Supporting Information

Pyrimidoaminotropanes as Potent, Selective and Efficacious Small Molecule Kinase Inhibitors of the Mammalian Target of Rapamycin (mTOR)

Anthony A. Estrada,*,[†] Daniel G. Shore,[†] Elizabeth Blackwood,[∞] Yung-Hsiang Chen,[Δ] Gauri Deshmukh,[Δ] Xiao Ding,[Δ] Antonio G. DiPasquale,[Ω] Jennifer A. Epler,[∞] Lori S. Friedman,[∞] Michael F. T. Koehler,[†] Lichuan Liu,[Δ] Shiva Malek,[§] Jim Nonomiya,[§] Daniel F. Ortwine,[†] Zhonghua Pei,[†] Steve Sideris,[§] Frederic St-Jean,[†] Lan Trinh,[§] Tom Truong,[∞] and Joseph P. Lyssikatos[†]

[†]Departments of Discovery Chemistry, [∞]Translational Oncology, [§]Biochemical and Cellular Pharmacology, and [Δ]Drug Metabolism and Pharmacokinetics, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, United States

[Ω]X-ray Crystallographic Facility, University of California Berkeley, 32 Lewis Hall Berkeley, California 94720, United States

*Corresponding Author. Tel: 650-225-4934, E-mail: estrada.anthony@gene.com

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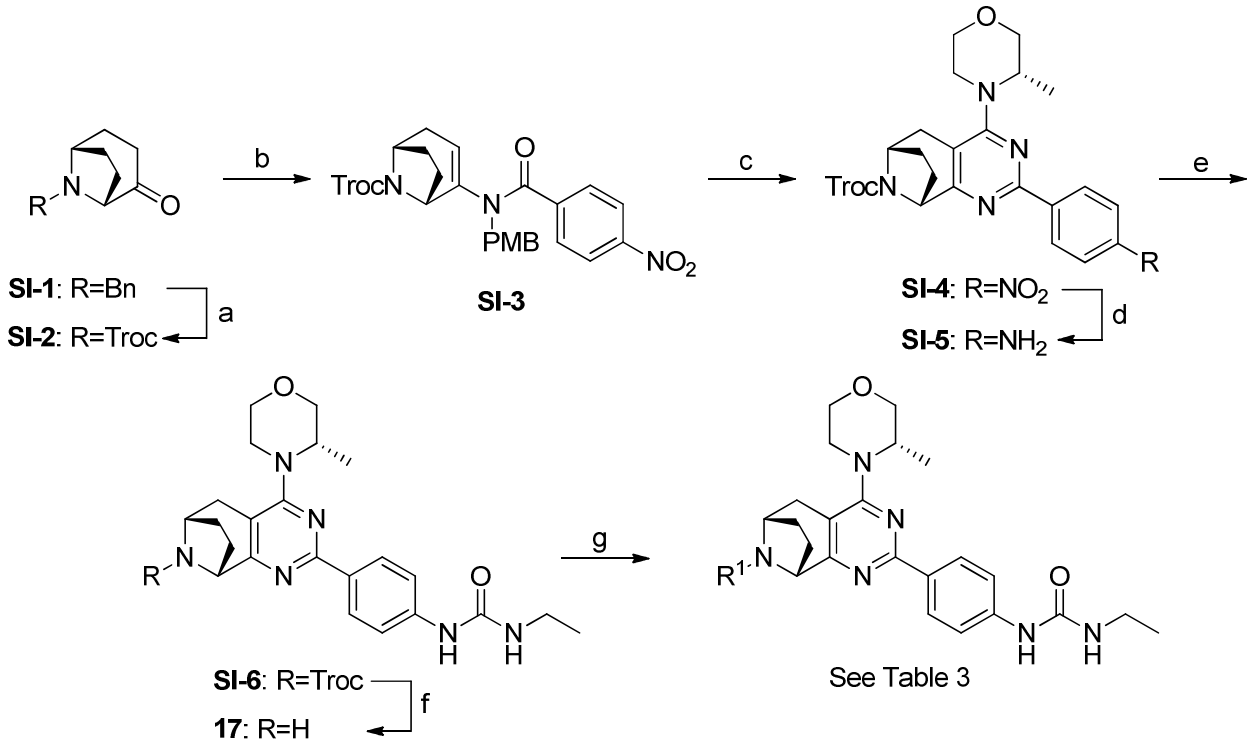
Supplementary Scheme 1. Synthesis of 7-PATs[a]



