

SUPPORTING INFORMATION

The Discovery of A-893, A New Cell-Active Benzoxazinone Inhibitor of Lysine Methyltransferase SMYD2.

Ramzi F. Sweis,^{*,†} Zhi Wang,[†] Mikkel Algire,[†] Cheryl H. Arrowsmith,^{‡, §} Peter J. Brown,[‡] Gary G. Chiang,[†] Jun Guo,[†] Clarissa G. Jakob,[†] Steven Kennedy,[‡] Fengling Li,[‡] David Maag,[†] Bailin Shaw,[†] Nirupama B. Soni,[†] Masoud Vedadi,^{‡,||} William N. Pappano[†]

[†] Discovery Research, AbbVie Inc., 1 North Waukegan Road, North Chicago, IL 60064

[‡] Structural Genomics Consortium, University of Toronto, Toronto, ON, M5G 1L7

[§] Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, Toronto, ON, M5G 2M9

^{||} Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, M5S 1A8

* **Corresponding Author:** Telephone: 847-937-0315 email: ramzi.sweis@abbvie.com

IN-VITRO BIOLOGY

SMYD2 Activity Assay:

SMYD2 activity assays were performed by monitoring the incorporation of the tritium labeled methyl group from *S*-adenosylmethionine (³H-SAM) to biotinylated peptide substrates using a Scintillation Proximity Assay (SPA). The reaction consisted of 5 nM SMYD2, 3 μM biotinylated p53 peptide (GSRAHSSHLKSKKGQSTSRH) and 70 nM ³H-SAM in reaction buffer containing 20 mM Tris pH 9.0, 2.5mM DTT, and 0.01% Triton X-100 in a final volume of 10 μL. Compounds were serially diluted in 10% DMSO followed by the addition of 1 μL to the reaction mixture resulting in 1% DMSO final. The reaction was carried out for 40 minutes at 23 °C before being quenched with 10 μL of 7.5 M guanidine hydrochloride. 180 μL of buffer was added, mixed, and transferred to a 96-well FlashPlate (Cat.# SMP103; Perkin Elmer; www.perkinelmer.com). After incubation the CPM was obtained using a Topcount plate reader (Perkin Elmer, www.perkinelmer.com) and the CPM counts in the absence of compound defined as 100% activity

while the CPM counts in the absence of the enzyme defined as background (0%). IC₅₀ values were determined using Graphpad Prism6.

Selectivity Assay:

Effect of A-893 on methyltransferase activity of G9a, EHMT1, SUV39H2, SETDB1, SETD8, SUV420H1, SETD7, MLL1 trimeric complex, EZH1 pentameric complex, EZH2 trimeric complex, PRMT1, PRMT3, PRMT5-MEP50 complex, PRMT6, PRMT7, PRMT8, PRDM9, SETD2, METTL21A, METTL21D, BCDIN3D, SMYD3 and DNMT1 was assessed by monitoring the incorporation of tritium-labeled methyl group to respective substrates using Scintillation Proximity Assay (SPA) as previously described (PMIDs: 25728001, 25136132). Assays were performed in a 20 μ l reaction mixture containing ³H-SAM (Cat.# NET155V250UC; Perkin Elmer; www.perkinelmer.com) at balanced conditions (substrate concentrations close to K_m values for each enzyme). Three concentrations (1 μ M, 10 μ M, and 50 μ M) of A-893 were used in all selectivity assays. To stop the enzymatic reactions, 7.5 M Guanidine hydrochloride was added, followed by 180 μ l of buffer (20 mM Tris, pH 8.0), mixed and then transferred to a 96-well FlashPlate (Cat.# SMP103; Perkin Elmer; www.perkinelmer.com). After mixing, the reaction mixtures in Flash plates were incubated for 1 hour and the CPM were obtained using Topcount plate reader (Perkin Elmer, www.perkinelmer.com). The CPM counts in the absence of compound for each data set were defined as 100% activity. In the absence of the enzyme, the CPM counts in each data set were defined as background (0%). For DNMT1 the dsDNA substrate was prepared by annealing two complementary strands (biotintlated forward strand: B-GAGCCCGTAAGCCCGTTCAGGTCG and reverse strand: CGACCTGAACGGGCTTACGGGCTC), synthesized by Eurofins MWG Operon.

For DOT1L, NSD1, NSD2, NSD3, ASH1L, SUV420H2, DNMT3A/3L, and DNMT3B/3L, a filter-based assay was used (PMIDs: 25728001, 25136132). In this assay, 20 μ l of reaction mixtures were incubated at RT for 1 hour, 100 μ l of 10% TCA was added, mixed and transferred to filter-plates (Millipore; cat.# MSFBN6B10; www.millipore.com). Plates were centrifuged at 2000 rpm (Allegra X-15R - Beckman Coulter, Inc.) for 2 min followed by 2 additional 10% TCA wash and one ethanol wash (180 μ l) followed by centrifugation. Plates were dried and 100 μ l MicroO (MicroScint-O; Cat.# 6013611, Perkin Elmer; www.perkinelmer.com) was added to each well, centrifuged and removed. 70 μ l of MicroO was added again and CPM was measured using Topcount plate reader.

Cellular Biology Methods:

Human A549 lung carcinoma cells (ATCC, Manassas, VA) were grown in DMEM + 10% Fetal Bovine Serum and plated at 5 million cells per 10 cm plate. The following day, compounds and DMSO control were added in triplicate at 10 mM each. Cells were incubated with compound for 24 hours and then lysed in complete lysis buffer containing protease and phosphatase inhibitors (Sigma, St. Louis, MO). Lysates were split and the Total p53 Assay Whole Cell Lysate Kit (MSD, Gaithersburg, MD) was performed according to manufacturer's directions to detect total levels of p53 protein. To detect cellular levels of p53K370me1, the remaining lysate was added to custom Mesoscale plates coated with anti-p53K370me1 antibody and signal was ultimately detected using sulfo-tagged mouse anti-total p53 according to manufacturer's instructions (MSD, Gaithersburg, MD). Rabbit anti-p53K370me1 (Genscript, Piscataway, NJ) was generated via peptide immunization and subsequent Protein A purification. Data presented represents the average of triplicate treatments and the error bars represent the standard deviation (n=3).

