and N,N-dimethylaminopyridine (17 mg, 0.14 mmol, 0.2 equiv) then cooled to 0 °C and added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (204 mg, 1.06 mmol, 1.5 equiv). The mixture was then heated to 40 °C for 22 hours. The solution was diluted with water (250 mL) and ethyl acetate (50 mL). The solution was extracted with ethyl acetate (5 x 25 mL) then washed the combined organic extracts with brine (10 mL). Dried the solution over magnesium sulfate, filtered, and concentrated. Purified the crude material by silica gel chromatography (gradient elution: 0-12% methanol in ethyl acetate) to give the title compound (80 mg, 0.19 mmol, 27%) as a white foam. $^1$H NMR (400 MHz, DMSO): ~60:40 mixture of rotamers * indicates minor amide rotamer (triazole tautomers also present) δ *13.56 (bs, 0.3H), 13.52 (bs, 0.7H), *8.35 (s, 0.3H), 8.13 (s, 0.6H), *8.11 (s, 0.4H), 7.76 (s, 0.7H), *7.29 (d, *J* = 7.2 Hz, 0.4H), 7.28 (d, *J* = 7.2 Hz, 0.6H), 7.18-7.07 (m, 4H), 4.55 (sex, *J* = 7.2 Hz, 1H), *4.46 (s, 0.8H), 4.42 (s, 1.2H), 3.70-3.63 (m, 2H), 3.19 (dd, *J* = 15.2, 6.8 Hz, 2H), 2.82 (dd, *J* = 15.2, 6.8 Hz, 2H), 2.71-2.52 (m, 4H), 2.44-2.33 (m, 2H), 1.70-1.59 (m, 2H), 1.55-1.43 (m, 2H). *LC*/MS (ESI$^+$): (*m/z*) 418 ($C_{23}H_{28}N_7O$ = $(M+1)^+$).

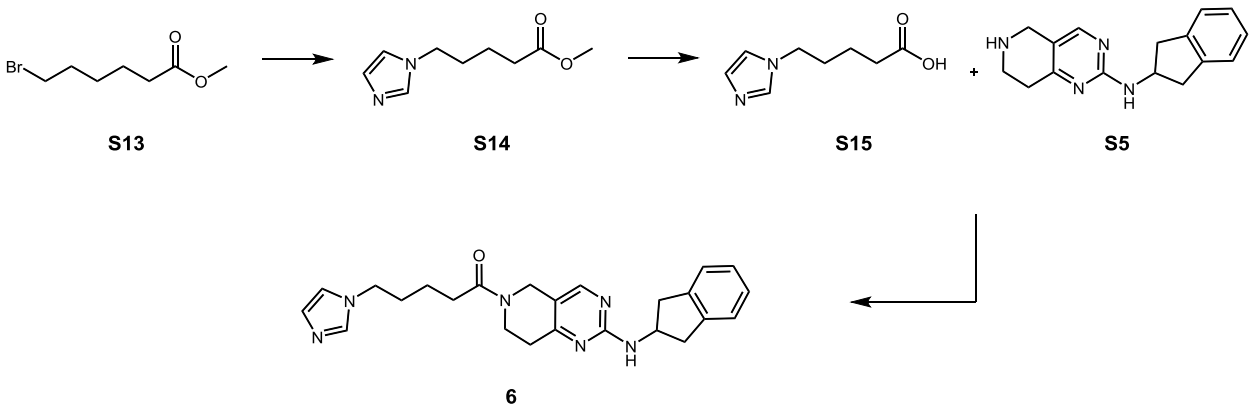



**S5** **S12** **5**

**6-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-6-oxohexanenitrile (S12)** To a flask containing N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (250 mg, 0.94 mmol, 1.00 equiv) was added 5-bromovaleric acid (204 mg, 1.13 mmol, 1.2 equiv), dichloromethane (2 mL) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.200 g, 1.03 mmol, 1.10 equiv) and then stirred at 25 °C for 90 minutes before purifying

directly by silica gel chromatography (gradient elution: 0-100% ethyl acetate in hexanes) to give the impure intermediate bromide (389 mg). To the bromide (389 mg) was added dimethylformamide (2 mL) and sodium cyanide (74.0 mg, 1.51 mmol, 1.60 equiv) then the reaction mixture was heated to 100 °C for 90 minutes. Diluted the reaction mixture with ethyl acetate (25 mL) and water (100 mL). Extracted the aqueous layer with ethyl acetate (3 x 25 mL). Washed the combined organic extracts over brine (10 ml), dried over magnesium sulfate, filtered and concentrated. Purified the crude material via silica gel chromatography (gradient elution: 0-100% ethyl acetate in hexanes) to give the title compound (0.148 g, 0.394 mmol, 42%) as a yellow glass. $^{1}$H NMR (400 MHz, DMSO): ~60:40 mixture of rotamers * indicates minor amide rotamer δ. 8.12 (bs, 0.6H), *8.10 (bs, 0.4H), *7.30 (d, *J* = 7.2 Hz, 0.4H), 7.28 (d, *J* = 7.2 Hz, 0.6H), 7.19-7.07 (m, 4H), 4.55 (sex, *J* = 7.2 Hz, 1H), *4.47 (s, 0.8H), 4.42 (s, 1.2H), *3.68 (t, *J* = 5.2 Hz, 0.8H), 3.67 (t, *J* = 5.2 Hz, 1.2H), 3.18 (dd, *J* = 16.0, 7.6 Hz, 2H), 2.81 (dd, *J* = 16.0, 7.6 Hz, 2H), 2.68 (t, *J* = 5.7 Hz, 1.2H), *2.58 (t, *J* = 5.7 Hz, 0.8H), 2.45-2.38 (m, 2H), 1.63-1.50 (m, 4H). *LC*/MS (ESI$^{+}$): (*m/z*) 376 ($C_{22}H_{26}N_5O$ = $(M+1)^{+}$).

**1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-5-(1H-tetrazol-5-yl)pentan-1-one (5)** To a flask containing 6-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-6-oxohexanenitrile (**S12**) (60 mg, 0.16 mmol, 1.00 equiv) was added toluene (0.8 mL), azidotrimethylsilane (0.213 mL, 1.6 mmol, 10 equiv), and dibutyloxostannane (10 mg, 0.040 mmol, 0.25 equiv). The flask was heated to 100 °C for six hours. Purified the crude material via reverse phase chromatography to give the title compound (70 mg, 0.16 mmol, 99%) as a white solid. $^{1}$H NMR (400 MHz, DMSO): ~60:40 mixture of rotamers * indicates minor amide rotamer δ 15.96 (bs (1H), 8.14 (s, 0.6H), *8.11 (s, 0.4H), *7.28 (d, *J* = 7.6 Hz, 0.4H), 7.26 (d, *J* = 7.6 Hz, 0.6H), 7.20-7.05 (m, 4H), 4.55 (sex, *J* =7.6 Hz, 1H), *4.47 (s, 0.8H), 4.42 (s, 1.2H), 3.70-3.64 (m, 2H), 3.19 (dd, *J* = 16.0, 7.2 Hz, 2H), ), 2.86 (t, J= 7.6 Hz, 2H), 2.83 (dd, *J* = 16.0, 7.2 Hz, 2H), 2.69 (t, *J* = 5.7 Hz, 1.2H), *2.58 (t, *J* = 5.7 Hz, 0.8H), 2.47-2.37 (m, 2H), 1.74-1.65 (m, 2H), 1. 58-1.48 (m, 2H). *LC*/MS (ESI$^{+}$): (*m/z*) 419 ($C_{22}H_{27}N_8O$ = $(M+1)^{+}$).