[a] Reagents and conditions: (a) Troc-Cl, MeCN, 25 °C, 24 h, 96%; (b) $PMBNH_2$, $Ti(OEt)_4$, CH_2Cl_2, 40 °C, 16 h, then Et_3N, *p*-NO_2BzCl, 0–25 °C, 74%; (c) (*S*)-3-methylmorpholine-4-carbonitrile, Tf_2O, 2-ClPyr, CH_2Cl_2, -78–25 °C, 42%; (d) Fe, NH_4Cl, $EtOH/H_2O$, Δ, 2 h; (e) EtNCO, Et_3N, THF, 25 °C, 62% (f) Zn, 1.0 M $NH_4OAc_{(aq)}$, THF, 25 °C, 48 h, 49%; (g) see experimental section.

Compounds 18, 19, 20, 21, 22, 23 and 24 were synthesized from a mixture of 17 and respective diastereomer. See below for experimental procedures for pure diastereomer (17).

(1*R*,5*S*)-8-benzyl-8-azabicyclo[3.2.1]octan-2-one (SI-1). Racemic **16**[1] (15.6 g, 72.5 mmol) was submitted to chiral SFC to yield the title compound as a pure enantiomer (7.07 g, 44% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 – 7.56 (m, 5H), 4.02 (s, 2H), 3.74 – 3.67 (m, 2H), 2.79 – 2.50 (m, 5H), 2.15 – 2.04 (m, 3H). LCMS, *m/z* = 216 $[M+H]^+$.

(1*R*,5*S*)-2,2,2-trichloroethyl 2-oxo-8-azabicyclo[3.2.1]octane-8-carboxylate (SI-2). To a solution of **SI-1** (1.15 g, 5.35 mmol) in acetonitrile (10 mL) was added 2,2,2-trichloroethyl chloroformate (3.8 mL, 28 mmol) and the reaction mixture was stirred for 24 h at rt. The reaction mixture was concentrated in vacuo and the crude residue was purified by flash column chromatography (0–15% EtOAc:DCM) to provide the title compound (1.55 g, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.84 – 4.76 (m, 1H), 4.75 – 4.66 (m, 1H), 4.58 (s, 1H), 4.52 (s, 1H), 2.57 – 2.22 (m, 5H), 1.57 – 1.52 (m, 1H). LCMS, *m/z* = 301 $[M+H]^+$.

(1*R*,5*S*)-2,2,2-trichloroethyl 2-(*N*-(4-methoxybenzyl)-4-nitrobenzamido)-8-azabicyclo[3.2.1]oct-2-ene-8-carboxylate (SI-3). To a solution of **SI-2** (1.55 g, 5.2 mmol) and 4-methoxybenzylamine (0.68 mL, 5.2 mmol) in methylene chloride (33 mL) was added titanium(IV) ethoxide (5.4 mL, 26 mmol). The reaction mixture was heated to reflux overnight and then cooled to 0 °C. To the cooled mixture was added triethylamine (1.33 mL, 9.5 mmol) and 4-nitrobenzoyl chloride (1.05 g, 5.7 mmol) sequentially and the mixture was stirred at 0 °C for 10 min then warmed to rt. After 1 h, an additional portion of 4-nitrobenzoyl chloride (1.05 g, 5.7 mmol) was added and the reaction mixture was stirred at rt for an additional 2 h. An additional portion of 4-nitrobenzoyl chloride (1.05 g, 5.7 mmol) was added and the reaction mixture was stirred at rt for 2 h. An additional portion of 4-nitrobenzoyl chloride (1.05 g, 5.7 mmol) was added and the reaction mixture was stirred at rt for 2 h, (4.4 equiv. of 4-nitrobenzoyl chloride were added in total). The reaction mixture was filtered through a short pad of celite, eluting with DCM. The filtrate was washed with sat. aq. ammonium chloride, the aqueous layer was extracted with DCM (2x) and the combined organic layers were dried over sodium sulfate, filtered and concentrated in vacuo. The crude residue was purified by flash column chromatography (20–50% EtOAc:Heptane) to provide the title compound (2.18 g, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 – 8.15 (m, 2H), 7.71 – 7.62 (m, 2H), 7.35 – 7.26 (m, 2H), 6.90 – 6.82 (m, 2H), 5.18 – 4.73 (m, 4H), 4.56 (d, *J* = 11.9 Hz, 1H), 4.46 – 4.31 (m, 2H), 3.81 (s, 3H), 2.61 – 2.44 (m, 1H), 2.19 – 2.06 (m, 1H), 1.99 – 1.84 (m, 1H), 1.84 – 1.67 (m, 2H), 1.42 – 1.30 (m, 1H). LCMS, *m/z* = 570 $[M+H]^+$.