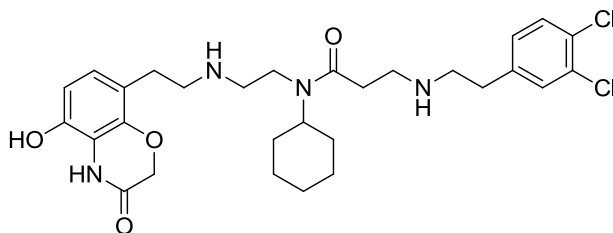
p53K370me1 / total p53 ratio			
	DMSO	A-893	AZ505
AVERAGE	0.097698	0.059956	0.022928
SD	0.00961	0.002629	0.001336
Total p53			
	DMSO	A-893	AZ505
AVE.	20935.67	19760.67	63852.67
SD	1202.17	1236.926	3782.972
p53K370me1			
	DMSO	A-893	AZ505
AVE.	2038	1182.667	1460.667
SD	89.94628	24.58319	2.516611

CHEMISTRY

General Experimental

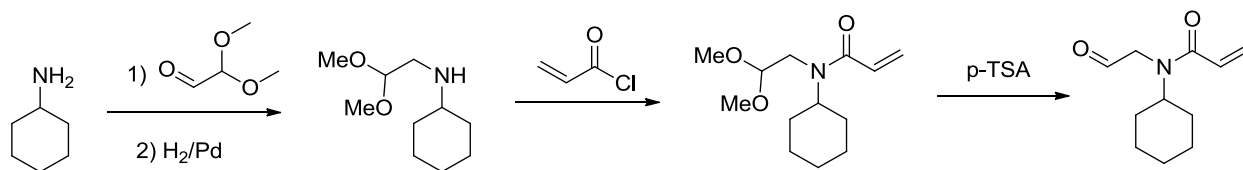
All reactions were carried out under an atmosphere of nitrogen. Reagents and solvents were used from commercial sources without additional purification. Hydrogenation reactions were run under a balloon. Sample purification was conducted on a Teledyne Isco or Analogix Intelliflash purification system using pre-packed commercially available silica gel columns. Reverse-phase purification was conducted on a Gilson preparative HPLC using a Phenomenex C18 column and eluting with a gradient of acetonitrile/water in 0.1% TFA. Purity of all final compounds was \geq 95% and determined by ^1H NMR (Varian Inova 400 NMR Spectrometer) and HPLC/MS (Agilent) via the following method: The gradient was 5% B for 0.1 min, 5-100% B in 5.1 min with a hold at 100% B for 0.5 min, then 100-5% B in 0.3 min (2.0 mL/min flow rate). Mobile phase A was 0.1% TFA in water, mobile phase B was HPLC grade MeCN. The column used for the chromatography was a 2.1 x 50 mm Phenomenex Luna Combi-HTS C8(2) column (5 μm particles) at a temperature of 65 $^\circ\text{C}$. Detection methods are with diode array (DAD) and evaporative light scattering (ELSD) detection under positive APCI ionization conditions.

N-cyclohexyl-3-((3,4-dichlorophenethyl)amino)-N-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)amino)ethyl)propanamide (1)



The title compound was made as described in WO 2008/075025 A1.

***N*-cyclohexyl-*N*-(2-oxoethyl)acrylamide.**



Step 1: *N*-(2,2-dimethoxyethyl)cyclohexanamine.

A mixture of 2,2-dimethoxyacetaldehyde (3.00 g, 50.4 mmol) and cyclohexanamine (5.00 g, 50.4 mmol) in methanol (50 ml) was stirred at room temperature for 16 hours to give a yellow solution. To this was then added Pd/C (0.537 g, 5.04 mmol). The mixture was hydrogenated for 24 hours at 2 bar. The mixture was filtered, and the filtrate was concentrated under reduced pressure to afford the title compound (8.0 g, 42.7 mmol, 85 % yield) as an oil, which was used without further purification.

Step 2: *N*-cyclohexyl-*N*-(2,2-dimethoxyethyl)acrylamide.