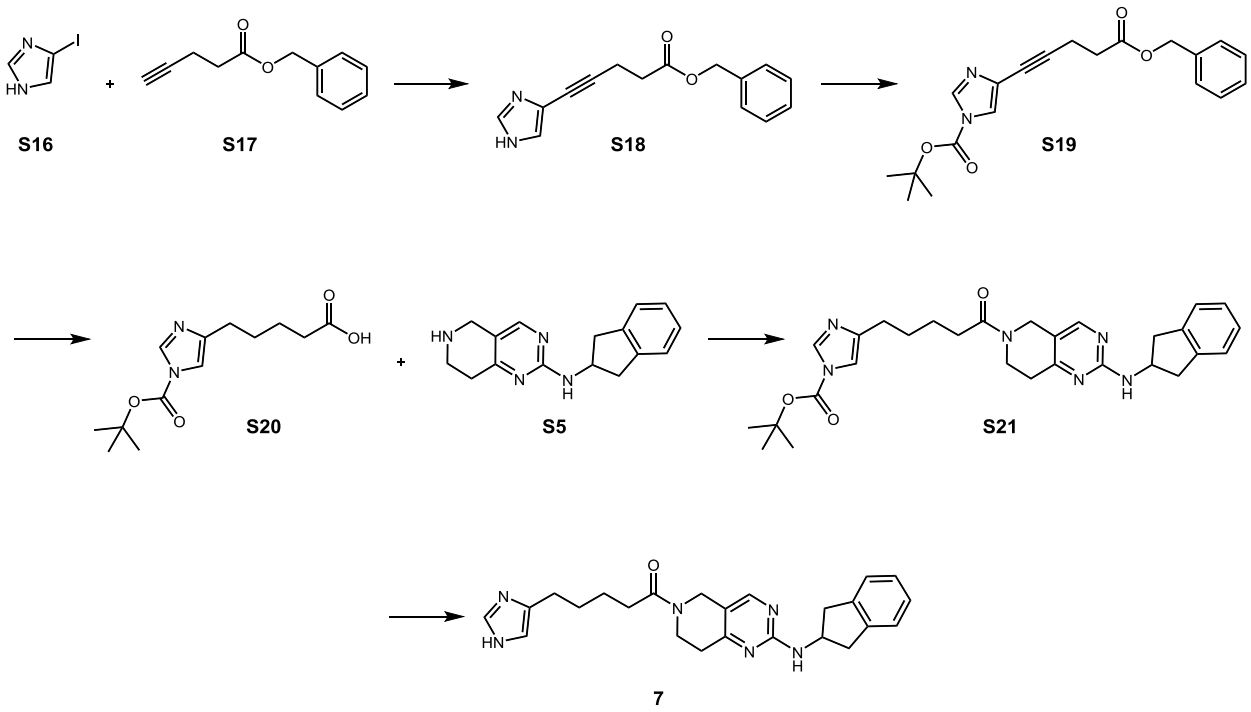



**Methyl 5-(1H-imidazol-1-yl)pentanoate (S14)** A flask containing 1*H*-imidazole (0.30 g, 4.4 mmol, 1.0 equiv) and methyl 5-bromopentanoate (**S13**) (1.03 g, 5.29 mmol, 1.2 equiv) and dimethylformamide (20 mL) was stirred at 25 °C for 18 hours before diluting with water (15 mL). The resulting solution was extracted with dichloromethane (50 mL) then washed with brine (20 mL). Dried the combined organic extracts over sodium sulfate, filtered, and concentrated. Purified the crude material by silica gel chromatography (gradient elution: 0-10% methanol in dichloromethane) to give the title compound (0.655 g, 3.59 mmol, 82%) as a yellow oil. *LC*/MS (ESI$^+$): (*m/z*) 183 ($C_9H_{15}N_2O_2$ = (M+1)$^+$).

**5-(1H-imidazol-1-yl)pentanoic acid (S15)** A flask containing methyl 5-(1H-imidazol-1-yl)pentanoate (**S14**) was added tetrahydrofuran (20 mL), water (15 mL) and lithium hydroxide (553 mg, 13.2 mmol, 4 equiv) and then stirred at 25 °C for 18 hours. Acidified the solution with hydrochloric acid (5 N in water, 3 mL, 15 mmol) then concentrated *in vacuo*. Purified the crude material directly by silica gel chromatography (gradient elution: 0-15% methanol in dichloromethane) to give the title compound (108 mg, 0.642 mmol, 19%) as an oil. *LC*/MS (ESI$^+$): (*m/z*) 169 ($C_8H_{13}N_2O_2$ = (M+1)$^+$).

**1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-5-(1H-imidazol-1-yl)pentan-1-one (6)** To a flask containing 5-(1H-imidazol-1-yl)pentanoic acid (**S15**) (150 mg, 0.90 mmol, 1.2 equiv) was added N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (200 mg, 0.75 mmol, 1.00 equiv), 1-hydroxybenzotriazole (0.20 g, 1.50 mmol, 2.00 equiv), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.29 g, 1.50 mmol, 2.00 equiv), dichloromethane ( 20 mL) and triethylamine (0.52 mL, 3.75 mmol, 5.00 equiv) then the mixture was stirred at 25 °C for 16 hours. The mixture was concentrated *in vacuo*. To the residue was added water (10 mL) and ethyl acetate (20 mL). After separation of the organic layer, the water layer was extracted with ethyl acetate (2 x 20 mL) then the combined organic extracts were dried over sodium sulfate,

filtered, and concentrated. The product was purified by silica gel chromatography (gradient elution: 0-10% methanol in dichloromethane) to give the title compound as an impure mixture (350 mg). The residue was purified by preparative HPLC (12 injections: Gemini 19*100mm c18, 5μm, Mobile Phase A: water(10mM $NH_4HCO_3$) B: ACN Gradient 35-55% B in 10min, stop at 15min: Flow Rate(ml/min) 24.00) to give the title compound (140 mg, 0.34 mmol, 45%) as a colorless oil. $^1$H NMR (400 MHz, $CDCl_3$): ~55:45 mixture of rotamers * indicates minor amide rotamer δ 7.94 (bs, 1H), 7.44 (bs, 1 H), 7.28-6.90 (m, 6H),5.66 (bs, 1H), 4.79 (sex, *J* = 6.0 Hz, 1H), 4.74 (s, 1.1H), *4.52 (s, 0.9H), 3.95-3.92 (m, 2H), *3.80 (t, *J* = 5.6 Hz, 0.9H), 3.64 (t, *J* = 5.6 Hz, 1.1H), 3.35-3.10 (m, 2H), 2.92-2.67 (m, 4H), 2.36 (t, *J* = 7.2 Hz, 2H), 1.95-1.65 (m, 4H). *LC*/MS (ESI$^+$): (*m/z*) 417 ($C_{24}H_{29}N_6O$ = $(M+1)^+$).




B**enzyl 5-(1H-imidazol-4-yl)pent-4-ynoate (S18)** To a flask containing 4-iodo-1H-imidazole (**S16**) (388 mg, 2.00 mmol, 1.00 equiv), and benzyl pent-4-ynoate (**S17**) (376 mg, 2.00 mmol, 1.00 equiv) was added dimethylformamide (8 mL), triethylamine (0.84 mL, 6.0 mmol, 3.0 equiv), and bis(triphenylphosphine)palladium(II) chloride (140 mg, 0.20 mmol, 0.1 equiv). Heated the solution to 75 °C for 16 hours, then diluted with water (30 mL) and extracted with ethyl acetate (3 x 30 mL). Washed the combined organic extracts with brine (20 ml), dried over magnesium sulfate, filtered, and concentrated. Purified the product by silica gel chromatography (gradient elution: 0-10% methanol in

dichloromethane) to give the title compound (0.350 g, 1.38 mmol, 69%) as a colorless oil. *LC*/MS ($ESI^+$): (*m/z*) 255 ($C_{15}H_{15}N_2O_2$ = $(M+1)^+$).

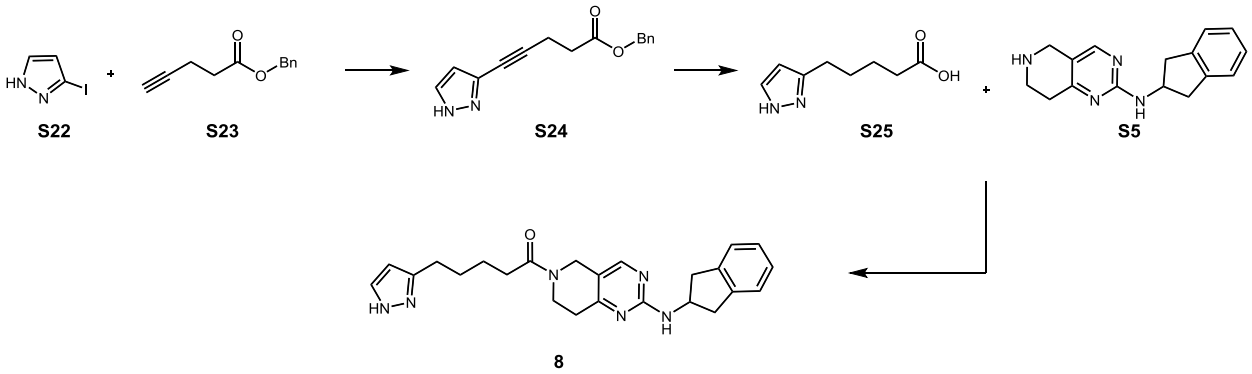
**Tert-butyl 4-(5-(benzyloxy)-5-oxopent-1-yn-1-yl)-1H-imidazole-1-carboxylate (S19)** To a flask containing benzyl 5-(1H-imidazol-4-yl)pent-4-ynoate (**S18**) (190 mg, 0.747 mmol, 1.00 equiv) was added di-t-butyldicarbonate (210 mg, 0.96 mmol, 1.29 equiv), dichloromethane (10 mL), triethylamine (0.21 mL, 1.49 mmol, 2.00 equiv) and DMAP (9 mg, 0.074 mmol, 0.1 equiv) then stirred at 25 °C for two hours. Concentrated the solution then purified directly by silica gel chromatography (25% ethyl acetate in hexanes) to give the title compound (0.170 g, 0.479 mmol, 64%) as a white solid. *LC*/MS ($ESI^+$): (*m/z*) 299 ($C_{16}H_{15}N_2O_4$ = $(M-tBu+1)^+$).

**5-(1-(tert-butoxycarbonyl)-1H-imidazol-4-yl)pentanoic acid (S20)** To a flask containing tert-butyl 4-(5-(benzyloxy)-5-oxopent-1-yn-1-yl)-1H-imidazole-1-carboxylate (**S19**) (170 mg, 0.48 mmol, 1.00 equiv) was added palladium (10% on carbon, 30 mg, 0.014 mmol) and methanol (13 mL). Stirred under an atmosphere of hydrogen for 16 hours. Filtered the reaction mixture, then concentrated to give the title compound (128 mg, 0.477 mmol, 99%) as a white solid. *LC*/MS ($ESI^+$): (*m/z*) 213 ($C_9H_{13}N_2O_4$ = $(M-tBu+1)^+$).

