Supplementary Information for

ACTIVATION OF PARKIN BY A MOLECULAR GLUE

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Supplementary Table 1. Isothermal titration calorimetry data collection and fitting

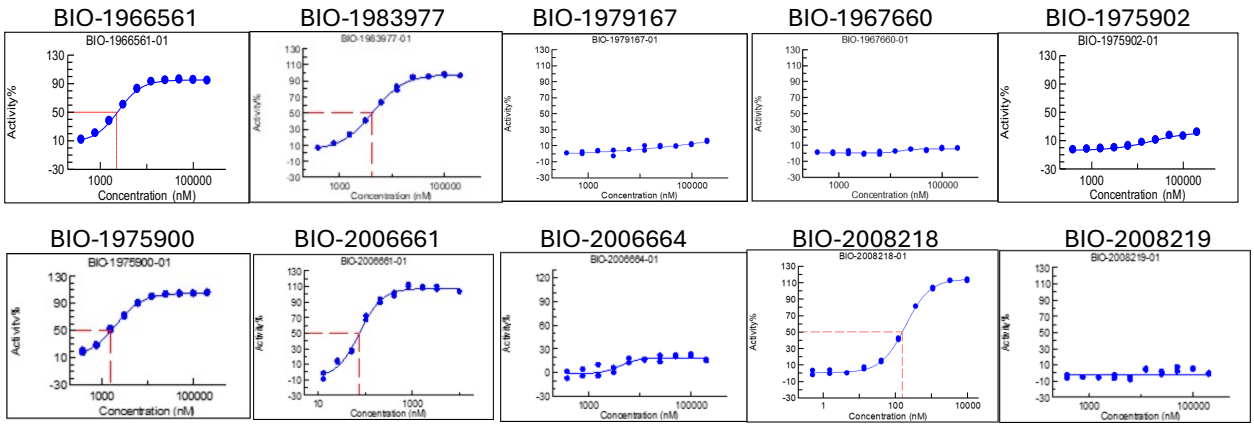
Cell sample[1]	Syringe sample	χ^2/DoF	Number of sites	Ka (M^{-1})	ΔH (cal/mol)	ΔS (cal/mol/deg)
R0RB (21 μM) + pUb (2×, 48 μM)	BIO-2007817 (165 μM)	3.22E4	1.07 ± 0.002	9.69E7 ± 1.7E7	-1.052E4 ± 47.6	0.664
R0RB (20 μM) + pUb (2×, 45 μM)	BIO-2007818 (166 μM)	705.9	1.20 ± 0.003	6.54E5 ± 1.58E4	-5368 ± 20.68	8.30
R0RB (20 μM)	BIO-2007817 (166 μM)	1.66E4	1 (fixed)	1.21E4 ± 1.6E3	-1.820E4 ± 1.65E3	-43.4
R0RB (K211N) (16 μM) + pUb (25 μM)	BIO-2007817 (166 μM)	1.82E4	1 (fixed)	2.55E4 ± 1.2E3	-2.894E4 ± 760	-78.5
R0RB (17 μM) + pUbl (25 μM)	BIO-2007817 (166 μM)	6173	1.13 ± 9.0E-4	9.09E7 ± 5.1E6	-1.001E4 ± 17.6	2.26
R0RB (20 μM) + pUbl-ACT (25 μM)	BIO-2007817 (166 μM)	5440	1.09 ± 0.003	8.08E6 ± 5.3E5	-5498 ± 23.9	12.9
R0RB (F146Y) (20 μM) + pUb (2×, 45 μM)	BIO-2007817 (166 μM)	2553	1.16 ± 0.003	1.13E6 ± 3.0E4	-5712 ± 20	8.21
R0RB (9 μM)	pUbl (200 μM)	1.4E4	0.806 ± 0.012	6.05E5 ± 3.5E4	-1.064E4 ± 214	-9.83
R0RB (4.5 μM) + BIO-2007817 (200 μM)	pUbl (100 μM)	6.7E4	0.99 ± 0.003	1.29E9 ± 6.6E8	-1.397E4 ± 95	-5.94

[1]Constructs derived from the sequence of rat parkin

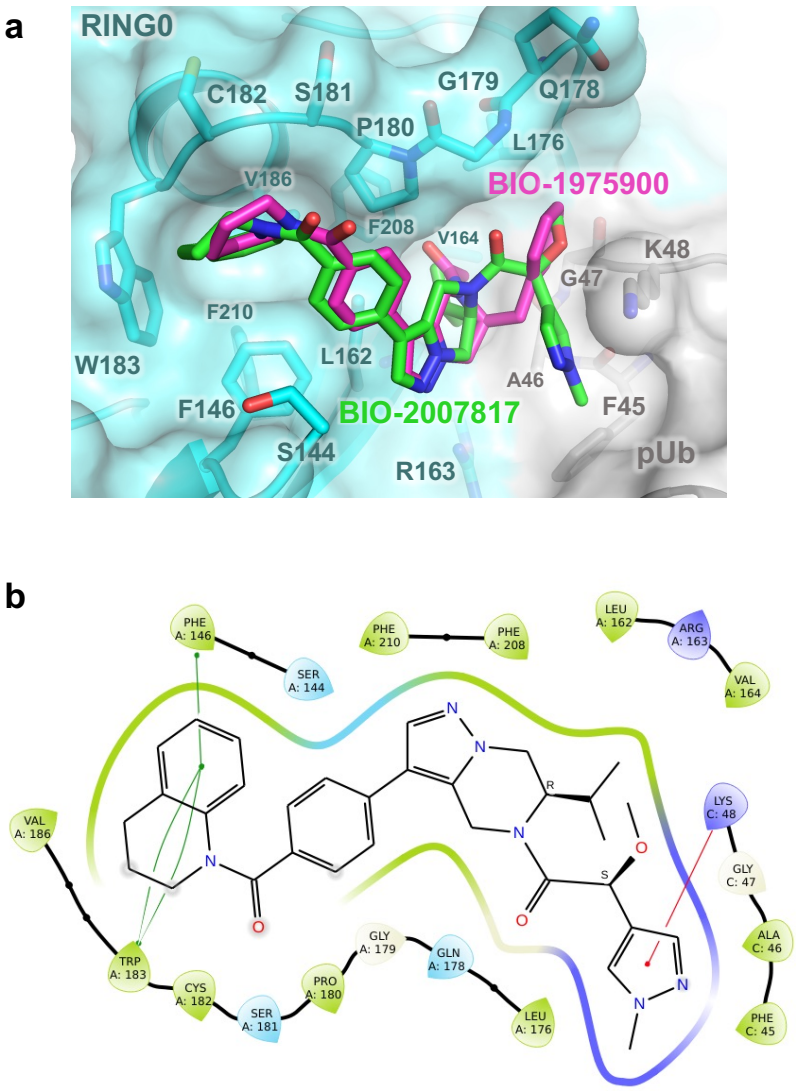
Supplementary Table 2. Data collection and refinement statistics