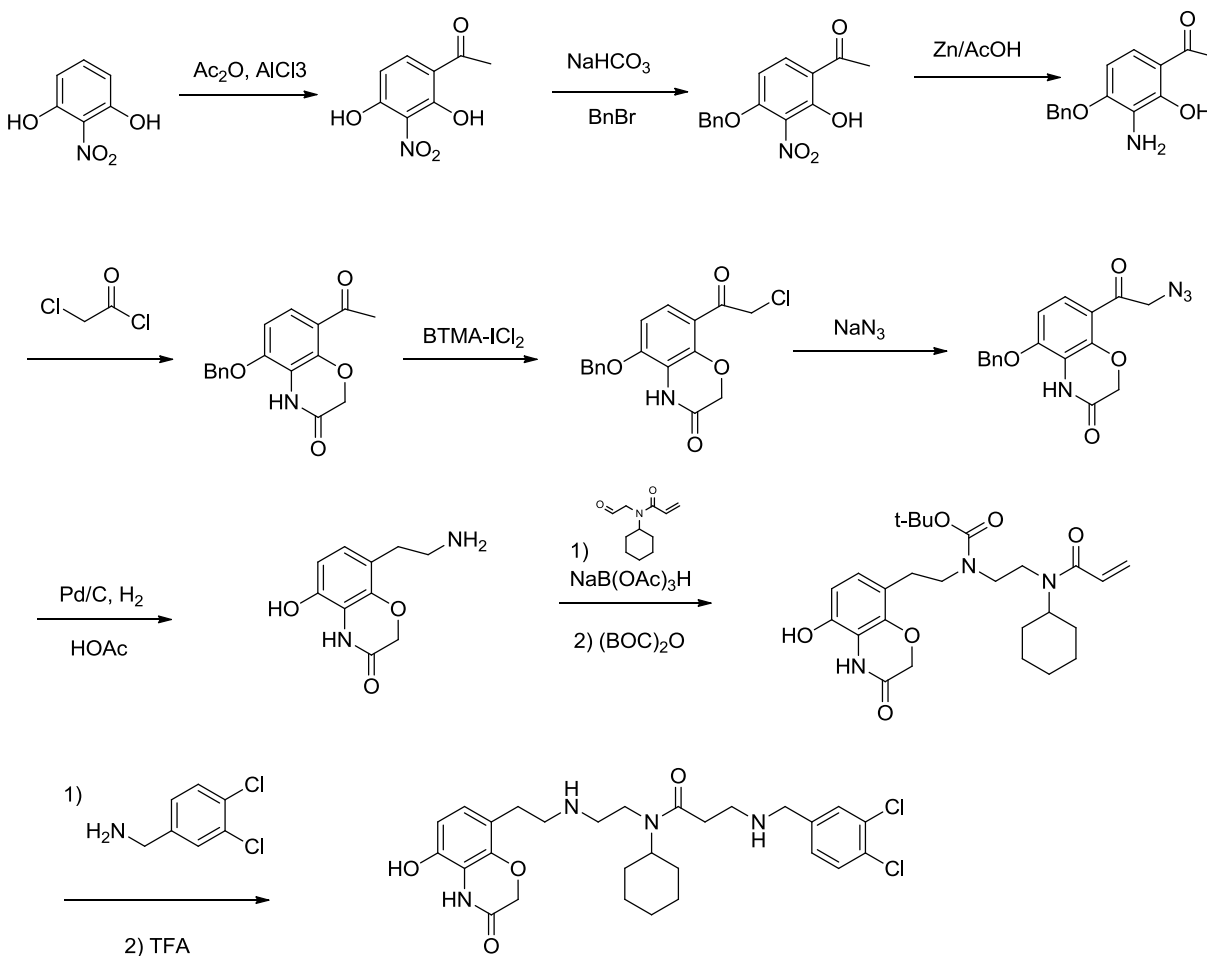
To a mixture of *N*-(2,2-dimethoxyethyl)cyclohexanamine (6.5 g, 34.7 mmol) and triethylamine (7.01 g, 69.4 mmol) in THF (50mL) was added acryloyl chloride (3.30 g, 36.4 mmol). The mixture was stirred at room temperature for 16 hours to give a yellow solution. The mixture was diluted with 100 mL of ethyl acetate. The organic phase was washed with 100 mL of sat. sodium bicarbonate and brine, dried over Na₂SO₄, and filtered. The filtrate was concentrated and the residue was purified by column chromatography on silica gel, eluting with pet. ether : EtOAc = 1:1 to afford the title compound (3.15 g, 13.1 mmol, 38 % yield) as an oil. MS (ESI+) 242 [M+H]⁺.

Step 3: *N*-cyclohexyl-*N*-(2-oxoethyl)acrylamide.

A mixture of *N*-cyclohexyl-*N*-(2,2-dimethoxyethyl)acrylamide (5.50 g, 22.8 mmol) and para-toluenesulfonic acid (3.92 g, 22.8 mmol) in dichloromethane (100 mL) was stirred at room temperature for 16 hours to give a yellow solution. The solution was diluted with additional dichloromethane, and washed with saturated aqueous sodium bicarbonate and brine. The organic layer was dried and concentrated to afford the crude product, which was purified via column

chromatography on silica gel (pet. ether : ethyl acetate= 1:1) to afford the title compound (1.10 g, 5.64 mmol, 25 % yield) as an oil. MS (ESI+) 196 [M+H]⁺.

***N*-cyclohexyl-3-((3,4-dichlorobenzyl)amino)-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[*b*][1,4]oxazin-8-yl)ethyl)amino)ethyl)propanamide (8)**



Step 1: 1-(2,4-dihydroxy-3-nitrophenyl)ethanone.

To a vigorously stirred solution of aluminum chloride (46.3 g, 348 mmol) in nitrobenzene (325 mL) was added 2-nitrobenzene-1,3-diol (24.5 g, 158 mmol) portionwise over 15 minutes. This was followed by the addition of acetic anhydride (15.6 mL) over 15 min. The resultant mixture was then heated to 100 °C for 5 hours. The reaction was cooled to room temperature and

carefully quenched with ice-cooled 2M hydrochloric acid (300 mL). The mixture was extracted with ether (2 × 500 mL) and the combined ether extracts were then washed with 2 M aqueous sodium hydroxide (2 × 400 mL). The combined aqueous layers were washed with ether and acidified to pH = 1 with 2 M hydrochloric acid (700 mL). The resulting precipitate was filtered, washed with water, and dried under vacuum at 40 °C to afford the title compound (29.5 g, 150 mmol, 95 % yield) as a brown solid. MS (ESI+) 198 [M+H]⁺.

Step 2: 1-(4-(benzyloxy)-2-hydroxy-3-nitrophenyl)ethanone.