**Tert-butyl 4-(5-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-5-oxopentyl)-1H-imidazole-1-carboxylate (S21)** Added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (92 mg, 0.48 mmol, 1.5 equiv) to a flask containing 5-(1-(tert-butoxycarbonyl)-1H-imidazol-4-yl)pentanoic acid (**S20**) (84 mg, 0.31 mmol, 1.00 equiv), N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (64 mg, 0.24 mmol, 0.77 equiv), 1-hydroxybenzotriazole (65 mg, 0.48 mmol, 1.5 equiv) and triethylamine (0.20 mL, 1.44 mmol, 4.5 equiv) in dichloromethane (15 mL) and stirred at 40 °C for 16 hours. Concentrated the reaction mixture then purified the product by silica gel chromatography (gradient elution: 0-10% methanol in dichloromethane) to give the title compound (40 mg, 0.077 mmol, 25%) as a white solid. *LC*/MS ($ESI^+$): (*m/z*) 517 ($C_{29}H_{37}N_6O_3$ = $(M+1)^+$).

**1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-5-(1H-imidazol-4-yl)pentan-1-one (7)** To a flask containing tert-butyl 4-(5-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-5-oxopentyl)-1H-imidazole-1-carboxylate (**S21**) (30 mg, 0.58 mmol, 1.00 equiv) was added trifluoroacetic acid (1.5 mL) and dichloromethane (8 mL). Stirred the solution at 25 °C for two hours. Concentrated the solution then added saturated aqueous sodium

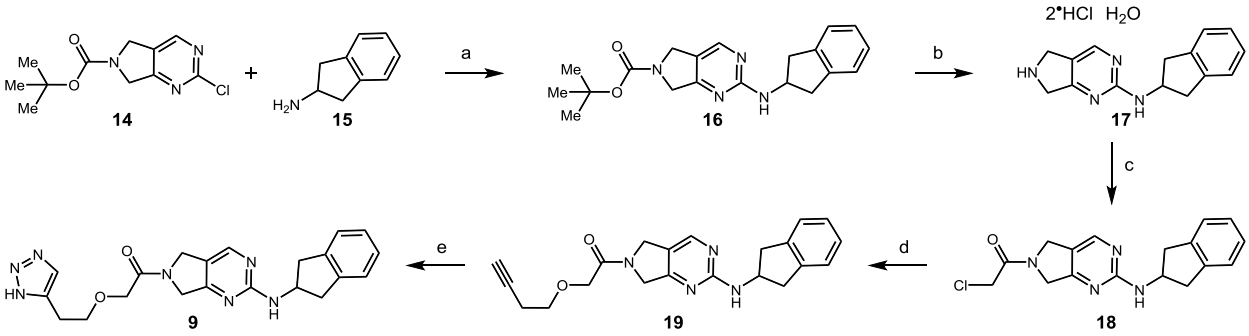
bicarbonate solution (10 mL), water (20 mL) and dichloromethane 20 mL. Separated the organic layer and further extracted the aqueous layer with dichloromethane (2 x 20 mL). Washed the combined organic extracts with brine (10 mL), dried over sodium sulfate, filtered, and concentrated. Purified the residue by silica gel chromatography (gradient elution: 0-10% methanol in dichloromethane) to give the title compound (20 mg, 83%) as a white solid. $^1$H NMR (400 MHz, $CD_3OD$): δ 8.10 (s, 1H), 7.65 (bs, 1H), 7.24-7.05 (m, 5H), 6.75 (bs, 1H), 4.69 (pent, *J* = 7.2 Hz, 1H), 4.55 (s, 2H), 3.82-3.70 (m, 2H), 2.89-2.44 (m, 10H), 1.83-1.77 (m, 4H). *LC*/MS (ESI$^+$): (*m/z*) 417 ($C_{24}H_{29}N_6O$ = (M+1)$^+$).




**benzyl 5-(1H-pyrazol-3-yl)pent-4-ynoate (S24)** To a flask containing 3-iodo-1H-pyrazole (**S22**) (388 mg, 2.00 mmol, 1.00 equiv) was added benzyl pent-4-ynoate (**S23**) (376 mg, 2.00 mmol, 1.00 equiv), bis(triphenylphosphine)palladium (II) chloride (140 mg, 0.200 mmol, 0.10 equiv), triethylamine (835 uL, 5.99 mmol, 3.00 equiv), copper (I) iodide (38.6 mg, 0.203 mmol, 0.10 equiv) and dimethylformamide (8 mL). The flask was purged with nitrogen then heated to 75 °C for 16 hours. The reaction mixture was transferred to a separatory funnel with water (30 mL) and extracted with ethyl acetate (2x20 mL). The combined organic extracts were washed with brine (20 mL), dried over magnesium sulfate, filtered, and concentrated. The crude mixture was purified by silica gel chromatography (1:10 methanol:dichloromethane) to give the title compound (360 mg, 1.42 mmol, 71%) as a colorless oil. *LC*/MS (ESI$^+$): (*m/z*) 255 ($C_{15}H_{15}N_2O_2$ = (M+1)$^+$).

**5-(1H-pyrazol-3-yl)pentanoic acid (S25)** To a flask containing benzyl 5-(1H-pyrazol-3-yl)pent-4-ynoate (**S24**) (450 mg, 1.77 mmol) was added methanol (40 mL) and palladium (5% on carbon, 50 mg). The reaction mixture was purged with hydrogen then heated to 60 °C for four hours. The mixture was filtered and the palladium residue washed with methanol (10 mL) and ethyl acetate (10 mL). The organic solution was then concentrated to give the title compound (200 mg, 1.19 mmol, 67%) as a colorless oil. *LC*/MS (ESI$^+$): (*m/z*) 169 ($C_8H_{13}N_2O_2$ = (M+1)$^+$).

**1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-5-(1H-pyrazol-3-yl)pentan-1-one (8)** To a flask containing 5-(1H-pyrazol-3-yl)pentanoic acid (**S25**) (190 mg, 1.13 mmol, 3.00 equiv) was added N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (100 mg, 0.38 mmol, 1.00 equiv), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (216 mg, 1.13 mmol, 3.00 equiv), 1-hydroxybenzotriazole (152 mg, 1.13 mmol, 3.00 equiv), triethylamine (471 uL, 3.38 mmol, 9.00 equiv) and dichloromethane (50 mL). The flask was stirred for 16 hours at room temperature. The reaction mixture was transferred to a separatory funnel with dichloromethane (20 mL) and water (20 mL) and then extracted with dichloromethane (3x20 mL). The combined organic extracts were washed with brine (30 mL), dried over magnesium sulfate, filtered, and concentrated to give crude product. The product was purified via HPLC (column, pHlex ODS, 21.2x250 mm, 10 μm, 100A, mobile phase A: water (10 mM $NH_4HCO_3$) B: acetonitrile, Gradient 30-50% B in 20 minutes, flow 24 mL/min, detection wavelength (214 nm, retention time 12.6 min, number of injections 16) to give the title compound (18 mg, 43 umol, 12%) as a white solid. $^1$H NMR (300 MHz, $CD_3OD$): ~70:30 mixture of rotamers * indicates minor amide rotamer δ 8.01 (s, 1H), 7.45 (s, 1H), 7.19-7.05 (m, 5H), 6.08 (bs, 0.7H), *6.03 (bs, 0.3H), 4.68 (pent, *J* = 7.2Hz, 1H), 4.53 (s, 2H), 3.84-3.71 (m, 2H), 3.35-3.27 (m, 2H), 2.89-1.99 (m, 8H), 1.72-1.62 (m, 4H). *LC*/MS (ESI$^+$): (*m/z*) 417 ($C_{24}H_{29}N_6O$ = $(M+1)^+$).




**Tert-butyl 2-((2,3-dihydro-1H-inden-2-yl)amino)-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidine-6-carboxylate (16)** Charged diisopropylethylamine (450 mL) into a flask cooled to 15 °C containing tert-butyl 2-chloro-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidine-6-carboxylate (**14**) (220 g, 860.4 mmol, 1.00 equiv), 2,3-dihydro-1H-inden-2-amine (**15**) (137 g, 1.03 mol, 1.20 equiv) in 1-methylpyrrolidin-2-one (3.6 L). Heated the resulting mixture to 80 °C for 16 hours, then cooled to 30 °C and transferred the resulting mixture into water (5 L) at 25 °C. Filtered the resulting solid and rinsed with water (2 x 300 mL). Reslurried the solid in ethyl acetate (350 mL) for 45 min at 15 °C. Filtered the resulting slurry then rinsed with 15 °C ethyl acetate (2 x 250 mL) to give the title compound (226 g, 641 mmol, 75%) as an off-white

solid. $^1$H NMR (400 MHz, DMSO): ~50:50 mixture of rotamers * indicates minor carbamate rotamer δ *8.24 (s, 0.5H), 8.23 (s, 0.5H), 7.47 (d, *J* = 7.2 Hz, 1H), 7.18-7.07 (m, 4H), 4.55 (sex, *J* = 7.2 Hz, 1H), 4.41 (bs, 1H), *4.37 (bs, 1H), *4.33 (bs, 1H), 4.29 (bs, 1H), 3.19 (dd, *J* = 15.6, 7.2 Hz, 2H), 2.82 (dd, *J* = 15.6, 7.2 Hz, 2H). *LC*/MS (ESI$^+$): (*m/z*) 353 ($C_{20}H_{25}N_4O_2$ = $(M+1)^+$).