	R0RB:2×pUb:BIO-1975900 PDB: 8W31
Data collection	
Space group	$P6_122$
Cell dimensions	
a, *b*, *c* (Å)	83.54, 83.54, 258.77
α, β, γ (°)	90, 90, 120
Resolution (Å)	48.22-2.50 (2.59-2.50)
R_{merge}	0.11 (2.94)
$I / \sigma I$	24.08 (1.72)
Completeness (%)	99.98 (100.00)
Redundancy	38.3 (40.7)
Refinement	
Resolution (Å)	2.50
No. reflections	19414 (1881)
R_{work} / R_{free}	0.240/0.294 (0.411/0.474)
No. atoms	3051
Protein	2970
Zn	6
BIO-1975900	37
Water	32
Glycerol	6
B-factors	
Protein	89.26
Zn	95.40
BIO-1975900	101.43
Water	71.38
Glycerol	90.03
R.m.s. deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.281

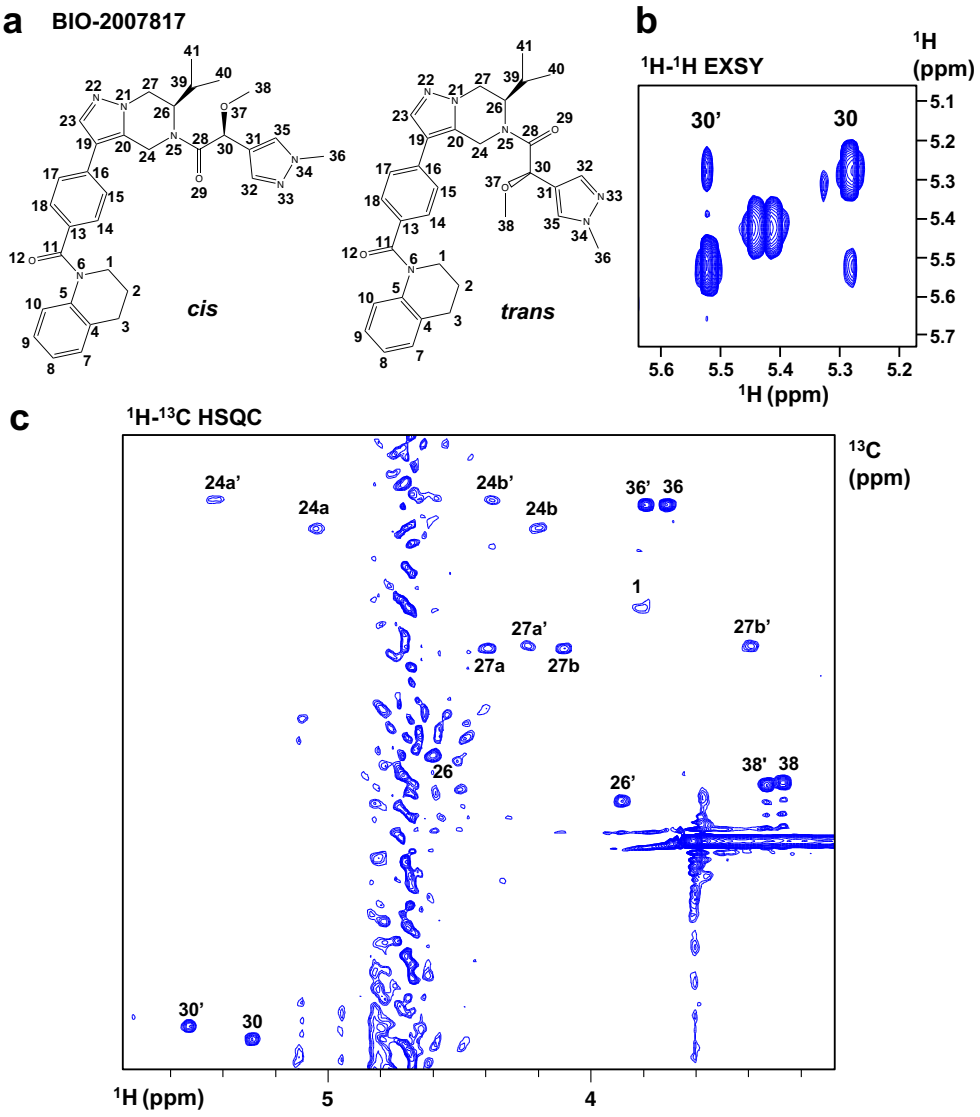
*A single crystal was used to determine this structure