To a solution of 1-(2,4-dihydroxy-3-nitrophenyl)ethanone (100 g, 508 mmol) in acetonitrile (700 mL) was added sodium bicarbonate (49.0 g, 583 mmol). The mixture was heated to 60 °C and benzyl bromide (75.6 mL) added. The mixture was then heated to reflux for 6.5 hours, and then cooled to 60 °C. Water (450 mL) was then added, and the mixture was cooled to below 45°C. At this point, methyl tert-butyl ether (450 mL) added and the mixture was cooled to 20°C and stirred for another 1.5 hours. The suspension was then filtered and washed with water (250 mL) then ethanol (250 mL) to yield the title compound. MS (ESI+) 288 [M+H]⁺.

Step 3: 1-(3-amino-4-(benzyloxy)-2-hydroxyphenyl)ethanone.

To a suspension of 1-(4-(benzyloxy)-2-hydroxy-3-nitrophenyl)ethanone (5.50 g 19.2 mmol) in acetic acid (55 mL) was added zinc dust (5.50 g, 84.1 mmol) portion-wise over 15 minutes, while maintaining the internal temperature below 40 °C with an ice bath. The mixture was allowed to warm to room temperature and stirred for a further 2 hours. The mixture was filtered through Celite and washed with acetic acid. The filtrate was then poured onto ice/water (500 mL). The resulting precipitate was filtered off, washed with water, and dried under vacuum at 40 °C to afford the title compound (4.80 g, 18.7 mmol, 97 % yield) as a light brown solid. MS (ESI+) 258 [M+H]⁺.

Step 4: 8-acetyl-5-(benzyloxy)-2H-benzo[b][1,4]oxazin-3(4H)-one.

2-Chloroacetyl chloride (1.77 mL) was added dropwise to a stirred mixture of 1-(3-amino-4-(benzyloxy)-2-hydroxyphenyl)ethanone (5.20 g, 20.2 mmol) and sodium hydrogen carbonate (3.74 g, 44.5 mmol) in DMF (30 mL) and then stirred for a further two hours. Cesium carbonate (7.90 g, 24.2 mmol) was added and the mixture was heated at 100 °C for 20 hours. The mixture

was cooled to room temperature, quenched with water (500 mL), extracted with ethyl acetate (2 × 200 mL), and the combined organic layers washed with water (3 × 300 mL) and brine. The organic layer was then dried over anhydrous sodium sulfate, filtered and evaporated in vacuo. The solid residue was treated with ether, filtered and the eluent dried to afford the title compound (5.70 g, 19.2 mmol, 95 % yield) as a beige solid. MS (ESI+) 298 [M+H]⁺.

Step 5: 5-(benzyloxy)-8-(2-chloroacetyl)-2H-benzo[b][1,4]oxazin-3(4H)-one.

Benzyltrimethylammonium dichloroiodate (14.2 g, 40.8 mmol) was added to a stirred solution of 8-acetyl-5-(benzyloxy)-2H-benzo[b][1,4]oxazin-3(4H)-one (5.50 g, 18.5 mmol) in a mixture of dichloromethane (100 mL), acetic acid (33 mL) and water (5.5 mL). The reaction mixture was stirred at 65 °C for 20 hours. The reaction was then cooled to room temperature, treated with aqueous sodium bisulphate (5.78 g, in 100 mL), and stirred for another 30 minutes. The mixture was diluted with ether (200 mL) and the resulting solid was filtered off, washed with water, then ether, and dried under vacuum at 40 °C to afford the title compound (5.60 g, 17.4 mmol, yield 94 % yield) as a yellow solid. MS (ESI+) 332 [M+H]⁺.

Step 6: 8-(2-azidoacetyl)-5-(benzyloxy)-2H-benzo[b][1,4]oxazin-3(4H)-one.

Sodium azide (1.18 g, 18.2 mmol) was added to a suspension of 5-(benzyloxy)-8-(2-chloroacetyl)-2H-benzo[b][1,4]oxazin-3(4H)-one (4.80 g, 14.5 mol) in DMF (50 mL) and the resulting mixture was stirred for 2 hours at room temperature. The mixture was poured onto ice water and the resulting solid was filtered off, washed with water and dried under vacuum at 40°C to afford the title compound (4.60 g, 13.6 mmol, 94 % yield) as an off-white solid. MS (ESI+) 339 [M+H]⁺.

Step 7: 8-(2-aminoethyl)-5-hydroxy-2H-benzo[b][1,4]oxazin-3(4H)-one.

A slurry of 10% palladium on carbon (1 gram) in acetic acid (20 mL) was added to a mixture of 8-(2-azidoacetyl)-5-(benzyloxy)-2H-benzo[b][1,4]oxazin-3(4H)-one (5.56 g, 16.4 mmol) in acetic acid (280 mL), followed by concentrated hydrochloric acid (14.3 mL). The mixture was stirred under a hydrogen atmosphere (5 bar) for 6 hours. Water (50 mL) was then added to dissolve any solid, followed by the addition of 10% palladium on carbon (1 g). The resultant mixture was stirred under a hydrogen atmosphere (5 bar) for another 20 hours. The mixture was

filtered through celite and the filtrate concentrated in vacuo. The solid residue was treated with ether, collected by filtration, and dried to afford the title compound (2.20 g, 10.6 mmol, 65 % yield) as a white solid. MS (ESI+) 209 [M+H]⁺.

Step 8: *tert*-butyl (2-(*N*-cyclohexylacrylamido)ethyl)(2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)carbamate.