(6*S*,9*R*)-2,2,2-trichloroethyl 4-((*S*)-3-methylmorpholino)-2-(4-nitrophenyl)-6,7,8,9-tetrahydro-5*H*-6,9-epiminocyclohepta[*d*]pyrimidine-10-carboxylate (SI-4). To a mixture of **SI-3** (2.17 g, 3.81 mmol), (*S*)-3-methylmorpholine-4-carbonitrile (0.53 g, 4.2 mmol) and 2-chloropyridine (0.43 mL, 4.6 mmol) in methylene chloride (24 mL) cooled to -78 °C was added trifluoromethanesulfonic anhydride (0.71 mL, 4.2 mmol) dropwise over 1 min and stirred for 5 min at -78 °C. The reaction mixture was then warmed to 0 °C for 5 min and then to rt and stirred for 30 min. The reaction mixture was diluted with DCM, washed with 1 N $NaOH_{(aq)}$ and the aqueous layer was extracted with DCM (2x). The combined organic layers were dried and concentrated in vacuo. The crude residue was purified by flash column chromatography (30% Acetone:Heptane) to provide the title compound (0.90 g, 42% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (d, *J* = 8.9 Hz, 2H), 8.28 (d, *J* = 8.8 Hz, 2H), 5.19 (s, 1H), 4.98 – 4.72 (m, 2H), 4.69 (d, *J* = 12.1 Hz, 1H), 4.02 (d, *J* = 7.3 Hz, 1H), 3.98 – 3.84 (m, 2H), 3.85 – 3.75 (m, 1H), 3.69 (d, *J* = 2.5 Hz, 2H), 3.49 – 3.34 (m, 2H), 2.43 – 2.22 (m, 3H), 2.14 – 2.00 (m, 1H), 1.72 – 1.58 (m, 1H), 1.48 (d, *J* = 6.8 Hz, 3H). LCMS, *m/z* = 556 $[M+H]^+$.

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[1] Denmark, S. E.; Matsuhashi, H. Chiral fluoro ketones for catalytic asymmetric epoxidation of alkenes with oxone. *J. Org. Chem.* **2002**, *67*, 3479–3486.

(6*S*,9*R*)-2,2,2-trichloroethyl 2-(4-aminophenyl)-4-((*S*)-3-methylmorpholino)-6,7,8,9-tetrahydro-5*H*-6,9-epiminocyclohepta[*d*]pyrimidine-10-carboxylate (SI-5). To a suspension of **SI-4** (0.90 g, 1.62 mmol) in ethanol (30 mL) and water (4.7 mL) was added ammonium chloride (0.35 g, 6.47 mmol) and iron powder (0.45 g, 8.09 mmol). The reaction mixture was stirred vigorously and heated to reflux for 2 h then diluted with DCM. The reaction mixture was washed with sat. aq. sodium bicarbonate and the aqueous layer was extracted with DCM (2x). The combined organic layers were dried over sodium sulfate, filtered and concentrated in vacuo to give the crude title compound which was used directly in the next step.

(6*S*,9*R*)-2,2,2-trichloroethyl 2-(4-(3-ethylureido)phenyl)-4-((*S*)-3-methylmorpholino)-6,7,8,9-tetrahydro-5*H*-6,9-epiminocyclohepta[*d*]pyrimidine-10-carboxylate (SI-6). To crude **SI-5** (0.88 g, 1.7 mmol) dissolved in tetrahydrofuran (8.11 mL) was added triethylamine (0.70 mL, 5.0 mmol) and ethylisocyanate (1.3 mL, 16.7 mmol). The reaction mixture was stirred overnight at rt. The reaction mixture was quenched by the addition of MeOH and concentrated in vacuo. The crude residue was purified by flash column chromatography (0–40% EtOAc:DCM) to provide the title compound (0.622 g, 62% yield, two steps). LCMS, *m/z* = 599 $[M+H]^+$.