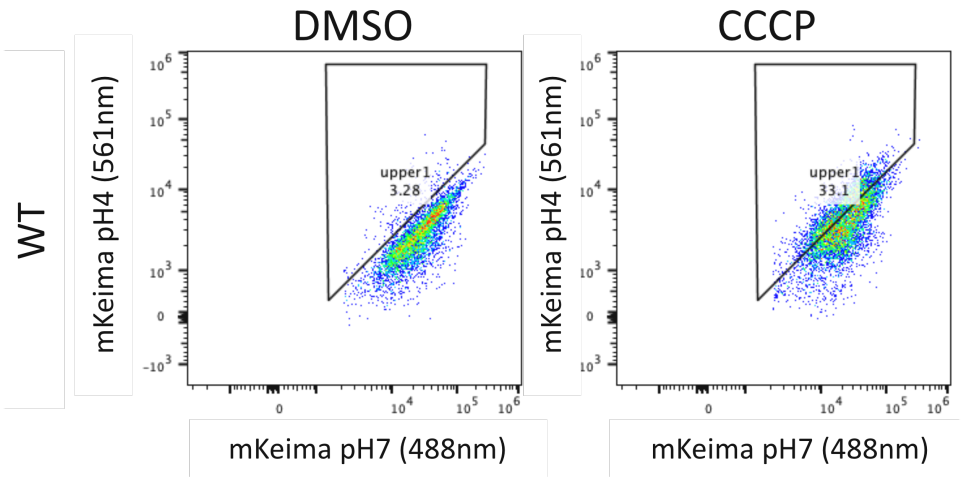
Supplementary Fig. 1. Autoubiquitination activity profiles for related compounds. Chemical structures and EC50 values are shown in main Figure 5.



Supplementary Fig. 2. BIO-2007817 binding to parkin. a, Docking of BIO-2007817 into the R0RB:2×pUb:BIO-1975900 crystal structure. **b**, Schematic of BIO-2007817 interactions.



Supplementary Fig. 3. NMR analysis of BIO-2007817 cis-trans isomerization. a, Chemical structure and atom numbering of BIO-2007817, with the chemical bond between atom #25 and 28 in the *cis* or *trans* configuration. **b**, ^{1}H-^{1}H EXSY spectrum of BIO-2007817 in water (10% D_2O) showing chemical exchange for the proton attached to the chiral center (#30), in proximity to the bond undergoing isomerization (‘). Mixing time = 500 msec. **c**, Selected region of the ^{1}H-^{13}C HSQC spectrum of BIO-2007817 (natural abundance) in water (10% D_2O). Peaks of ^{1}H-^{13}C pairs in proximity to the isomerization center show doublets, suggesting that the molecule adopts two distinct conformations.



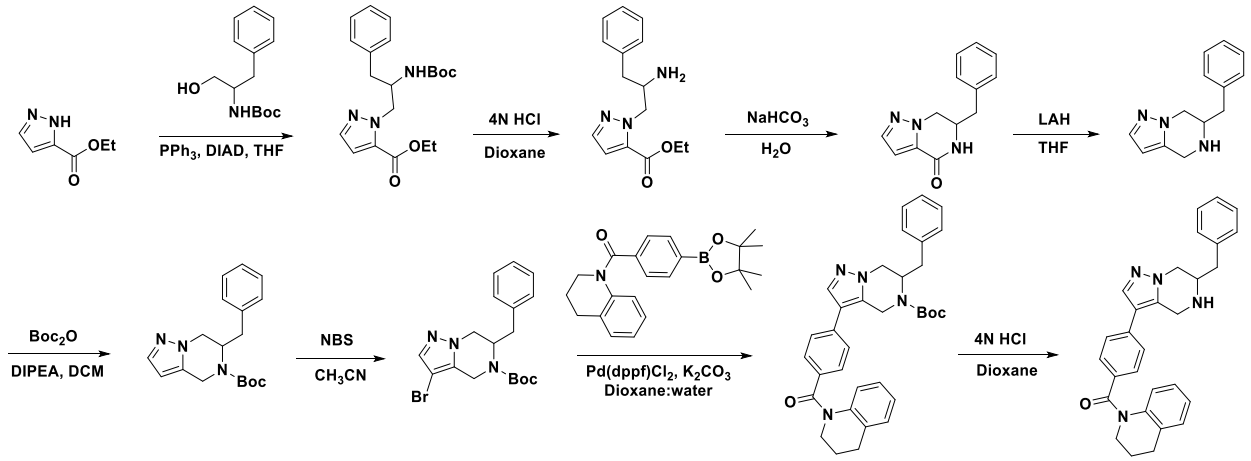
Supplementary Fig. 4. Flow cytometry measurements of mitophagy. Measurement of mitoKeima was made using a dual-excitation ratiometric pH calculation where pH 7 was detected through the excitation at 405 nm and pH 4 at 561 nm. For each sample, GFP-Parkin-positive, mt-Keima-405 nm-positive cells were gated and the percentage of cells with an increase in the 405:561 nm ratio in mt-Keima quantified. The percentage in treated cells minus the percentage in untreated cells was calculated as the induced parkin-mediated mitophagy and normalized to WT in each experimental set.

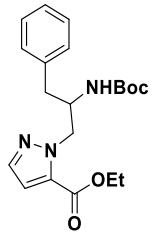
Supplementary Methods

Synthesis of compounds

All solvents and chemicals used were reagent grade. Anhydrous solvents were purchased from Sigma-Aldrich and used as received. Analytical thin layer chromatography (TLC) and silica gel column chromatography were performed on Merck silica gel 60 (230-400 mesh). Removal of solvents was conducted by using a rotary evaporator and residual solvents were removed from non-volatile compounds using a vacuum manifold maintained at approximately 1 Torr. NMR spectra were recorded on a Bruker Advance 400 MHz, 500 MHz and 600 MHz NMR spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to residual undeuterated solvent as internal reference and coupling constants (J) are reported in hertz (Hz). Splitting patterns are indicated as follows: s = singlet; d = doublet; t = triplet; q = quartet; qn = quintet; dd = doublet of doublets; dt = doublet of triplets; tt = triplet of triplets; m = multiplet; br = broad peak. All yields reported are isolated yields. All final compounds were purified to ≥95% purity as determined by LC/MS analysis (using a lineal gradient of elution: 90% water in trifluoroacetic acid (containing 0.1% v/v)/10% CH_3CN in trifluoroacetic acid (containing 0.1% v/v) to 10% water in trifluoroacetic acid (containing 0.1% v/v) and 90% CH_3CN in trifluoroacetic acid (0.1% v/v) for 2 minutes and S2 then holding at 10% water in trifluoroacetic acid (0.1% v/v) and 90% CH_3CN in trifluoroacetic acid (0.1% v/v) up to 3 minutes at a flowrate of 3 mL/min (injection volume 5µL and using a Waters Sunfire C18 3.5 uM 4.6x20mm IS column)). MS mode: MS:ESI+ scan range 100-1000 daltons. PDA detection 210-400 nm. Final compounds were analyzed using UPLC (Water's Acquity (Waters Milford, MA)) coupled with an AB Sciex 6600 Triple-TOF mass spectrometer (AB Sciex Framingham, MA). A Water's Acquity HSS T3 (1.7um beads, 2.1x50mm) column was used for separation. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The flow rate was 0.45 mL/min and the following gradient was used from 0-0.2 minutes 5% B and increased linearly to 65% B at 5 minutes and 90% at 6.1 min and remained there for 0.4 minutes, dropped back to 5% B over 0.1 minutes and remained there for 0.4 minutes. The mass spectrometer was operated in positive ion mode. An electrospray ionization source was used with the following parameters: Ion spray voltage floating 4500 V, ion source gas 1 50 (arbitrary units), ion source gas 2 50 (arbitrary units), curtain gas 30 (arbitrary units), and temperature 500 °C