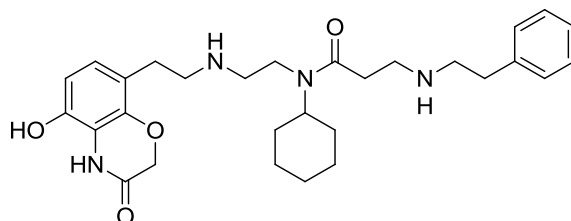
In a mixture of NMP (20 mL), and water (2 mL) was dissolved 8-(2-aminoethyl)-5-hydroxy-2H-benzo[b][1,4]oxazin-3(4H)-one (2.10 g, 10.1 mmol) and *N*-cyclohexyl-*N*-(2-oxoethyl)acrylamide (2.00 g, 10.3 mmol). To this was added sodium bicarbonate (793 mg, 9.44 mmol) and the reaction was stirred for 15 minutes. Sodium triacetoxyborohydride (2.73 g, 12.9 mmol) was then added, and the resulting mixture was stirred for another 20 hours. The mixture was diluted with ethyl acetate (50 mL) and a solution of sodium bicarbonate (3.61 g) in water (50 mL) was added, followed by di-*tert*-butyl dicarbonate (2.25 g, 10.3 mmol). After stirring for 2 hours more, the mixture was diluted with ethyl acetate (100 mL) and washed with water and brine. The organic phase was dried over anhydrous sodium sulfate and evaporated in vacuo. The residue was purified by column chromatography on silica gel (eluting with pet. ether / ethyl acetate = 2:1) to afford the title compound (870 mg, 1.78 mmol, 17 % yield) as a white solid. MS (ESI+) 488 [M+H]⁺.

Step 9: *N*-cyclohexyl-3-((3,4-dichlorobenzyl)amino)-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)amino)ethyl)-propanamide.

Tert-butyl(2-(*N*-cyclohexylacrylamido)ethyl)(2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[b][1,4]-oxazin-8-yl)ethyl)carbamate (100 mg, 0.205 mmol) and (3,4-dichlorophenyl)methanamine (72 mg, 0.41 mmol) were dissolved in ethanol (1 mL) and heated at 50 °C for 20 hours. The solvent was evaporated in vacuo and the residue dissolved in dichloromethane (1 mL). Trifluoroacetic acid (1 mL, 13 mmol) was added and the mixture stirred for 2 hours, and then concentrated under reduced pressure. The residue was purified by prep-HPLC with (eluting with acetonitrile and 0.2% aqueous trifluoroacetic acid) to afford the title compound (125 mg, 0.155 mmol, 76 % yield) as a white solid. ¹H NMR (400 MHz, DMSO-d₆) δ 10.01 (s, 1H), 9.94 – 9.84 (m, 1H), 8.84 – 8.77 (m, 2H), 8.43 (bs, 1H), 7.83 (m, 1H), 7.77 (m, 1H), 7.55 – 7.47 (m, 1H), 6.70 – 6.57 (m, 1H), 6.47 (m, 1H), 4.54 (s, 2H), 4.27 – 4.20 (m, 2H), 3.57 – 3.44 (m, 1H), 3.18 – 2.91 (m,

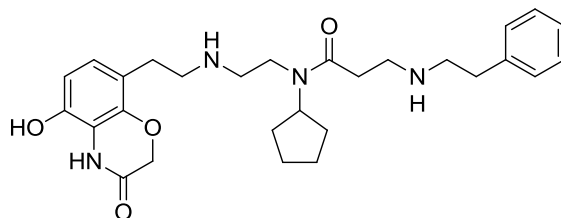
6H), 2.84 – 2.66 (m, 4H), 1.83 – 1.73 (m, 2H), 1.71 – 1.57 (m, 2H), 1.59 – 0.99 (m, 5H). MS (ESI+) 563.3 [M+H]⁺.

***N*-cyclohexyl-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[*b*][1,4]oxazin-8-yl)ethyl)amino)ethyl)propanamide (9)**



The title compound was prepared by a procedure analogous to that reported for *N*-cyclohexyl-3-((3,4-dichlorobenzyl)amino)-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[*b*][1,4]oxazin-8-yl)ethyl)amino)ethyl)propanamide, substituting 2-phenylethanamine for (3,4-dichlorophenyl)methanamine. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.02 – 9.96 (m, 2H), 8.49 (bs, 3H), 7.37 – 7.30 (m, 2H), 7.29 – 7.21 (m, 3H), 6.64 (d, *J* = 8.2 Hz, 1H), 6.46 (d, *J* = 8.3 Hz, 1H), 4.52 (s, 2H), 3.28 – 2.84 (m, 10H), 2.86 – 2.68 (m, 4H), 1.83 – 1.16 (m, 9H), 1.12 – 1.00 (m, 1H). MS (ESI+) 509.2 [M+H]⁺.

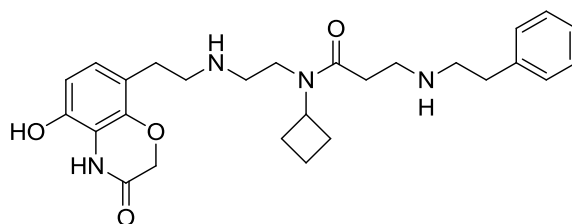
***N*-cyclopentyl-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[*b*][1,4]oxazin-8-yl)ethyl)amino)ethyl)propanamide (10)**



The title compound was prepared by a procedure analogous to that reported for *N*-cyclohexyl-3-((3,4-dichlorobenzyl)amino)-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[*b*][1,4]oxazin-8-yl)ethyl)amino)ethyl)propanamide, substituting cyclopentylamine for cyclohexylamine and 2-phenylethanamine for (3,4-dichlorophenyl)methanamine. ¹H NMR (400 MHz, DMSO-*d*₆) δ

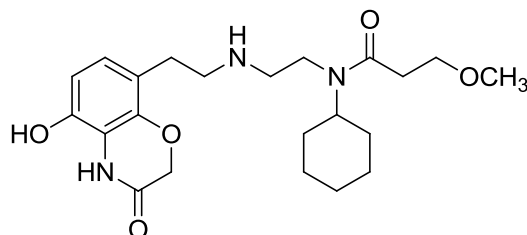
10.04 – 9.92 (m, 2H), 8.55 – 8.40 (m, 4H), 7.44 – 7.31 (m, 2H), 7.34 – 7.23 (m, 3H), 6.67 (m, 1H), 6.48 (m, 1H), 4.54 (s, 2H), 3.28 – 2.87 (m, 11H), 2.88 – 2.67 (m, 4H), 1.55 (m, 2H). MS (ESI+) 495.2 [M+H]⁺.