**N-(2,3-dihydro-1H-inden-2-yl)-6,7-dihydro-5H-pyrrolo[3,4-d]pyrimidin-2-amine dihydrochloride hydrate (17)** To a flask containing tert-butyl 2-((2,3-dihydro-1H-inden-2-yl)amino)-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidine-6-carboxylate (**16**) (226 g, 641 mmol, 1.00 equiv) was added tetrahydrofuran (2 L) then cooled to 17 °C before adding hydrochloric acid (5 M in water, 670 mL) with cooling to keep the temperature below 26 °C during the addition. Heated the resulting solution to 50 °C for 16 hours, cooled to 25 °C then diluted with water (500 mL) and tert-butylmethylether ( 500 mL). Separated the organic layer then washed the aqueous layer with tert-butylmethylether (3 x 1 L). Concentrated the aqeuous phase to a volume of 200 mL and filtered the resulting slurry. Rinsed the filter cake with tert-butylmethylether (2 x 200 mL) and dried to give the title compound (177 g, 516 mmol, 80%) as a light brown solid. $^1$H NMR (400 MHz, DMSO): δ 10.38 (bs, 2H), 8.37 (bs, 2H), 7.25-7.10 (m, 4H), 6.50 (bs, 3H), 4.65 (pent, *J* = 7.2 Hz, 1H), 4.4.40 (t, *J* = 4.8 Hz, 2H), 4.36-4.29 (m, 2H), 3.26 (dd, *J* = 15.6, 7.2 Hz, 2H), 2.89 (dd, *J* = 15.6, 7.2 Hz, 2H). LC/MS (ESI$^+$): (*m/z*) 253 ($C_{15}H_{17}N_4$ = $(M-2HCl-H2O+1)^+$).

**2-chloro-1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidin-6-yl)ethan-1-one (18)** Stirred a suspension of N-(2,3-dihydro-1H-inden-2-yl)-6,7-dihydro-5H-pyrrolo[3,4-d]pyrimidin-2-amine dihydrochloride hydrate (**17**) (14.4 g, 41.9 mmol, 1.00 equiv) and triethylamine (19.7 mL, 141 mmol, 3.37 equiv) in dichloromethane (200 mL) for 10 minutes at 23 °C. Cooled the reaction mixture to -30 °C then added 2-chloroacetyl chloride (3.86 mL, 48.6 mmol, 1.16 equiv) over two minutes then warmed to 23 °C over 10 minutes. Added methanol (5 mL) to the mixture, then concentrated *in vacuo*. Slurried the crude reaction mixture in methanol (30 mL), added 50 g silica gel and then removed the solvent *in vacuo*. Loaded the resulting silica gel onto a loading column and purified via silica gel chromatography (gradient elution: 50-100% ethyl acetate in hexanes, then 0-10% methanol in ethyl acetate ) to give the title compound (11.5 g, 35.5 mmol, 84%). $^1$H NMR (400 MHz, $CDCl_3$): ~50:50 mixture of rotamers * indicates minor amide rotamer δ 8.19 (bs, 0.5H), *8.18 (bs, 0.5H), 7.22-7.12 (m, 4H), *4.82 (s, 1H), 4.77 (pent, *J* = 7.2 Hz, 1H), *4.70 (s, 1H), 4.68 (s, 1H), 4.61 (s, 1H), *4.08 (s, 1H), 4.06 (s, 1H), 3.36 (dd, *J* = 16.0, 7.2 Hz, 2H), 2.85 (dd, *J* = 16.0, 5.0 Hz, 2H). LC/MS (ESI$^+$): (*m/z*) 329 ($C_{17}H_{18}ClN_4O$ = $(M+1)^+$).

**2-(but-3-yn-1-yloxy)-1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidin-6-yl)ethan-1-one (19)** To a solution of sodium hydride (60 wt% in mineral oil, 2.06 g, 51.4 mmol, 2.00 equiv) in tetrahydrofuran (86 mL) at 0 °C was added 3-butyn-1-ol (4.64 g, 64.3 mmol, 2.50 equiv), then stirred at 23 °C for 15 minutes. Added this solution to 2-chloro-1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidin-6-yl)ethan-1-one (**18**) (8.45 g, 25.7 mmol, 1.00 equiv) in tetrahydrofuran (86 mL) at 0 °C and stirred for five minutes. Poured the reaction mixture into 50% saturated aqueous sodium bicarbonate solution. Separated the organic layer and further extracted the aqueous layer with ethyl ether (2 x 50 mL) and ethyl acetate (2 x 50 mL). Combined the organic extracts and washed with brine (20 mL), dried over anhydrous sodium sulfate, filtered, and concentrated. Combined the reaction mixture with a second reaction run under identical stoichiometry and conditions employing 2-chloro-1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidin-6-yl)ethan-1-one (**18**) (3.0 g, 9.1 mmol) and purified by silica gel chromatography (gradient elution: 25-100% ethyl acetate in hexanes) to give the title compound (2.90 g, 8.00 mmol, 23%).$^{1}$H NMR (400 MHz, $CDCl_3$): ~50:50 mixture of rotamers * indicates minor amide rotamer δ 8.16 (bs, 0.5H), *8.13 (bs, 0.5H), 7.23-7.10 (m, 5H), 4.83-4.75 (m, 1H), *4.76 (s, 1H), 4.66 (s, 1H), 4.65 (s,1H), *4.59 (s, 1H), *4.21 (s, 1H), 4.18 (s, 1H), 3.67 (dt, *J* = 6.6, 2.4 Hz, 2H), 3.35 (dd, *J* = 16.0, 7.2 Hz, 2H), 2.84 (dd, *J* = 16.0, 5.0 Hz, 2H), 2.51 (dt, *J* = 6.6, 1.7 Hz, 1H), *2.50 (dt, *J*= 6.6, 1.7 Hz, 1H)), *1.96 (t, *J* = 2.7, 0.5H), 1.95 (t, *J* = 2.7 Hz, 0.5H). LC/MS (ESI$^{+}$): (*m/z*) 363 ($C_{21}H_{23}N_4O_2$ = $(M+1)^{+}$).

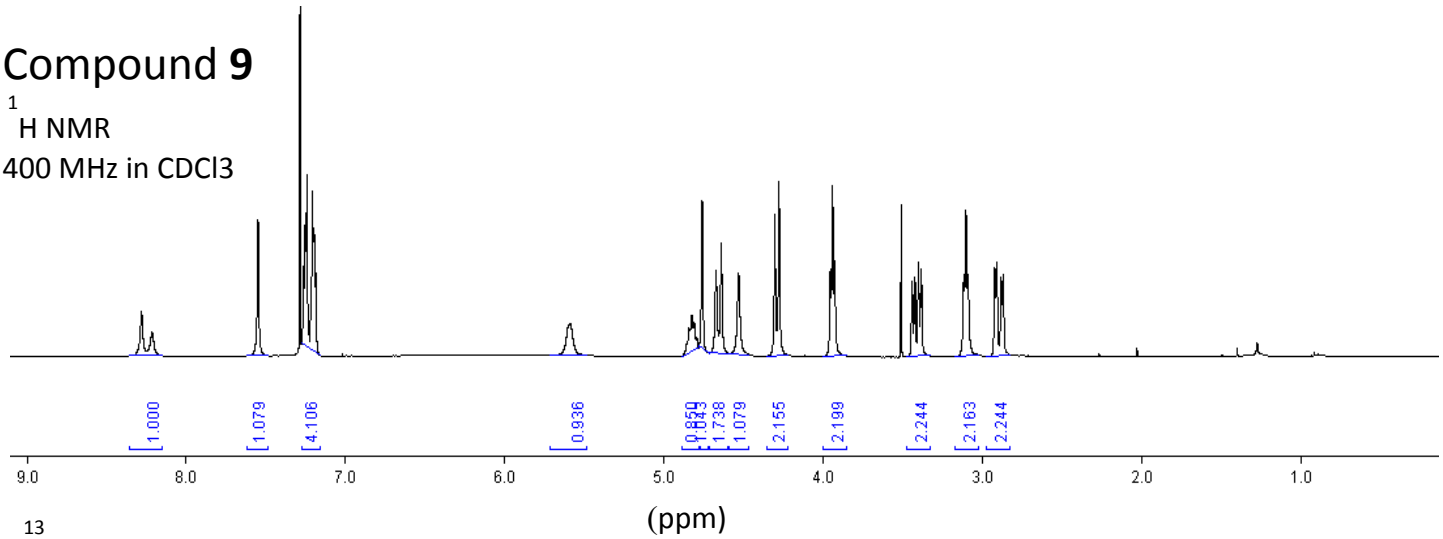
**2-(2-(1H-1,2,3-triazol-5-yl)ethoxy)-1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidin-6-yl)ethan-1-one (9)** Added dimethylformamide (27 mL) and water (27 mL) to a flask containing 2-(but-3-yn-1-yloxy)-1-(2-((2,3-dihydro-1H-inden-2-yl)amino)-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidin-6-yl)ethan-1-one (**19**) (2.90 g, 8.00 mmol, 1.00 equiv). Added copper(II) sulfate pentahydrate (400 mg, 1.60 mmol, 0.2 equiv) and L-ascorbic acid sodium salt (3.17 g, 16.0 mmol, 2.00 equiv) then evacuated and backfilled with nitrogen (x2). Added azidotrimethylsilane (8.53 mL, 64.0 mmol, 8.00 equiv) and heated to 90 °C for 70 minutes. Cooled the reaction to 23 °C and removed all the solvent *in vacuo.* Purified the resulting residue by silica gel chromatography (gradient elution: 0-9% methanol in ethyl acetate ) to give the title compound (980 mg, 2.42 mmol, 30%).$^{1}$H NMR (400 MHz, $CDCl_3$): 60:40 mixure of rotamers * indicates minor rotamer δ 8.18 (bs, 0.6H), *8.13 (bs, 0.4H), 7.49 (s, 1H), 7.21-7.09 (m, 4 H), 5.70-5.50 (m, 1H), 4.87-4.78 (m, 1H), 4.75 (s, 1.2H), *4.67 (s, 0.8H), 4.64 (s, 1.2H) *4.53 (s, 0.8H), *4.30 (s, 0.8H), 4.28 (s, 1.2H), 3.93 (t, *J* = 5.6 Hz, 2H), 3.43 (dd, *J* = 16.2, 7.1 Hz, 2H), 3.10 (t, *J* = 5.6 Hz, 2H), 2.89 (dd, *J* = 16.2, 4.9 Hz, 2H). $^{13}$C NMR (400 MHz, $CDCl_3$): * indicates minor δ *169.3,