General procedure A:

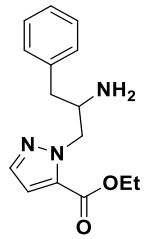




ethyl -1-(2-((tert-butoxycarbonyl)amino)-3-phenylpropyl)-1H-pyrazole-5-carboxylate

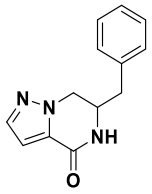
To a solution of ethyl 1H-pyrazole-5-carboxylate (5.00 g, 35.68 mmol) and Triphenylphosphine (12.17 g, 46.38 mmol) in THF (50 mL) was added DIAD (9.38 g, 46.38 mmol, 9.11 mL) at 0 °C. After 30 minutes, a solution of tert-butyl (1-hydroxy-3-phenylpropan-2-yl)carbamate (8.97 g, 35.68 mmol) in dry THF (2 mL) was added and stirred at 25 °C for 16 hours. The mixture was concentrated and the residue was purified by chromatography on silica gel (eluted with EtOAc in petroleum ether:15%) to give ethyl-1-(2-((tert-butoxycarbonyl)amino)-3-phenylpropyl)-1H-pyrazole-5- (11.47 g, 86 % yield) as a colorless oil.

^{1}H NMR: (400MHz, METHANOL-d4) δ ppm 7.55 - 7.46 (m, 1H), 7.31 - 7.19 (m, 5H), 6.94 - 6.80 (m, 1H), 4.74 - 4.64 (m, 1H), 4.54 - 4.48 (m, 1H), 4.40 - 4.23 (m, 3H), 2.89 - 2.62 (m, 2H), 1.38 - 1.33 (m, 3H), 1.32 - 1.20 (m, 9H).



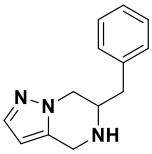
ethyl -1-(2-amino-3-phenylpropyl)-1H-pyrazole-5-carboxylate

To a solution of ethyl 2-[2-(tert-butoxycarbonylamino)-3-phenyl-propyl]pyrazole-3-carboxylate (11.47 g, 30.71 mmol) in Dioxane (100 mL) was added HCl/Dioxane (4N solution, 50 mL) and stirred at 25 °C for 16 hours. The mixture was filtered and the filter-cake was washed with EtOAc (30 mL) to provide ethyl 2-(2-amino-3-phenyl-propyl)pyrazole-3-carboxylate (8.50 g, crude HCl salt) as a white solid. Material was used as is in the next step. UPLC-MS: m/z = 274.5 $[M+H]^+$.



6-benzyl-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one

A solution of ethyl 2-(2-amino-3-phenyl-propyl)pyrazole-3-carboxylate (8.50 g, 31.10 mmol, Hydrochloride) in saturated $NaHCO_3$ in water (100 mL) was stirred at 25 °C for 16 hours. The solution was extracted with DCM (4 x 150 mL). The combined organic layers were dried over Na_2SO_4, filtered and the filtrate was concentrated to afford 6-benzyl-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one (5.30 g) as a white solid and was used as is in the next step. UPLC-MS: m/z= 228.2 $[M+H]^+$.



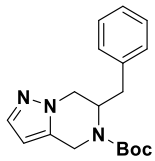
6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazine

To a solution of $LiAlH_4$ (5.30 g, 23.32 mmol) in dry THF (100 mL) was added 6-benzyl-6,7-dihydro-5H-pyrazolo[1,5-a]pyrazin-4-one (5.30 g, 23.32 mmol) dropwise under inert atmosphere at 0 °C. After 10 minutes, the mixture was heated to 65 °C and stirred for 16 hours. The mixture was cooled down, quenched with water (3 mL), 15% NaOH in water (3 mL) and water (9 mL).

The suspension was stirred at 25 °C for 1 hour. The solution was filtered and the filtrates were concentrated to afford 6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazine (4.50 g) as a white solid.

UPLC-MS: m/z= 214.1 $[M+H]^+$.

^{1}H NMR: (500MHz, METHANOL-d4) δ ppm 7.41 (d, J = 2.0 Hz, 1H), 7.37 - 7.22 (m, 5H), 6.05 (s, 1H), 4.14 (d, J = 16.0 Hz, 1H), 4.04 - 4.01 (m, 1H), 3.94 (d, J = 16.0 Hz, 1H), 3.74 (t, J = 11.5 Hz, 1H), 3.42 - 3.78 (m, 1H), 2.99 - 2.95 (m, 1H), 2.87 - 2.78 (m, 1H).