1-ethyl-3-(4-((6*S*,9*R*)-4-((*S*)-3-methylmorpholino)-6,7,8,9-tetrahydro-5*H*-6,9-epiminocyclohepta[*d*]pyrimidin-2-yl)phenyl)urea (17). To a solution of **SI-6** (0.62 g, 1.04 mmol) in tetrahydrofuran (10 mL) at rt was added zinc dust (2.72 g, 41.6 mmol) and 1.0 M $NH_4OAc_{(aq)}$ (9.9 mL). The reaction mixture was stirred vigorously for 48 h at rt. The reaction mixture was diluted with EtOAc and sat. aq. sodium bicarbonate and filtered through celite, eluting with EtOAc. The layers were separated, the aqueous layer was extracted with EtOAc (2x) and the combined organics were dried over sodium sulfate, filtered and concentrated in vacuo. The crude residue was purified by preparative HPLC to give the title compound as a pure diastereomer (0.215 g, 49% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, *J* = 8.6 Hz, 2H), 7.35 (d, *J* = 8.6 Hz, 2H), 6.26 (s, 1H), 4.66 (t, *J* = 5.2 Hz, 1H), 4.32 (d, *J* = 5.5 Hz, 1H), 4.08 – 3.99 (m, 1H), 3.99 – 3.94 (m, 1H), 3.94 – 3.87 (m, 1H), 3.84 – 3.76 (m, 2H), 3.72 (dd, *J* = 11.3, 2.9 Hz, 1H), 3.64 (dd, *J* = 11.3, 2.2 Hz, 1H), 3.42 – 3.35 (m, 1H), 3.35 – 3.28 (m, 2H), 3.10 (dd, *J* = 16.2, 5.1 Hz, 1H), 2.29 (d, *J* = 16.1 Hz, 1H), 2.13 – 1.99 (m, 3H), 1.42 (d, *J* = 6.7 Hz, 3H), 1.17 (t, *J* = 7.2 Hz, 3H). LCMS, *m/z* = 423 $[M+H]^+$.

<u>In vitro microsome metabolic stability experiments were carried out as described in:</u>

Halladay, J. S.; Wong, S.; Jaffer, S. M.; Sinhababu, A. K.; Khojasteh-Bakht, S. C. Metabolic stability screen for drug discovery using cassette analysis and column switching. *Drug Metab. Lett*. **2007**, *1*, 67–72.

<u>Time-dependent CYP inhibition experiments were carried out as described in:</u>

(a) Mukadam, S.; Tay, S.; Tran, D.; Wang, L.; Delarosa, E. M.; Khojasteh, S. C.; Halladay, J.; Kenny, J. R. Evaluation of time-dependent cytochrome P450 inhibition in a high-throughput, automated assay: Introducing a novel area under the curve shift approach. *Drug Metab. Lett*. **2012**, *6*, 45–53.

(b) Obach, R. S.; Walsky, R. L.; Venkatakrishnan, K. Mechanism-based inactivation of human cytochrome P450 enzymes and the prediction of drug-drug interactions. *Drug Metab. Dispos.* **2007**, *35*, 246–255.

<u>In vitro protein binding experiments were carried out as follows</u>:

Binding in plasma was determined using a 48-well rapid equilibrium dialysis (RED) device using a dialysis membrane with molecule weight cut-off value of 8000 Da (Pierce Biotechnology, Rockford, IL). Blank plasma was spiked with testing compound for a concentration of 5 μM. 300 μL plasma containing compound was added to the donor side and 500 μL buffer was added to the receiver side. The plate was placed on a rocking platform at 400 rpm with 1 mm radius agitation for 4 hr at 37 °C. 20 μL of buffer and plasma samples were transferred to a 96-well plate and 20 μL of opposite blank matrix was added. Plasma proteins were precipitated using 65% acetonitrile containing internal standard (0.1 μM propranolol) and the unbound fraction (f_u) was calculated as the ratio of the buffer concentration versus the plasma concentration.