169.2, 167.0, *166.8, *162.4, 162.2, 152.8, *152.3, 141.1, 137.8, 130.9, 126.7, 124.9, 115.9, 69.8, 69.3, *69.0, 52.7, *52.5, 51.2, 49.0, *47.9, 40.1, 24.7. LC/MS ($ESI^+$): (*m/z*) 406 ($C_{21}H_{24}N_7O_2$ = $(M+1)^+$).

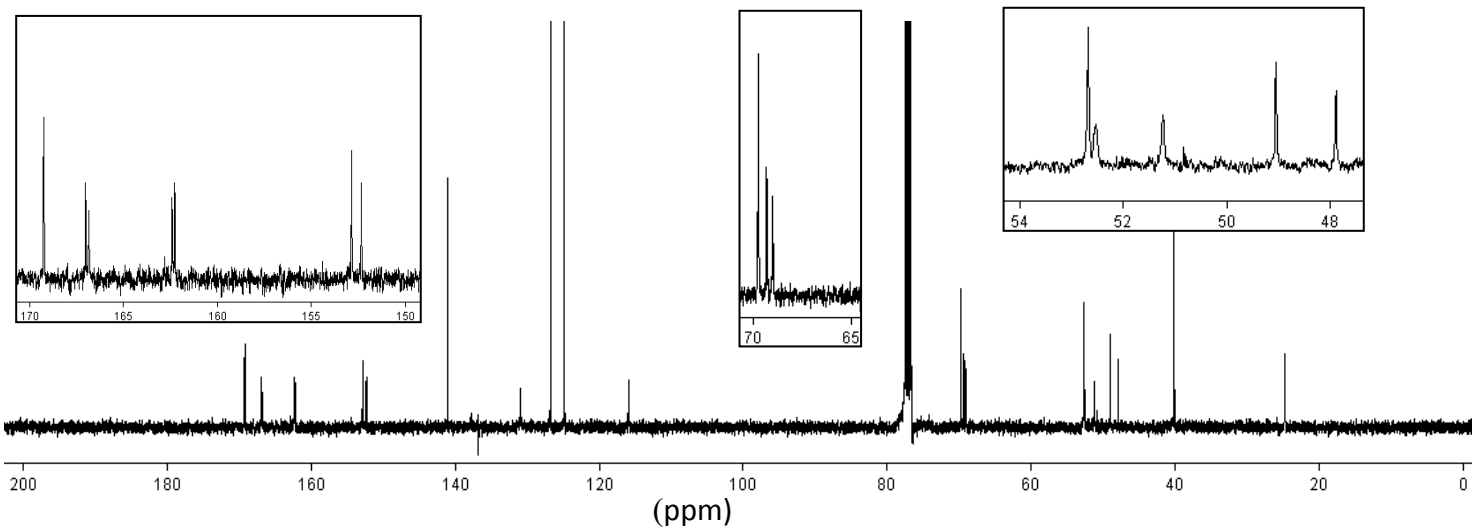
## Compound **9**

$^{1}$H NMR

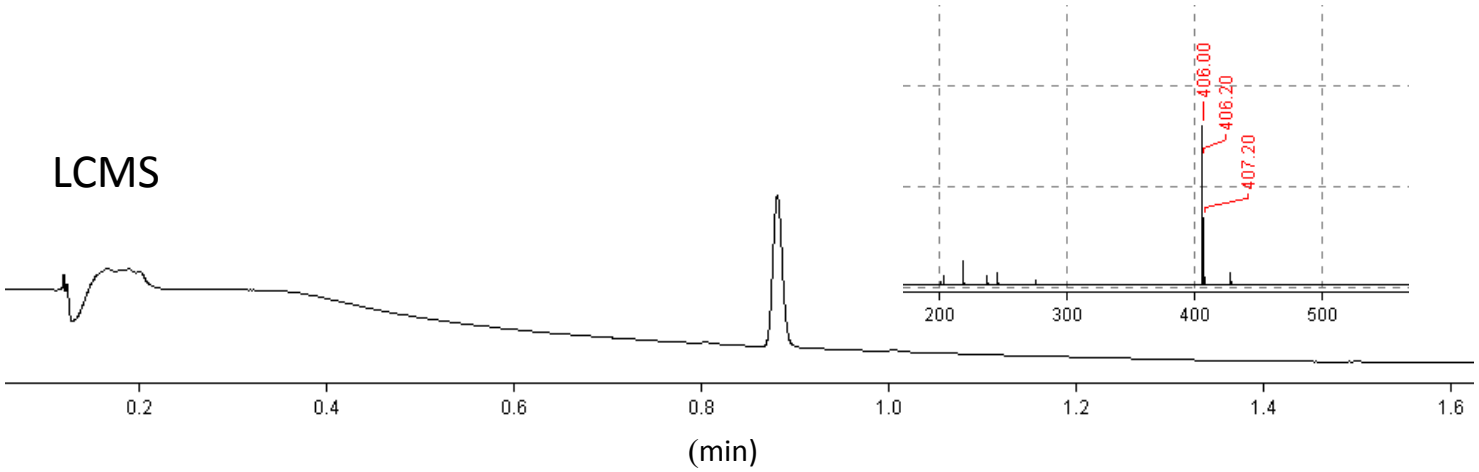
400 MHz in CDCl3



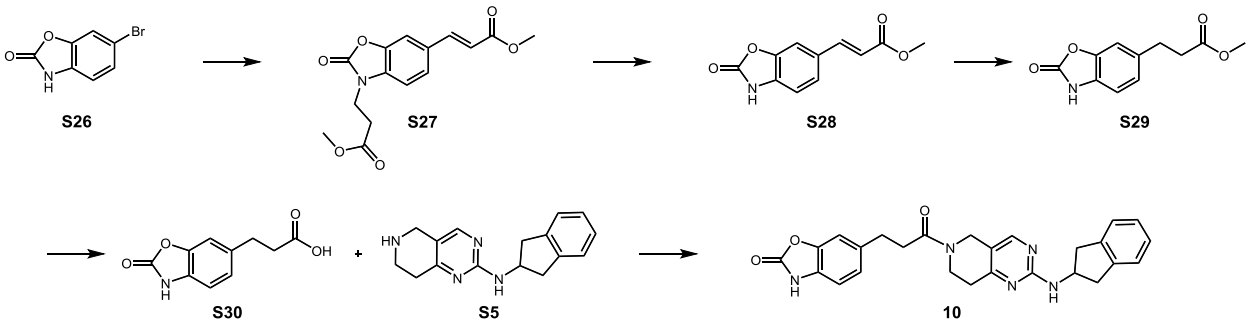

$^{13}$C NMR, 400 MHz in CDCl3




LCMS




2x50 Xbridge C18 3.5um column, 5-95%B gradient in 1.5 minutes with a 0.25 minute hold at 95%B, A: 10mM NH4HCO3 in water pH=9, B: ACN. Flow Rate = 1.2ml/min. Column Temp = 50 degrees