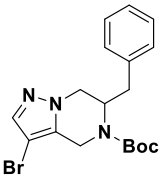


tert-butyl -6-benzyl-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-carboxylate

To a solution of 6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazine (4.50 g, 21.10 mmol) in DCM (50 mL) was added triethylamine (6.41 g, 63.30 mmol, 8.78 mL), Boc_2O (5.53 g, 25.32 mmol, 5.82 mL) at 25 °C and stirred for 2 hours. The mixture was concentrated and purified by column chromatography on silica gel (eluted with EtOAc in petroleum ether：20%) to give tert-butyl 6-benzyl-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazine-5-carboxylate (5.30 g, 55% yield over 3 steps) as a colorless oil.

LCMS: m/z = 256.1 $[M+H]^+$.

^{1}H NMR: (500MHz, METHANOL-d4) δ ppm 7.53 (d, J = 2.0 Hz, 1H), 7.34 - 7.10 (m, 5H), 6.24 (s, 1H), 4.96 - 4.94 (m, 2H), 4.47 -4.45 (m, 1H), 4.29 - 4.11 (m, 2H), 2.72 -2.71 (m, 2H), 1.46 - 1.28 (m, 9H).



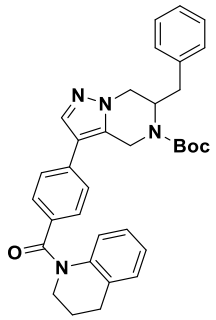
tert-butyl -6-benzyl-3-bromo-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-carboxylate

To a solution of tert-butyl 6-benzyl-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazine-5-carboxylate (5.30 g, 16.91 mmol) in DCM (80 mL) was added NBS (3.31 g, 18.60 mmol) and was stirred at 25 °C for 16 hours. The mixture was poured into water (100 mL) and extracted with DCM (200 mL x 2).

The combined organic layers were washed with brine (100 mL), dried over Na_2SO_4, and filtered. The residue was concentrated and purified by column chromatography on silica (gel eluted with EtOAc in petroleum ether 30%) to give tert-butyl 6-benzyl-3-bromo-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazine-5-carboxylate (4.10 g, 62% yield) as a yellow oil.

LCMS: m/z = 392 $[M+H]^+$.

^{1}H NMR: (500MHz, METHANOL-d4) δ ppm 7.56 (s, 1H), 7.35 - 7.11 (m, 5H), 5.01 - 4.88 (m, 1H), 4.86 - 4.71 (m, 1H), 4.35 - 4.11 (m, 3H), 2.73 - 2.71 (m, 2H), 1.49 - 1.25 (m, 9H).

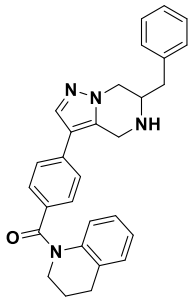


tert-butyl-6-benzyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-carboxylate

To a solution of tert-butyl 6-benzyl-3-bromo-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazine-5-carboxylate (1.00 g, 2.55 mmol) in Dioxane/H_2O (4:1, 20 mL) was added 3,4-dihydro-2H-quinolin-1-yl-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]methanone (1.85 g, 5.10 mmol), K_2CO_3 (1.06 g, 7.65 mmol), $Pd(dppf)Cl_2$ (93.26 mg, 127.46 umol) under nitrogen atmosphere and was stirred at 75 °C for 2 hours. The mixture was concentrated and purified by chromatography on silica gel (eluted with EtOAc in petroleum ether: 40%) to give tert-butyl 6-benzyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-carboxylate (1.16 g, 83% yield) as a yellow solid.

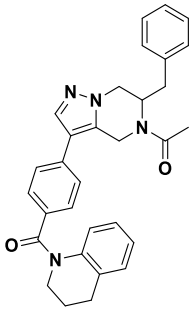
LCMS: m/z = 549.4 $[M+H]^+$.

^{1}H NMR: (400MHz, METHANOL-d4) δ ppm δ = 7.87 (s, 1H), 7.73 - 7.55 (m, 1H), 7.46 - 7.40 (m, 3H), 7.35 - 7.15 (m, 6H), 7.08 - 6.83 (m, 2H), 6.80 - 6.70 (m, 1H), 4.98 - 4.90 (m, 1H), 4.67 - 4.55 (m, 1H), 4.36 - 4.16 (m, 2H), 3.96 - 3.87 (m, 2H), 3.75 (s, 1H), 2.93 - 2.74 (m, 4H), 2.12 - 2.03 (m, 2H), 1.47 - 1.27 (m, 9H).



(4-(6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)phenyl)(3,4-dihydroquinolin-1(2H)-yl)methanone

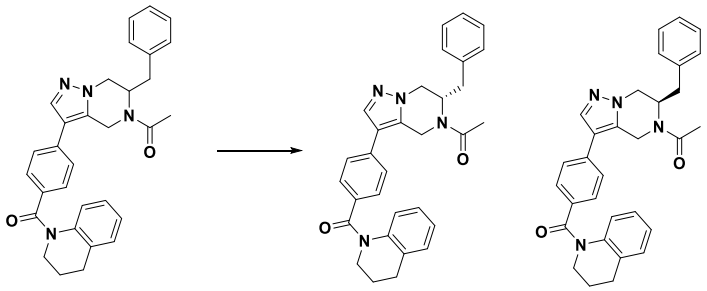
A solution of tert-butyl 6-benzyl-3-[4-(3,4-dihydro-2H-quinoline-1-carbonyl)phenyl]-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazine-5-carboxylate (1.16 g, 2.11 mmol) in 4N HCl in Dioxane (20 mL) was stirred for 3 hours at room temperature. The mixture was concentrated to give [4-(6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)phenyl]-(3,4-dihydro-2H-quinolin-1-yl)methanone (1.30 g, crude, HCl salt) as a yellow solid, and used as is in the next step. UPLC-MS: m/z = 449.2 $[M+H]^+$.