In-life PK/PD study experiments were carried out as follows:

Human prostate cancer NCI-NCI-PC3 cells (National Cancer Institute, Frederick, MD) were implanted subcutaneously into the right hind flanks of female NCR nude mice (5×10^6 cells in 100 μL Hank's Balanced Salt Solution). Tumors were monitored until they reached a mean tumor volume of approximately 500 mm^3, then similarly sized tumors were randomly assigned to groups (n=4). Compounds **8**, **20** and **1** were formulated as suspensions in 0.5% methylcellulose/0.2% Tween 80 (MCT) and dosed orally at 25, 50 and 100 mg/kg for **8**, 1.25, 2.5, and 5 mg/kg for **20** and 80 mg/kg for **1** (100 μL dose / 25 g animal). Plasma samples were harvested at 1, 6 and 10 hours post-dose for **8** and 6 hours post-dose for **20** and **1**.

Blood samples were harvested by terminal cardiac puncture and collected into tubes containing K_2EDTA as an anticoagulant. Samples were kept chilled on ice until centrifugation at 1500-2000xg for 5 minutes; plasma was transferred to a new vial and snap frozen. Samples were stored at -80 °C prior to PD biomarker evaluation.

Xenograft tumor lysates were prepared as follows:

Frozen tumors were lysed in ice-cold extraction buffer by mechanical disruption and agitation with garnet/ceramic sphere matrix in a FastPrep-24 (MP Biomedicals, Solon, OH; five 20-second cycles with rest periods on wet ice). Extraction buffer (Biosource; Carlsbad, CA) was supplemented with protease inhibitors (F. Hoffman-La Roche; Mannheim, Germany), 1 mM phenylmethysufonyl fluoride, and Phosphatase Inhibitor Cocktails 1 and 2 (Sigma-Aldrich; St. Louis, MO). After thorough disruption, tumor lysates were clarified by centrifugation at 4 °C. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce; Rockford, IL) and normalized to a standard concentration of 5 mg/ml.

PD biomarker assays were carried out as follows:

The Meso Scale Discovery Multi-Spot Biomarker Detection System (Meso Scale Discovery; Gaithersburg, MD) was used to determine the levels of Akt phosphorylated at serine 473 (pAkt) and S6RP phosphorylated at serine 235/236 (pS6RP). These double-determinant immunoassays quantify protein levels on the basis of measurements of electrochemoluminescence intensity. In the case of pS473 and total Akt, both of these were measured in the same well using a duplex assay plate. All other markers required separate plates to measure phosphorylated and total protein. Protein was loaded at 20 μg/well for Akt and 10 μg/well for S6RP.

Levels of phosphoprotein were normalized to the matched total protein signal (expressed as a ratio). Percent inhibition was calculated by comparison of treated tumor lysates with an average of the time-matched vehicle controls.