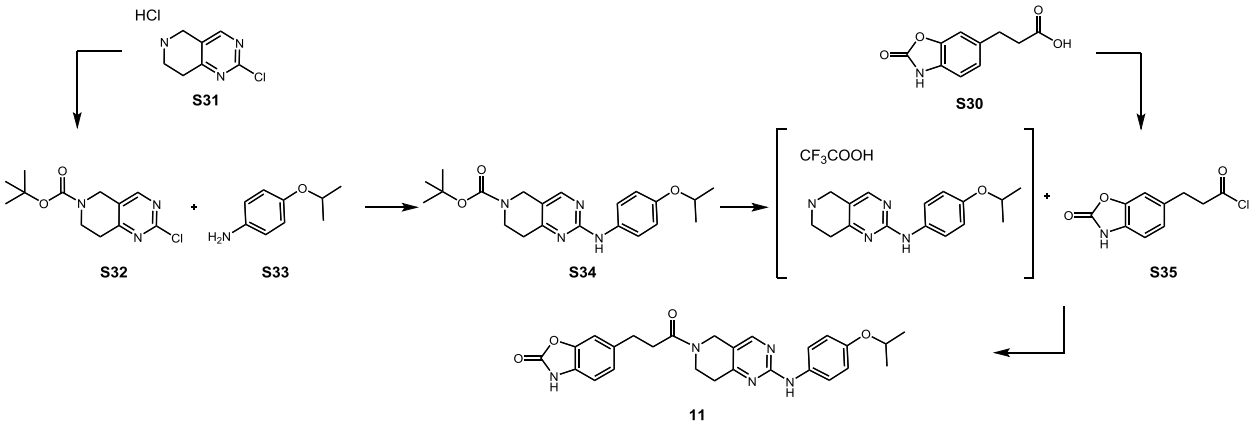



**Methyl (E)-3-(3-(3-methoxy-3-oxopropyl)-2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)acrylate (S27)** To a microwave vial was added 6-bromobenzo[d]oxazol-2(3H)-one (**S26**) (500 mg, 2.34 mmol, 1.00 equiv), triphenylphosphine (110 mg, 421 umol, 0.18 equiv), palladium (II) acetate (52.4 mg, 234 umol, 0.10 equiv), diisopropylethylamine (1.22 mL, 7.00mmol, 3.0 equiv), dimethylformamide (9 mL) and methyl acrylate (2.11 mL, 23.4 mmol, 10.0 equiv). The vial was heated to 150 °C for 45 minutes. Diluted the reaction mixture with water (20 mL) then extracted with ethyl acetate (3x20 mL). Washed the combined organic extracts with water (2x10mL), and brine (10mL) then dried over magnesium sulfate, filtered, and concentrated. Purified the crude product via silica gel chromatography (gradient elution: 0-40% ethyl acetate in dichloromethane) to give the title compound (620 mg, 2.03 mmol, 87%) as a light yellow solid. $^1$H NMR (400 MHz, $CDCl_3$): δ 7.66 (d, *J* = 15.3 Hz, 1H), 7.37 (d, *J*= 1.6 Hz, 1H), 7.35 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 6.35 (d, *J* = 15.3 Hz, 1H), 4.11 (t, *J* = 6.6 Hz, 2H), 3.79 (s, 3H), 3.66 (s, 3H), 2.83 (t, *J*= 6.6 Hz, 2 H). LC/MS (ESI$^+$): (*m/z*) 306 ($C_{15}H_{16}NO_6$ = $(M+1)^+$).

**methyl (E)-3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)acrylate (S28)** To a flask containing Methyl (E)-3-(3-(3-methoxy-3-oxopropyl)-2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)acrylate (**S27**) (510mg, 1.67 mmol, 1.00 equiv) was added tetrahydrofuran (20 mL). To the solution was added potassium tert-butoxide (206 mg, 1.84 mmol, 1.10 equiv ) in one portion, then the reaction was heated to 60 °C for 90 minutes. Half-saturated ammonium chloride (3 mL) and brine (10 mL) and dichoromethane (150 mL) were added to the reaction mixture. Separated the organic layer then dried over magnesium sulfate, filtered, and concentrated. Suspended the residue in dichloromethane (5 mL) and filtered, rinsing the solid with dichloromethane (1 mL) to give the desired product as an impure white solid (418 mg, multiple contaminants, unknown purity). Used the crude material without further purification. LC/MS (ESI$^+$): (*m/z*) 220 ($C_{11}H_{10}NO_4$ = $(M+1)^+$).

**methyl 3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propanoate (S29)** To a flask containing methyl (E)-3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)acrylate (**S28**) (418 mg), was added palladium (10% on carbon, 400 mg) then the flask was purged with nitrogen before adding a 1:1 mixture of dichloromethane:methanol (40 mL). The flask was purged with hydrogen and stirred at room temperature for two hours before concentrating to give the title compound as an impure solid (285 mg). Used the crude material without further purification. LC/MS ($ESI^+$): (*m/z*) 222 ($C_{11}H_{12}NO_4$ = $(M+1)^+$).

**6-(3-(2-((2,3-dihydro-1H-inden-2-yl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-3-oxopropyl)benzo[d]oxazol-2(3H)-one (10)** To a flask containing methyl 3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propanoate (**S29**) (61 mg, 275 umol, 1.00 equiv) was added tetrahydrofuran (1.6 mL) and lithium hydroxide (1.0 M in water, 551 uL, 2.00 equiv) then stirred at room temperature for four hours. Added hydrochloric acid (5 M in water, 137 uL, 2.5 equiv) to the reaction mixture, then concentrated until dryness, coevaporating with toluene (2x2 mL) to give crude 3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propanoic acid (**S30**) (~60 mg) as a white solid. To a vial containing the crude acid **S30** was added N-(2,3-dihydro-1H-inden-2-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine (**S5**) (88.1 mg, 331 umol, 1.20 equiv), N,N-dimethylaminopyridine (1.7 mg, 14 umol, 0.05 equiv), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (317 mg, 1.66 mmol, 6.00 equiv) and dichloromethane (2 mL) then stirred at room temperature for 10 minutes. Exchanged the reaction solvent to DMF to enhance solubility by concentrating *in vacuo* then added dimethyl formamide (2 mL). Heated the reaction mixture to 60 °C for 20 minutes. Diluted the reaction with brine (5 mL) and ethyl acetate (20 mL). Washed the organic layer with brine (2x10 mL) then dried over magnesium sulfate, filtered, and concentrated. Purified the crude product by silica gel chromatography (gradient elution: 0-8% methanol in dichloromethane) to give the title compound (71 mg, 156 umol, 57%) as a colorless foam. $^1$H NMR (400 MHz, DMSO): ~50:50 mixture of rotamers * indicates minor amide rotamer δ 11.48 (s, 0.5H), *11.44 (s, 0.5H), 8.12 (s, 0.5H), *8.03 (s, 0.5H), 7.92 (s, 2H), 7.30-7.24 (m, 1H), 7.20-7.05 (m, 5H), 7.02-6.88 (m, 2H), 4.53 (sex, *J* = 6.7 Hz, 1H), 4.42 (s, 2H), *3.67 (t, *J* = 6.6 Hz, 1H), 3.66 (t, *J* = 6.6 Hz, 1H), 3.18 (dd, *J* = 15.5, 7.7 Hz,2H), 2.85 (dd, *J* = 15.5, 7.7 Hz, 2H), 2.83-2.75 (m, 2H), 2.70-2.50 (m, 2H). LC/MS ($ESI^+$): (*m/z*) 456 ($C_{26}H_{26}N_5O_3$ = $(M+1)^+$).

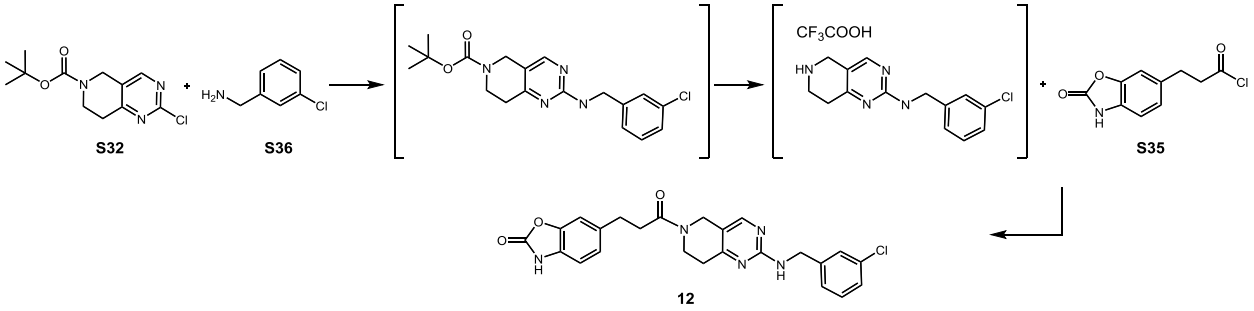



**Tert-butyl 2-chloro-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate (S32)** To a flask containing commercially available 2-chloro-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidine hydrochloride (**S31**) ( 4.00g, 19.4 mmol, 1.00 equiv) was added water (50 mL), ethyl acetate (100 mL) and sodium bicarbonate (3.26 g, 38.8 mmol, 2.00 equiv), then stirred the reaction at room temperature until the solids had dissolved. Di-tert-butyldicarbonate (4.66 g, 21.35 mmol, 1.10 equiv) was added to the reaction mixture in one portion then stirred at room temperature for 75 minutes. Transferred the reaction mixture to a separatory funnel, separated the organic layer then extracted the aqueous layer with ethyl acetate (2 x 30 mL). Dried the combined organic extracts over magnesium sulfate, filtered, and concentrated. Purified the crude product by flash column chromatography (gradient elution: hexanes to ethyl acetate) to give the title compound (4.33 g, 16.05 mmol, 83%) as a white solid. $^1$H NMR (400 MHz, DMSO): δ 8.59 (s, 1H), 4.52 (s, 2H), 3.62 (t, *J* = 5.8 Hz, 2H), 2.83 (t, *J* = 5.8 Hz, 2H), 1.39 (s, 9H). LC/MS (ESI$^+$): (*m/z*) 270 ($C_{12}H_{16}ClN_3O_2$ = $(M+1)^+$).