1-(6-benzyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)ethan-1-one BIO-1967878

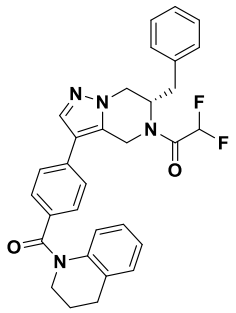
To a solution of [4-(6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)phenyl]-(3,4-dihydro-2H-quinolin-1-yl)methanone (100 mg, 223 umol), TEA (0.1 mL) in DCM (1 mL) was added acetyl chloride (55 mg, 701 umol, 50 uL) at 0 °C and was stirred at 25 °C for 2 hours. The mixture was concentrated, and the residue was purified by prep-HPLC (column: Phenomenex Synergi C18 150*30mm*4um; condition: water (0.05%HCl)-ACN; from 55%-75%) to give 1-[6-benzyl-3-[4-(3,4-dihydro-2H-quinoline-1-carbonyl)phenyl]-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-5-yl]ethanone (48 mg, 44% yield) as a white solid.

^{1}H NMR: (500MHz, METHANOL-d4) δ ppm 7.94 - 7.89 (m, 1H), 7.48 - 7.45 (m, 2H), 7.44 - 7.40 (m, 2H), 7.34 - 7.26 (m, 3H), 7.23 - 7.15 (m, 3H), 7.03 (t, J = 7.5 Hz, 1H), 6.90 - 6.89 (m, 1H), 6.76 (br s, 1H), 5.49 - 5.33 (m, 1H), 4.95 - 4.90 (m, 1H), 4.74 - 4.66 (m, 1H), 4.36 (s, 2H), 4.10 - 3.91 (m, 2H), 2.91 - 2.64 (m, 4H), 2.16 (s, 1H), 2.09 - 2.04 (m, 2H), 1.83 (m, 2H).
HPLC: purity: 99.4 %.
UPLC-MS: m/z = 491.1 $[M+H]^+$.



(S)-1-(6-benzyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)ethan-1-one BIO-1975900

1-(6-benzyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)ethan-1-one (25 mg) was separated by chiral SFC using CHIRALPAK IB 30x250mm, 5um Method: 40% EtOH w/ 0.1% DEA in CO_2 (flow rate: 100 mL/min, ABPR 120 bar, MBPR 40psi, column temp 40°C). First elution product Rf E1: 3.05min provided BIO-1975900 (6.9 mg, %ee = 100%). Second elution product Rf E2: 3.60min provided BIO-1975902 (8.2 mg, %ee = 97%). The stereochemistry of the products was not determined but was assigned based on the biological activities observed in our program for this subseries. Analogs prepared chiraly as the (S)-configuration displayed activity while the match pair (R)-configuration were inactive.



(S)-1-(6-benzyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)-2,2-difluoroethan-1-one BIO-2008218

(S)-(4-(6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)phenyl)(3,4-dihydroquinolin-1(2H)-yl)methanone was prepared following the general procedure A using (S)-2-amino-3-phenylpropan-1-ol in the first step.

To a solution of [4-[(6S)-6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl]phenyl]-(3,4-dihydro-2H-quinolin-1-yl)methanone (120 mg, 267 umol) in EtOAc (2 mL) was added TEA (81 mg, 802 umol), T_3P (170 mg, 267 umol, 50% purity) and 2,2-difluoroacetic acid (39 mg, 401 umol, 25 uL) . The mixture was stirred at 20°C for 16 hours, then was concentrated and purified by prep-HPLC (column:Phenomenex Synergi C18 150*30mm*4um; condition : water (0.05% HCl)-ACN; from : 50%-80%) to give 1-[(6S)-6-benzyl-3-[4-(3,4-dihydro-2H-quinoline-1-carbonyl)phenyl]-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-5-yl]-2,2-difluoro-ethanone (21 mg, 15% yield) as white solid.

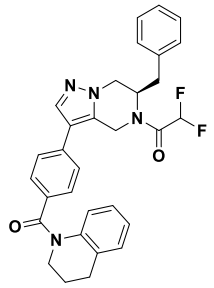
1HNMR: (500 MHz, METHANOL-d4) ppm: 7.87-7.94 (m, 1H), 7.39-7.52 (m, 4H), 7.15-7.36 (m, 6H), 7.04 (t, J=7.50 Hz, 1H), 6.84-6.94 (m, 1H), 6.69-6.83 (m, 1H), 6.18-6.65 (m, 1H), 4.90-5.48 (m, 2H), 4.69-4.77 (m, 1H), 4.19-4.33 (m, 2H), 3.90 (t, J=6.50 Hz, 2H), 2.81-3.01 (m, 4H), 2.04-2.10 (m, 2H).

UPLC-MS: m/z = 527.4 $[M+H]^+$.

HPLC: purity: 95.9 %.

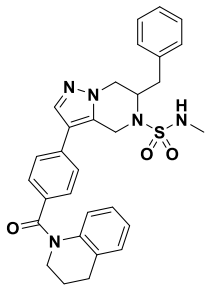
SFC: purity: %ee = 100.00 %, Rt = 6.26 min. Chiralpak AD-3 150 Á4.6mm I.D., 3um

Mobile phase: 40% of ethanol (0.05% DEA) in CO_2 Flow rate: 2.5mL/min



(R)-1-(6-benzyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)-2,2-difluoroethan-1-one BIO-2008219

Was prepared similarly to BIO-2008218 using (R)-2-amino-3-phenylpropan-1-ol and general procedure A. purity: ee = 100.00 %, Rt = 4.66 min. Chiralpak AD-3 150 Á4.6mm I.D., 3um Mobile phase: 40% of ethanol (0.05% DEA) in CO_2 Flow rate: 2.5mL/min



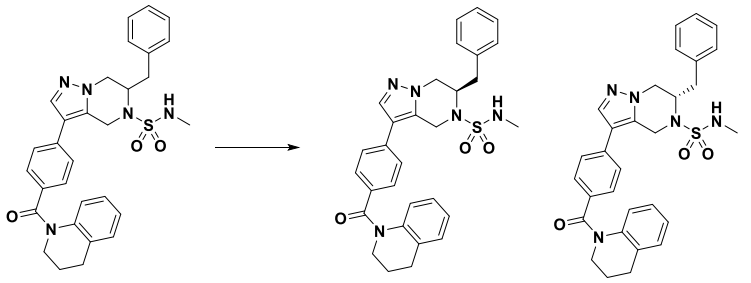
6-benzyl-N-methyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-sulfonamide BIO-2005981

To a solution of (4-(6-benzyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)phenyl)(3,4-dihydroquinolin-1(2H)-yl)methanone (40 mg, 0.089 mmol) and TEA (13.5 mg, 0.13 mmol) in THF (1.0 mL) was added methylsulfamoyl chloride (12 mg, 0.089 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 hours. The mixture was concentrated and purified by prep-HPLC (Column: Phenomenex Synergi C18 150*30mm*4um; condition: water (0.05% HCl)-ACN, 51~71%; FlowRate (ml/min): 25) to give 6-benzyl-N-methyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-sulfonamide (14 mg, 27% yield, HCl salt) as a white solid.