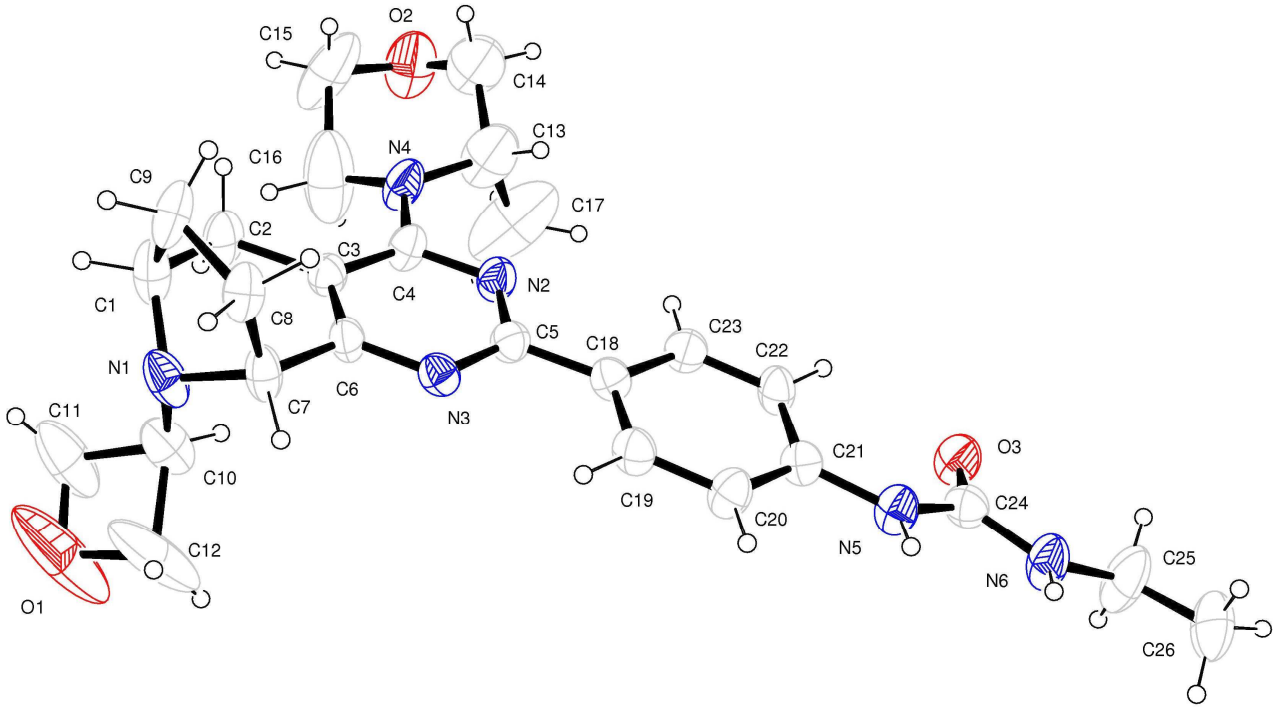
Tumor growth inhibition assays were carried out as follows:

Tumor volumes were determined using digital calipers (Fred V. Fowler Company, Inc.) using the formula (L × W × W)/2. Tumor growth inhibition (%TGI) was calculated as the percentage of the area under the fitted curve (AUC) for the respective dose group per day in relation to the vehicle, such that $\%TGI = 100 \times 1-(AUC_{treatment}/day)/(AUC_{vehicle}/day)$. Curve fitting was applied to Log_2 transformed individual tumor volume data using a linear mixed-effects model using the R package nlme, version 3.1–97 in R v2.12.0.

MCF7neo/HER2 tumor model experiments were carried out as follows:

Three days prior to cell inoculation, all mice received subcutaneous placement of a 0.36 mg sustained release estradiol 17-beta pellet (Innovative Research). Under general Isoflurane anesthesia (for restraint only), pellets were administered using a 10 or 12 gauge trochar needle. The pellet is placed either at the right shoulder or right flank, 1-2 cm from the skin entry point and the skin was closed using a single wound clip. Once the estrogen pellet was administered, topical analgesic was applied once skin entry point was made. Topical Lidocane HCL Jelly Usp 2% or Topical Emla Cream without Tegaderm was used as the analgesic. Animals were observed during recovery.

Taconic nude mice were inoculated in right upper flank, SC, with 5 million MDA-MD-361-X1 (aka MCF7 neo/Her2) cells in HBSS/matrigel (1:1), volume of 100 μL. When the tumors reached a mean volume of about 200 mm^3, 60 mice with similarly sized tumor were selected and grouped into 12 efficacy groups, n=5.



Supplementary Figure 1. ORTEP plot of **20**: CCDC 921296

Small molecule X-ray collection data and analysis (**20**, CCDC 921296):

X-ray quality crystals were grown from a saturated 1,2-dichloroethane solution with the slow diffusion of diisopropyl ether to reduce solubility and deposit the crystals. A colorless plate 0.06 x 0.04 x 0.02 mm in size was mounted on a Cryoloop with Paratone oil. Data were collected in a nitrogen gas stream at 100(2) K using phi and omega scan. Crystal-to-detector distance was 60 mm and exposure time was 5 seconds per frame using a scan width of 1.0°. Data collection was 98.3% complete to 62.99° in □. A total of 49601 reflections were collected covering the indices, -27<=*h*<=30, -17<=*k*<=17, -21<=*l*<=21. 8930 reflections were found to be symmetry independent, with an R_{int} of 0.0297. Indexing and unit cell refinement indicated a C-centered, monoclinic lattice. The space group was found to be C2 (No. 5). The data were integrated using the Bruker SAINT software program and scaled using the SADABS software program. Solution by direct methods (SIR-2008) produced a complete heavy-atom phasing model consistent with the proposed structure. All non-hydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-97). All hydrogen atoms were placed using a riding model. Their positions were constrained relative to their parent atom using the appropriate HFIX command in SHELXL-97. SQUEEZE was used to treat the disordered diisopropyl ether that cocrystallized with the target molecule. Absolute stereochemistry was unambiguously determined to be *R* at C7 and C33, respectively and *S* at C1, C13, C27, and C39, respectively.