***N*-cyclobutyl-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)amino)ethyl)-3-(phenethylamino)propanamide (11)**



The title compound was prepared by a procedure analogous to that reported for *N*-cyclohexyl-3-((3,4-dichlorobenzyl)amino)-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)amino)ethyl)propanamide, substituting cyclobutylamine for cyclohexylamine and 2-phenylethanamine for (3,4-dichlorophenyl)methanamine. ¹H NMR (400 MHz, DMSO-d₆) δ 10.03 – 9.86 (m, 2H), 8.66 – 8.51 (m, 4H), 7.40 – 7.32 (m, 2H), 7.31 – 7.25 (m, 3H), 6.67 (d, *J* = 8.2 Hz, 1H), 6.47 (d, *J* = 8.0 Hz, 1H), 4.56 – 4.49 (m, 2H), 3.27 – 3.03 (m, 5H), 2.97 – 2.87 (m, 4H), 2.78 – 2.69 (m, 3H), 2.42 – 1.86 (m, 5H), 1.86 – 1.53 (m, 2H). MS (ESI+) 481.1 [M+H]⁺.

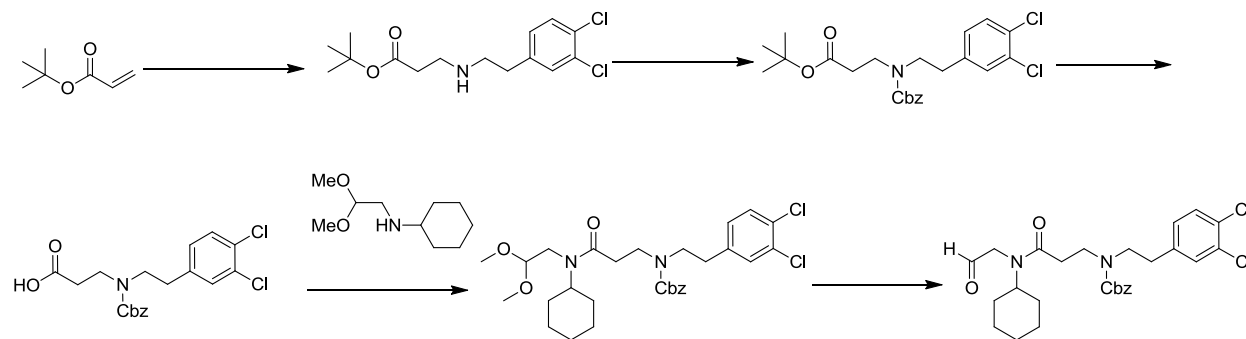
***N*-cyclohexyl-*N*-(2-((2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)amino)ethyl)-3-methoxypropanamide (12)**



The title compound was prepared by a procedure analogous to that reported for *tert*-butyl (2-(*N*-cyclohexylacrylamido)ethyl)(2-(5-hydroxy-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)carbamate (from Step 8 above), and by stirring the compound in methanol / dichloromethane followed by Cbz deprotection to furnish the title compound. ¹H NMR (500 MHz, DMSO-d₆) δ 9.48 (bs, 1H), 8.69 – 8.31 (m, 2H), 6.67 (d, *J* = 8.3 Hz, 1H), 6.49 (d, *J* = 8.3 Hz, 1H), 4.54 (s, 2H), 4.09 – 3.50 (m, 3H), 3.50 – 3.43 (m, 2H), 3.15 – 2.96 (m, 4H), 2.91 – 2.78

(m, 2H), 2.64 – 2.57 (m, 1H), 1.82 – 1.18 (m, 9H), 1.14 – 0.98 (m, 1H). MS (ESI+) 420.1 [M+H]⁺.

Benzyl 3-(cyclohexyl(2-oxoethyl)amino)-3-oxopropyl(3,4-dichlorophenethyl) carbamate



Step 1: *tert*-Butyl 3-(3,4-dichlorophenethylamino)propanoate.

A mixture of *tert*-butyl acrylate (21.92 g, 171 mmol), 2-(3,4-dichlorophenyl)ethanamine (25 g, 132 mmol) in EtOH (250 mL) was stirred at room temperature overnight. The progress of the reaction was monitored by LC/MS. The mixture was then concentrated under reduced pressure, to furnish *tert*-butyl 3-((3,4-dichlorophenethyl)amino)propanoate (38.6 g, 112 mmol, 85 % yield) as the residue, which was used in the next step without further purification. MS (ESI+) 320 [M+H]⁺.

Step 2: *tert*-Butyl 3-((benzyloxycarbonyl)(3,4-dichlorophenethyl)amino) propanoate.