UPLC-MS: m/z = 542.4 $[M+H]^+$.

HPLC: purity = 100.00%.

^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm: 7.90 (s, 1H), 7.39-7.46 (m, 4H), 7.30-7.35 (m, 2H), 7.20-7.29 (m, 4H), 7.02-7.06 (m, 1H), 6.67-6.84 (m, 1H), 6.73-6.80 (m, 1H), 4.90-4.92 (m, 1H), 4.68 (d, *J*=17.0 Hz, 1H), 4.54-4.61 (m, 1H), 4.25-4.31 (m, 1H), 4.15-4.21 (m, 1H), 3.88-3.92 (m, 2H), 3.04-3.10 (m, 1H), 2.86-2.96 (m, 3H), 2.28 (s, 3H), 2.04-2.10 (m, 2H).



(R)-6-benzyl-N-methyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-sulfonamide BIO-2006664

(S)-6-benzyl-N-methyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-sulfonamide BIO-2006661

The racemic 6-benzyl-N-methyl-3-(4-(1,2,3,4-tetrahydroquinoline-1-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazine-5(4H)-sulfonamide (30 mg, 0.055 mmol) was separated by chiral SFC (Condition: DAICEL CHIRALCEL OD-H (250mm*30mm,5um); Column: 0.1%NH_3H_2O EtOH; Begin B: 50%; End B: 50%; Flow Rate (ml/min): 70) to give the desired product. The stereochemistry was not assigned but derived from SAR observed in our program and BIO-2008218 data.

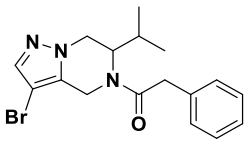
E1 (BIO-2006661), 11.7 mg as a white solid. Rt = 1.97 min.

LCMS: m/z = 542.3 $[M+H]^+$. HPLC: Purity: 100%. SFC: ee = 98.3%.

E2 (BIO-2006664), 6.9 mg as a white solid. Rt = 2.35 min

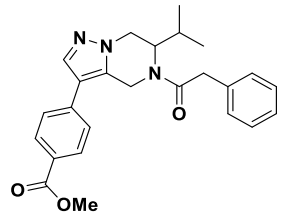
LCMS: m/z = 542.3 $[M+H]^+$. HPLC: Purity: 100%. SFC: ee = 100%.

BIO-1966561, BIO-2007817 and BIO-2007818 were prepared as described in *iScience*, **2022**, 25(1), 103650.



1-(3-bromo-6-isopropyl-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)-2-phenylethan-1-one

Material was prepared using methods described in *iScience*, 2022, 25(1), 103650 using racemic 2-amino-3-methylbutan-1-ol.

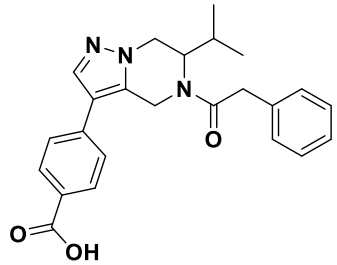


methyl (R)-4-(6-isopropyl-5-(2-phenylacetyl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)benzoate

A solution of 1-(3-bromo-6-isopropyl-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-5-yl)-2-phenyl-ethanone (1.15 g, 3.17 mmol), (4-methoxycarbonylphenyl)boronic acid (0.86 g, 4.76 mmol), $Pd(PPh_3)_4$ (0.18 g, 158.73 umol) and K_2CO_3 (2 M in water, 4.8 mL) in dioxane:water (30:10 mL) was stirred at 85°C for 2h. The solution was cooled down, diluted in water followed by extraction with DCM. The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography on silica gel (0 to 100% AcOEt in heptane) to afford methyl 4-[6-isopropyl-5-(2-phenylacetyl)-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-3-yl]benzoate (1.10 g, 83 % yield).

UPLC-MS: m/z = 418 $[M+H]^+$.

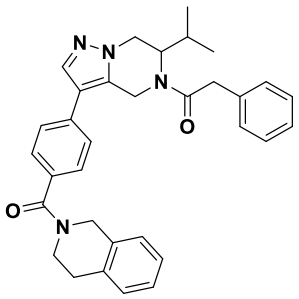
^1H NMR (500 MHz, DMSO-d_6) δ = 8.05 - 7.92 (m, 3H), 7.70 - 7.57 (m, 2H), 7.38 - 7.16 (m, 5H), 5.61 - 5.27 (m, 1H), 4.77 - 4.59 (m, 1H), 4.45 - 3.83 (m, 8H), 1.82 - 1.70 (m, 1H), 1.63 - 1.46 (m, 1H), 0.99 - 0.87 (m, 3H), 0.76 (m, 3H).



4-(6-isopropyl-5-(2-phenylacetyl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)benzoic acid

A solution of methyl 4-[6-isopropyl-5-(2-phenylacetyl)-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-3-yl]benzoate (1.05 g, 2.51 mmol) and lithium hydroxide (1M in water, 5 mL) in THF:MeOH:water (2:1:1, 25 mL) was stirred at 55°C overnight. The solution was cooled down and diluted in water (pH 14). The aqueous layer was washed with DCM (2x), then was acidified to pH2 with 6N HCl followed by extraction with DCM. The combined organic layers were dried

over Na_2SO_4, filtered and concentrated to provide 4-[6-isopropyl-5-(2-phenylacetyl)-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-3-yl]benzoic acid (915 mg, 90% yield) which was used crude in the next step. UPLC-MS: m/z = 404 $[M+H]^+$.