Table 1. Crystal data and structure refinement for **20**.

X-ray ID	**20**

Empirical formula	C26 H34 N6 O3	
Formula weight	478.59	
Temperature	100(2) K	
Wavelength	1.54178 Å	
Crystal system	Monoclinic	
Space group	C2	
Unit cell dimensions	a = 27.2948(5) Å	□= 90°.
	b = 15.3732(3) Å	□= 132.5200(10)°.
	c = 18.4555(4) Å	□ = 90°.
Volume	5707.7(2) $Å^3$	
Z	8	
Density (calculated)	1.114 Mg/m^3	
Absorption coefficient	0.604 mm^{-1}	
F(000)	2048	
Crystal size	0.06 x 0.04 x 0.02 mm^3	
Crystal color/habit	colorless plate	
Theta range for data collection	3.25 to 62.99°.	
Index ranges	-27<=h<=30, -17<=k<=17, -21<=l<=21	
Reflections collected	49601	
Independent reflections	8930 [R(int) = 0.0297]	
Completeness to theta = 62.99°	98.3 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.9880 and 0.9647	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	8930 / 1 / 635	
Goodness-of-fit on F^2	1.076	
Final R indices [I>2sigma(I)]	R1 = 0.0792, wR2 = 0.2286	
R indices (all data)	R1 = 0.0925, wR2 = 0.2409	
Absolute structure parameter	0.14(8)	

Largest diff. peak and hole 0.427 and -0.233 e.Å^{-3}

Supplementary Table 1. Analytical SFC conditions

compd	Method	Instrument	Column brand	Column diameter (mm)	Column length (mm)	Column particle size (µm)	Column temperature (℃)	Mobile phase: A	Mobile phase: B	Gradient	Flow rate (mL/min)	UV Detection Wavelength (nm)	run length (min)	Mass spectrometer	Ionization	Scan range (AMU)
8, **9**	SFC1	Berger Analytical SFC/MS	Chiralpak IC	4.6	100	5.0	40	Carbon dioxide	Ethanol w/ 0.1%TEA	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800
10	SFC2	Berger Analytical SFC/MS	Chiralpak AS	4.6	100	5.0	40	Carbon dioxide	Methanol w/ 0.1%DEA	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800
11, **12**	SFC3	Berger Analytical SFC/MS	Lux Cellulose-1	4.6	100	5.0	40	Carbon dioxide	Methanol w/ 0.1%DEA	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800
17	SFC4	Berger Analytical SFC/MS	Chiralpak AS	4.6	100	5.0	40	Carbon dioxide	Methanol	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800
18, **19**	SFC5	Berger Analytical SFC/MS	Lux Cellulose-1	4.6	100	5.0	40	Carbon dioxide	Methanol w/ 0.1%TEA	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800
21	SFC6	Berger Analytical SFC/MS	Chiralpak IC	4.6	100	5.0	40	Carbon dioxide	Methanol	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800
22	SFC7	Berger Analytical SFC/MS	Lux Cellulose-1	4.6	100	5.0	40	Carbon dioxide	Methanol	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800
23	SFC8	Berger Analytical SFC/MS	Lux Cellulose-1	4.6	100	5.0	40	Carbon dioxide	Methanol	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800
24	SFC9	Berger Analytical SFC/MS	Lux Cellulose-1	4.6	100	5.0	40	Carbon dioxide	Ethanol w/ 0.1%DEA	5-50% in 4.5 min, hold at 55% for 0.5 min, reequilibrate to 10% for 1 min	5.0	220 and 254	6.0	Waters ZQ quadrupole	ESI+	140 - 800