Benzyl chloroformate (23.00 g, 135 mmol) was added to a mixture of *tert*-butyl 3-(3,4-dichlorophenethylamino)propanoate (39g, 123 mmol) and Et₃N (34.2 ml, 245 mmol) in dichloromethane (300 mL), then the mixture was stirred overnight. The progress of the reaction was monitored by LC/MS. Ice-water (200 mL) was added to the reaction and the mixture was extracted with dichloromethane (500 mL x 2). The combined organic phases were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure to give the residue. This was then purified by column chromatography on silica gel by using (dichloromethane/petroleum ether = 2:1) to give the title compound (43 g, 67.5 mmol, 71.0 % yield). MS (ESI+) 476.1 [M+Na]⁺

Step 3: 3-((Benzyloxycarbonyl)(3,4-dichlorophenethyl)amino)propanoic acid.

To the solution of *tert*-butyl 3-((benzyloxycarbonyl)(3,4-dichlorophenethyl)amino) propanoate (30.0 g, 66.3 mmol) in dichloromethane (50 mL) was added TFA (50 mL) dropwise. The reaction was stirred at room temperature for 3h. The progress of the reaction was monitored by LCMS and TLC. The reaction was concentrated under reduced pressure to give the crude product. Water (50 mL) was then added and extracted twice with ethyl acetate (150 mL X 2). The combined organic solvent was washed by water (50 mL X 2) and dried over Na₂SO₄. After the removal of the solvent, the title compound was isolated as the residue (23.5 g, 53.4 mmol, 80 % yield) and used without further purification. MS (ESI+) 397 [M+H]⁺

Step 4: Benzyl 3-(cyclohexyl(2,2-dimethoxyethyl)amino)-3-oxopropyl (3,4-dichlorophenethyl) carbamate

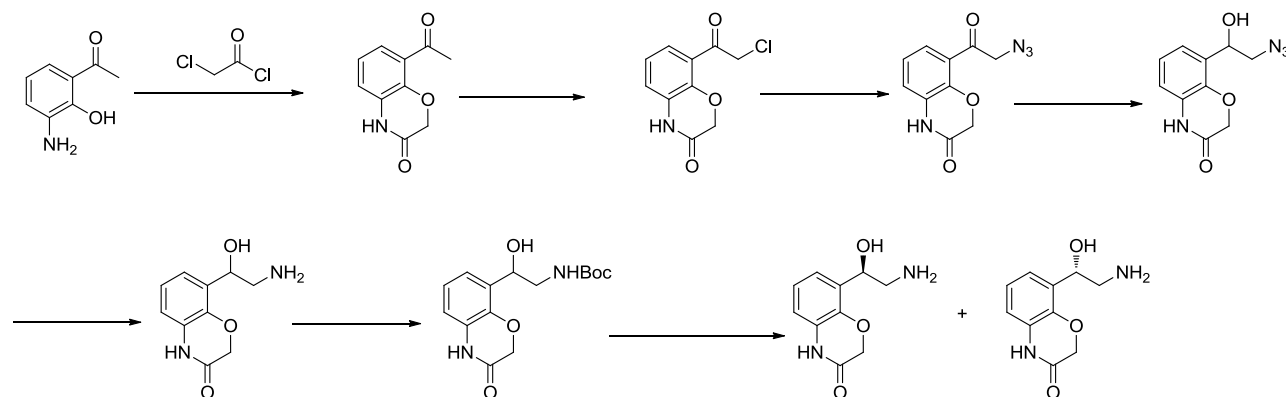
A mixture of 3-((benzyloxycarbonyl)(3,4-dichlorophenethyl)amino)propanoic acid (38 g, 96 mmol), HOBT (16.15 g, 105 mmol) and di-isopropylethylamine (36.8 ml, 211 mmol) in DMF (100 mL) was stirred at room temperature for 40 min. To this was added *N*-(2,2-dimethoxyethyl)cyclohexanamine (25.1 g, 134 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (20.22 g, 105 mmol). The reaction then was stirred at room temperature overnight. The progress of the reaction was monitored by LC/MS. Next, ice-water (150 mL) was added and stirred for 30 minutes. The mixture was extracted three times with dichloromethane (300 mL x 2). The combined organic phase was washed by 1N HCl (100 mL) and a saturated solution of sodium bicarbonate (150 mL), respectively. The organic layer was dried over Na₂SO₄, filtered, and concentrated under reduced pressure to give the crude product. The residue was purified by column chromatography on silica gel using a solvent gradient of pet. ether/ethyl acetate from 5/1 to 2/1 to furnish the title compound (33g, 58.4 mmol, 60.9 % yield) as pale yellow oil. MS (ESI+) 567 [M+H]⁺

Step 5: Benzyl 3-(cyclohexyl(2-oxoethyl)amino)-3-oxopropyl(3,4-dichlorophenethyl) carbamate.

To a solution of benzyl 3-(cyclohexyl(2,2-dimethoxyethyl)amino)-3-oxopropyl (3,4-dichlorophenethyl)carbamate (15g, 26.5 mmol) in dichloromethane (50 mL) was added paratoluenesulfonyl acid monohydrate (15.14 g, 80 mmol). The reaction was then stirred at room temperature overnight. The progress of the reaction was monitored by LC/MS. A saturated

solution of sodium bicarbonate was then added to adjust to a pH=8. The reaction was extracted three times with dichloromethane (150 mL x 3). The combined organic layers were washed with brine, dried over magnesium sulfate, and concentrated in vacuo to give the title compound (13g, 22.27 mmol, 84% yield) which was used further without purification. MS (ESI+) 521 [M+H]⁺.

(R)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one & (S)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one



Step 1: 8-acetyl-2H-benzo[b][1,4]oxazin-3(4H)-one.