**Tert-butyl 2-((4-isopropoxyphenyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate (S34)** To a microwave vial was added tert-butyl 2-chloro-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate (**S32**) (955 mg, 3.54 mmol, 1.00 equiv), 4-isopropoxyaniline (**S33**) (2.62 mL, 17.7 mmol, 5.00 equiv), isopropyl alcohol (1 mL), and triethylamine (493 uL, 3.54 mmol, 1.00 equiv) then heated to 160 °C in the microwave for 60 minutes. Concentrated the reaction mixture, then purified directly by chromatography (gradient elution: hexanes to 50% ethyl acetate in hexanes) to give the title compound (787 mg, 2.05 mmol, 58 %) as a light yellow foam. $^1$H NMR (400 MHz, DMSO): δ 9.29 (s, 1H), 8.23 (s, 1H), 7.55 (d, *J* = 9.0 Hz, 2H), 6.79 (d, *J*= 9.0 Hz, 2H), 4.47 (sept, *J* = 5.8 Hz, 1H), 4.37 (s, 2H), 3.59 (t, *J* = 6.0 Hz, 2H), 2.68 (t, *J* = 6.0 Hz, 2H), 1.40 (s, 9H), 1.20 (d, *J* = 6.0 Hz, 6H). LC/MS (ESI$^+$): (*m/z*) 385 ($C_{21}H_{29}N_4O_3$ = $(M+1)^+$).

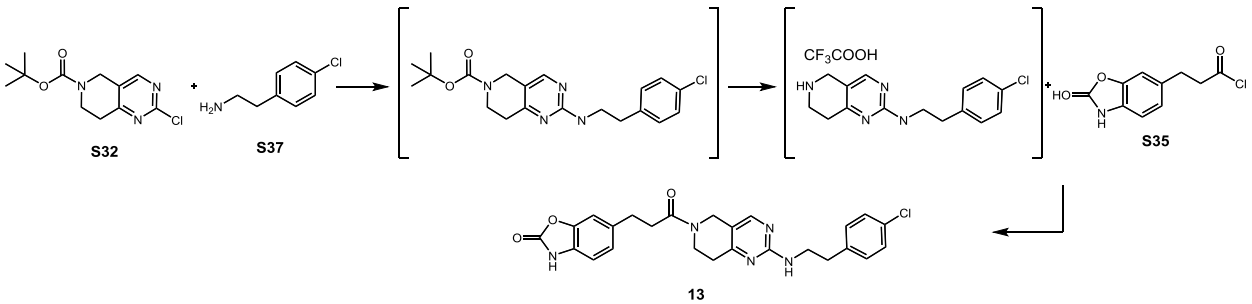
**3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propanoyl chloride (S35)** To a vial containing 3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propanoic acid (**S30**) (1.5 g, 7.24 mmol, 1.00 equiv) was added dichloromethane (4 mL), and oxalyl chloride (2.51 mL, 28.96 mmol, 4.00 equiv) then stirred at room temperature for 20 minutes then at 40 °C for 40 minutes. Concentrated the reaction mixture on the rotovap, coevaporating with toluene to give the title compound (1.32 g , 5.85 mmol, 81%) as a cream colored solid. Used the crude material without further characterization.

**6-(3-(2-((4-isopropoxyphenyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-3-oxopropyl)benzo[d]oxazol-2(3H)-one (11)** To a vial containing tert-butyl 2-((3-isopropoxyphenyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate (**S34**) (100 mg, 261 mmol, 1.18 equiv) was added dichloromethane (0.5 mL) and trifluoroacetic acid (0.5 mL) then stirred at room temperature for 3.5 hours. Removed the solvent on the rotovap, coevaporating with toluene (1 mL). Dissolved the residue in dichloromethane (1 mL) then added imidazole (53.1 mg, 780 umol, 3.00 equiv) and 3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propanoyl chloride (**S35**) (49.9 mg, 221 umol, 1.00 equiv), then stirred at room temperature for 68 hours. Purified the crude material directly with flash column chromatography (Gradient elution: ethyl acetate to 7% methanol in ethyl acetate) to give the title compound (44 mg, 93 umol, 36%) as a white solid. $^1$H NMR (400 MHz, DMSO) ~60:40 mixture of amide rotamers* indicates minor amide rotamer: δ 11.47 (bs, 1H), 9.28 (s, 1H), 8.24 (s, 0.6H), *8.15 (s, 0.4H), 7.56 (d, *J* = 9.0 Hz, 2H), 7.07-6.97 (m, 3H), 6.79 (d, *J* = 9.0 Hz, 2H), 4.49 (s, 2H), 4.48 (sept, *J* = 6.3 Hz, 1H), *3.71 (t, J = 6.2 Hz, 0.8H), 3.69 (t, *J* = 6.2 Hz, 1.2H), 2.83-2.62 (m, 6H), 1.20 (d, *J* = 6.3 Hz, 6 H). LC/MS (ESI$^+$): (*m/z*) 474 ($C_{26}H_{28}N_5O_4$ = $(M+1)^+$).




**6-(3-(2-((3-chlorobenzyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-3-oxopropyl)benzo[d]oxazol-2(3H)-one (12)** To a microwave vial containing tert-butyl 2-chloro-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate (**S32**) (103 mg, 0.382 mmol, 1.00 equiv) was added 3-

chlorobenzylamine (**S36**) (70.3 mg, 0.496 mmol, 1.30 equiv) and diisopropylethylamine (0.087 mL, 0.498 mmol, 1.30 equiv). The mixture was then heated to 130 °C for three hours, then cooled to room temperature to give the crude tert-butyl 2-((3-chlorobenzyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate which was not isolated. To the crude tert-butyl 2-((3-chlorobenzyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate was added dichloromethane (0.3 mL) and trifluoroacetic acid (0.5 mL) and stirred at room temperature for two hours. Transferred the mixture to a separatory funnel with dichloromethane (5 mL) and basified with sodium hydroxide (aqueous solution, 4.0 mL, 5.0 N). To the mixture was added brine (2 mL) and water (5 mL) then extracted with dichloromethane (4x5 mL). Dried the combined organic extracts over magnesium sulfate, filtered, and concentrated to give crude N-(3-chlorobenzyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine 2,2,2-trifluoroacetate which was used without further purification. To the crude N-(3-chlorobenzyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine 2,2,2-trifluoroacetate was added imidazole (78 mg, 1.15 mmol, 3.0 equiv) and dichloromethane (5 mL) and stirred at room temperature until homogeneous. Cooled the reaction mixture to -30 °C, then added 3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propanoyl chloride (**S35**) (86.2 mg, 0.382 mmol, 1.00 equiv) in one portion. Warmed the reaction mixture to room temperature for three hours. Purified the reaction solution directly by flash column chromatography (gradient elution: ethyl acetate to 5% methanol in ethyl acetate) to give the title compound (111 mg, 0.239 mmol, 63%) as a yellow glass. $^1$H NMR (400 MHz, DMSO) ~55:45 mixture of rotamers* indicates minor amide rotamer : δ 11.4 (bs, 1H), 8.09 (s, 0.55H), *8.00 (s, 0.45H), 7.63-7.53 (m, 1H), 7.30-7.14 (m, 4H), 7.00-6.88 (m, 3H), 4.43 (d, *J* = 6.5 Hz, 2H), 4.41 (s, 2H), 3.68-3.63 (m, 2H), 2.83-2.75 (m, 2H), 2.70-2.50 (m, 4H). LC/MS (ESI$^+$): (*m/z*) 464 ($C_{24}H_{23}ClN_5O_3$ = $(M+1)^+$).




**6-(3-(2-((4-chlorophenethyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidin-6(5H)-yl)-3-oxopropyl)benzo[d]oxazol-2(3H)-one (13)** To a microwave vial containing tert-butyl 2-chloro-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate (**S32**) (103 mg, 0.382 mmol, 1.00 equiv) was added 4-chlorophenethylamine (**S37**) (77.3 mg, 0.496 mmol, 1.30 equiv) and diisopropylethylamine (0.087 mL,

0.498 mmol, 1.3 equiv) then heated to 130 °C for four hours. Cooled the reaction mixture to room temperature to give tert-butyl 2-((4-chlorophenethyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate which was not isolated. To the crude tert-butyl 2-((4-chlorophenethyl)amino)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxylate was added dichloromethane (2 mL) and trifluoroacetic acid (0.5 mL) and stirred at room temperature for 14 hours. Transferred the solution to a separatory funnel with dichloromethane (5 mL) and basified with sodium hydroxide (aqueous solution, 6.0 mL, 5.0 N). Added brine (2 mL) and water (5 mL) then extracted with dichloromethane (4x5 mL). Dried the combined organic extracts over magnesium sulfate, filtered, and concentrated to give crude N-(4-chlorophenethyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine 2,2,2-trifluoroacetate which was used without further purification. To the N-(4-chlorophenethyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-amine 2,2,2-trifluoroacetate was added imidazole (78.0 mg, 1.15 mmol, 3.0 equiv) and dichloromethane (5 mL) and stirred at room temperature until homogeneous. Cooled the reaction mixture to -30 °C, then added 3-(2-oxo-2,3-dihydrobenzo[d]oxazol-6-yl)propanoyl chloride (**S35**) (86.2 mg, 0.382 mmol, 1.00 equiv) in one portion, then warmed to room temperature for three hours. Purified the solution directly by flash column chromatography (gradient elution: ethyl acetate to 5% methanol in ethyl acetate) to give the title compound (111 mg, 0.239 mmol, 61%) as a yellow glass. $^{1}$H NMR (400 MHz, DMSO) ~50:50 mixture of rotamers * indicates minor amide rotamer: δ *12.0 (bs, 0.5H), 11.46 (bs, 0.5H), *8.09 (s, 0.5H), 7.99 (s, 0.5H), 7.29 (d, *J*= 8.5 Hz, 2H), 7.24-7.14 (m, 2H), 7.05-6.88 (m, 4H), 4.41 (s, 2H), 3.68-3.62 (m, 2H), 3.41 (q, *J*= 6.6 Hz, 2H), 2.84-2.72 (m, 4H), 2.71-2.50 (m, 4H). LC/MS (ESI$^{+}$): (*m/z*) 478 ($C_{25}H_{25}ClN_5O_3$ = $(M+1)^{+}$).