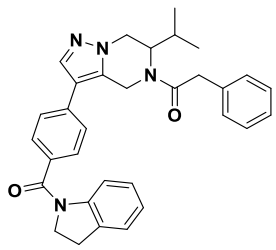


1-(6-isopropyl-3-(4-(1,2,3,4-tetrahydroisoquinoline-2-carbonyl)phenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)-2-phenylethan-1-one BIO-1967660

A solution of 4-[6-isopropyl-5-(2-phenylacetyl)-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-3-yl]benzoic acid (15 mg, 37 umol), 1,2,3,4-tetrahydroisoquinoline (9.9 mg, 74.3 umol)), DIPEA (26 uL, 148.7 umol) and HATU (17 mg, 44.6 umol) in DCM (2 mL) was stirred at room temperature for 3h. The solution was diluted in water followed by extraction with DCM. The combined organic layers were filtered through a 0.45uM filter, then was evaporated and purified by prep HPLC (Waters SunFire Prep C18 5um OBD 19x100mm, 5 to 70% CH_3CN in water, TFA buffer) to afford 1-[3-[4-(3,4-dihydro-1H-isoquinoline-2-carbonyl)phenyl]-6-isopropyl-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-5-yl]-2-phenyl-ethanone (16 mg, 82% yield).

UPLC-MS: m/z = 519 $[M+H]^+$.

^1H NMR (500 MHz, DMSO-d_6) δ = 7.99 (m, 1H), 7.71 - 7.15 (m, 13H), 5.67 - 5.34 (m, 1H), 4.95 - 2.86 (m, 12H), 1.88 - 1.51 (m, 1H), 1.09 - 0.88 (m, 3H), 0.82 m, 3H).

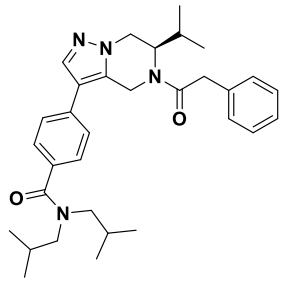


1-(3-(4-(indoline-1-carbonyl)phenyl)-6-isopropyl-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)-2-phenylethan-1-one BIO-1979167

A solution of 4-[6-isopropyl-5-(2-phenylacetyl)-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-3-yl]benzoic acid (15 mg, 37.1 umol), indoline (9 mg, 74.3 umol)), DIPEA (20 ul, 111.5 umol) and HATU (21 mg, 55.8 umol) in DMF (0.1 M) was stirred at room temperature for 16h. The solvent was removed under vaccum. The residue was diluted in AcOEt (3 mL), washed with water then was concentrated under vaccum. The residue was purified by prep HPLC (Waters SunFire Prep C18 5um OBD 19x100mm, 20 to 75% CH_3CN in water, TFA buffer) to afford 1-(3-(4-(indoline-1-carbonyl)phenyl)-6-isopropyl-6,7-dihydropyrazolo[1,5-a]pyrazin-5(4H)-yl)-2-phenylethan-1-one (15.2 mg, 66% yield, TFA salt).

UPLC-MS: m/z = 505.2 $[M+H]^+$.

^1H NMR (500 MHz, DMSO-d_6) δ = 8.12 - 7.50 (m, 6H), 7.41 - 6.92 (m, 8H), 5.61 - 5.30 (m, 1H), 4.80 - 3.05 (m, 10H), 1.83 - 1.49 (m, 1H), 1.01 - 0.88 (m, 3H), 0.77 (m, 3H).



(R)-N,N-diisobutyl-4-(6-isopropyl-5-(2-phenylacetyl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)benzamide BIO-1983977

A solution of 4-[6-isopropyl-5-(2-phenylacetyl)-6,7-dihydro-4H-pyrazolo[1,5-a]pyrazin-3-yl]benzoic acid (15 mg, 37.1 umol), N-isobutyl-2-methyl-propan-1-amine (9 mg, 74.3 umol), DIPEA (20 ul, 111.5 umol) and HATU (21 mg, 55.8 umol) in DMF (0.1 M) was stirred at room temperature for 16h. The solvent was removed under vaccum. The residue was diluted in AcOEt (3 mL), washed with water then was concentrated under vaccum. The residue was purified by prep HPLC (Waters SunFire Prep C18 5um OBD 19x100mm, 20 to 75% CH_3CN in water, TFA buffer) to afford N,N-diisobutyl-4-(6-isopropyl-5-(2-phenylacetyl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)benzamide (19 mg, 70% yield).

UPLC-MS: m/z = 515.3 $[M+H]^+$.

^{1}H NMR (500 MHz, DMSO-d_6) δ = 7.92 (d, *J* = 1.2 Hz, 1H), 7.61 - 7.46 (m, 2H), 7.42 - 6.89 (m, 7H), 5.57 - 5.29 (m, 1H), 4.75 - 4.58 (m, 1H), 4.43 - 3.83 (m, 5H), 3.43 - 3.00 (m, 2H), 2.79 - 2.51 (m, 2H), 2.13 - 1.45 (m, 3H), 1.06 - 0.58 (m, 18H).

N,N-diisobutyl-4-(6-isopropyl-5-(2-phenylacetyl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazin-3-yl)benzamide (19 mg) was separated by chiral SFC using CHIRALPAK AD-H 30x250mm, 5um, Method: 40% MeOH w/ 0.1% DEA in CO_2 (flow rate: 100 mL/min, ABPR 120bar, MBPR 40psi, column temp 40°C). First elution product Rf E1: 1.89 min provided BIO-1983977 (2.2 mg, %ee = 100%). Second elution product Rf E2: 2.73 min provided BIO-1983978 (2.4 mg, %ee = 100%). The stereochemistry of the products was not determined but was assigned based on the biological activities observed in our program for this subseries. Analogs prepared chiraly as the (R)-configuration displayed activity while the match pair (S)-configuration were inactive.