**Crystallization and Structure Determination:**

Recombinant rat Autotaxin with a C-terminal His tag was expressed in HEK293E cells and purified by Ni-NTA and size exclusion chromatography (SEC). Protein was concentrated to 12.3mg/ml in the final SEC buffer (10mM Tris pH 8.0, 100mM NaCl) prior to adding 2mM compound (100mM DMSO stock). Vapor diffusion crystallization was setup as 2μl + 1μl (protein + well solution) hanging drops with well solution containing 14% PEG 10,000, 0.2M Ammonium Acetate, and 0.1M Tris pH 8.5. Microseeding was employed to achieve the most suitable crystals for data collection. Resulting crystals were flash frozen in liquid nitrogen following a quick dip in well solution with 25 % glycerol added as a cryogenic agent.

Diffraction data were collected at LRL-CAT 31-ID (Advanced Photon Source, Argonne, IL). Structures were solved using a previously determined structure (unpublished) as a model. Structure refinement was initiated with rigid body refinement followed by iterative rounds of restrained refinement and model building with Buster[2], REFMAC5[3] and COOT[4]. Ligand coordinates and dictionaries were generated and fit to density using RHOFIT[5]. A structure with **PF-8380** was obtained from Proteros biostructures GmbH as a Gallery Structure.

**Inhibition of Autotaxin as Measured by Choline Release**

The purpose of this assay is to detect autotaxin inhibition using a choline release assay.

Test compounds (10 mM stocks in 100% DMSO) are serially diluted in 100% DMSO resulting in 10 concentrations of 100X inhibitor in half area 96 well plates (Corning 3992). Each of these 10 wells in 100% DMSO is diluted 1:33.33 in assay buffer in round bottom 96 well plates (Fisher 12565502) resulting in 3X concentrations in well containing 3% DMSO. The assay buffer is 50 mM Tris pH8.0, 5 mM KCl, 1 mM $CaCl_2$, 1 mM MgCh, 0.01% TRITON™ X-100 (Sigma T9284) and 0.01% fatty acid free bovine serum albumin (Sigma A8806). A 20 μL aliquot of each 3X test compound is then added to black flat bottom 96 well plates (Corning 3991) in singlicate. A 20 μL aliquot per well 15 of 3X recombinant human autotaxin (full length human autotaxin with a C-terminal His tag transfected into 293E cells and purified via nickel chelate and size exclusion chromatography) is then added to every well except for the no enzyme control wells. A 20 μL aliquot per well of assay buffer is added to the no enzyme control wells. A 20 μL aliquot of a 3X cocktail containing choline oxidase (Sigma C5896), horseradish 20 peroxidase (Sigma P8125), amplex ultrared (Invitrogen A36006) and the autotaxin substrate lysophosphatidylcholine (LPC) 16:0 (Avanti Polar Lipids 855675P) is added to each well while avoiding exposure to light. The final concentrations in the well of choline oxidase, horseradish peroxidase, amplex ultrared and LPC 16:0 are 0.4 units/mL, 4 units/mL, 40 uM and 30 uM respectively. The plate is then sealed with aluminum foil 25 seals and incubated at 37°C for 1 hour in a Labline Imperial III

[2] Bricogne G., Blanc E., Brandl M., Flensburg C., Keller P., Paciorek W., Roversi P, Sharff A., Smart O.S., Vonrhein C., Womack T.O. (2011). BUSTER version 2.11.5. Cambridge, United Kingdom: Global Phasing Ltd.
[3] Murshudov, G. N.; Skubak, P.; Lebedev, A. A.; Pannu, N. S.; Steiner, R. A.; Nicholls, R. A.; Winn, M. D.; Long, F.; Vagin, A. A. *Acta Cryst. D* **2011**, 67, 355.
[4] Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. *Acta Cryst. D* **2010**, 66, 486.
[5] Womack, TO, Smart OS, Sharff A, Flensburg C, Keller P, Paciorek W, Vonrhein C and Bricogne G (2011). RHOFIT, version 1.2.1. Cambridge, United Kingdom: Global Phasing Ltd

incubator. During this incubation, LPC is cleaved by autotaxin resulting in lysophosphatidic acid (LPA) 16:0 and choline. The choline that is released is oxidized by choline oxidase resulting in betaine and hydrogen peroxide. The hydrogen peroxide reacts with the horseradish peroxide and amplex ultrared to form the fluorescent molecule resorufin. The resorufin on the plates is measured with a SpectraMax Gemini EM fluorometer at excitation emission wavelengths of 530-590 nm using SoftMax Pro 4.8 software. $IC_{50}$s are calculated using 4 parameter curve fits with the internal Lilly software OLO curve fitting tool. Based on numerous replicates of PF-8380 and internal compounds, the minimum significant ratio[6] was determined to be 1.38.

**Inhibition of endogenous autotaxin in plasma**

This assay is a tool that can be used to identify selective autotaxin-mediated LPA inhibitor compounds when it is used to test compounds that have been identified as autotaxin inhibitors. LPA biosynthesis through autotaxin is believed to be the source of LPA for LPA 1 mediated neuropathic pain.[7] Targeted inhibition of the autotaxin mediated LPA biosynthesis is supported by the results of this assay. Units of plasma from healthy human female donors collected in sodium heparin (Lampire Biologicals) are pooled, aliquoted and stored at -80 °C. On the day of assay, aliquots of the plasma are thawed and spun for 10 minutes at 3000 RPMs at 4 °C in a centrifuge to remove debris. A 90 μL aliquot of plasma is added to each well of a 96 well round bottom polypropylene plate. A 10 μL aliquot of 10X test compound containing 10% DMSO in assay buffer (50 mM Tris pH8.0, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) is added to each well except for the control wells which contain no test compound. This results in 10 1X concentrations of test compound in singlicate with a final concentration of 1% DMSO in 90% plasma. A 10 μL aliquot of 10% DMSO in assay buffer without test compound is added to the 0 hour (n=8) and 3 hour no test compound controls (n=8) wells. A 10 μL aliquot of 500 mM ethylenediaminetetraacetic acid (EDTA) is added to each of the 0 hour no test compound control wells to chelate endogenous autotaxin. The entire 5 contents of the 0 hour no test compound control wells are transferred to a new 96 well round bottom polypropylene plate and frozen at -80°C. The plate containing plasma+/- test compounds (minus the 0 hour no inhibitor control wells) is then incubated for 3 hours at 37 °C in a Robbins

[6] Eastwood, B. J., Farmen, M. W., Iversen, P. W., Craft, T. J., Smallwood, J. K., Garbison, K. E., Delapp, N. W., Smith, G. F., The minimum significant ratio: a statistical parameter to characterize the reproducibility of potency estimates from the concentration-response assays and estimation by replicate-experiment studies, *J. Biomol. Screen,* **2006**, *11*, 253.

[7] Inoue, M., Ma, L., Aoki, J., Chun, J., Ueda, H.; Autotaxin, a synthetic enzyme of lysophosphatidic acid (LPA), mediates the induction of nerve-injured neuropathic pain, *Mol. Pain*, **2008**, *4*, 6.

Scientific™ model 400 hybridization incubator while rocking at 14,000 RPMs. During this 3 hour incubation, lecithin cholesterol acyltransferases present in the plasma cleave phosphatidylcholine resulting in higher plasma levels of the autotaxin substrate lysophosphatidylcholine (LPC). The increased endogenous LPC levels are cleaved by endogenous autotaxin resulting in higher plasma concentrations of endogenous lysophosphatidic acid (LPA).[8] This increase in LPA in the 3 hour incubation can be inhibited by autotaxin inhibitors. Following the 3 hour incubation, 10 μL of 500 mM EDT A is added to all of the remaining wells (test compound containing wells and 3 hour no test compound control wells) to chelate the endogenous autotaxin. The entire contents of these wells are then added to the plate containing the 0 hour no test compound control plasma that had previously been stored at -80°C (without thawing the 0 hour plasma). The plate is then re-covered with an aluminum foil seal and placed back at -80°C until extraction for mass spec analysis. On the day of extraction, the plates are thawed on ice and 25 uL of plasma from each well is transferred to a 2 mL True Taper™ square 96 deep well plate (Analytical Sales and Products #968820). A 400 μL aliquot of extraction buffer (50% methanol, 49.9% acetonitrile, 0.1% acetic acid) containing LPA internal standards (50 ng/ml D5 deuterium LPA 16:0 and 50 ng/ml D5 deuterium LPA 18:0) is added to each well and the total LPA in each sample is determined by mass spec analysis. Percent reduction of LPA is calculated according to the following formula: (3 hour plasma + test compound- 0 hour plasma no test compound control) / (3 hour plasma no test compound control - 0 hour plasma no test compound control) X 100. $IC_{50}$ values are calculated using 4 parameter curve fitting.

[8] Nakamura, K., Ohkawa, R., Okubo, S., Tozuka, M., Okada, M., Aoki, S., Aoki, J., Arai, H., Ikeda, H., Yatomi, Y.; Measurement of lysophospholipase D/autotaxin activity in human serum samples, *Clin. Biochem.* **2007**, *40*, 274-277.