To a stirred mixture of 1-(3-amino-2-hydroxyphenyl)ethanone (5.00 g, 33.1 mmol) and sodium bicarbonate (6.11 g, 72.8 mmol) in DMF (30 mL) was added 2-chloroacetyl chloride (2.89 mL, 36.4 mmol) dropwise. The mixture was stirred for 2 hours. Cesium carbonate (12.9 g, 39.7 mmol) was then added and the resulting mixture was heated at 100°C for 20 hours. The mixture was cooled to ambient temperature, quenched with water, and then extracted with ethyl acetate. The combined organic layers were washed with water and brine, dried over anhydrous Na₂SO₄, filtered and then evaporated in vacuo. The residue was washed with ether and dried to afford the title compound. (5.80 g, 30.4 mmol, 84 % yield) as a beige solid. ¹H NMR (400 MHz, DMSO-d₆): δ 10.88 (s, 1H), 7.25 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.09 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.03 (t, *J* = 7.7 Hz, 1H), 4.72 (s, 2H), 2.55 (s, 3H). MS (ESI+) 192.1 [M+H]⁺

Step 2: 8-(2-chloroacetyl)-2H-benzo[b][1,4]oxazin-3(4H)-one.

Benzyltrimethylammonium dichloroiodate (20.0 g, 57.5 mmol) was added to a stirred solution of 8-acetyl-2H-benzo[b][1,4]oxazin-3(4H)-one (5.00 g, 26.2 mmol) in dichloromethane (100 mL),

AcOH (33 mL) and water (5.5 mL). The resultant mixture was stirred at 65 °C for 20 hours. It was then cooled to ambient temperature, quenched via addition of an aqueous NaHSO₃ solution (5.78 g in 100 mL) and stirred further for 30 minutes. The mixture then was diluted with Et₂O (200 mL) and the resulting solid was collected with filtration, washed with water, extracted with diethyl ether, concentrated, and dried under vacuum at 40 °C to afford the title compound as a light brown solid (5.30 g, 23.5 mmol, 90 % yield). ¹H NMR (400 MHz, DMSO-d₆): δ 10.93 (s, 1H), 7.37 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.14 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.08 (t, *J* = 7.7 Hz, 1H), 5.02 (s, 2H), 4.73 (s, 2H). MS (ESI+) 226.1 [M+H]⁺

Step 3: 8-(2-azidoacetyl)-2H-benzo[b][1,4]oxazin-3(4H)-one

To a suspension of 8-(2-chloroacetyl)-2H-benzo[b][1,4]oxazin-3(4H)-one (4.00 g, 17.7 mmol) in DMF (50 mL) was carefully added sodium azide (1.44 g, 22.2 mmol). After addition, the resulting mixture was stirred for 2 hours and then poured onto ice/water. The precipitated product was collected with filtration, washed with water and then dried in vacuo at 40 °C to afford the title compound (4.00 g, 17.2 mmol, 97 % yield) as a light brown solid. ¹H NMR (400 MHz, DMSO-d₆) δ 10.93 (s, 1H), 7.42 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.14 (dd, *J* = 7.6, 1.5 Hz, 1H), 7.09 (t, *J* = 7.7 Hz, 1H), 4.71 (d, *J* = 11.4 Hz, 4H). MS (ESI+) 233.1 [M+H]⁺

Step 4: 8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one

A suspension 8-(2-azidoacetyl)-2H-benzo[b][1,4]oxazin-3(4H)-one (10 g, 43.1 mmol) in a mixture of MeOH (60 mL) and THF (60 mL) was treated with sodium borohydride (2.49 g, 65.9 mmol), and the resultant mixture was stirred at room temperature for 2 hours. The mixture was partitioned between EtOAc and brine. The organic layer was then separated, dried, filtered and concentrated in vacuo. The residue was triturated with acetone (20 mL) to yield 11 g of intermediate, of which 7 g was re-dissolved into ethanol (150 mL). To this was added Pd/C (10%, 89% wet, 700 mg). The resulting mixture was briefly evacuated and then back-filled with hydrogen. This operation was repeated three times. The resulting mixture was then stirred under a hydrogen atmosphere (balloon pressure) at room temperature for 12 hours. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The residue was washed with ether and then dried to give the title compound (6.0 g, 85% purity, 24.4 mmol, 89 % yield over two steps) as a white solid. MS (ESI+) 209.2 [M+H]⁺

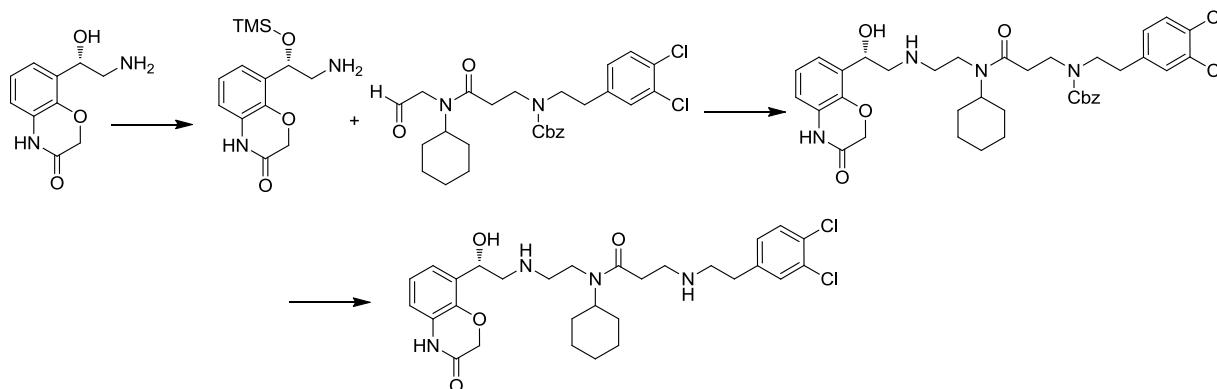
Step 5: *tert*-Butyl 2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl carbamate

To a solution of 8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one (7.0 g, 33.6 mmol) in DMF (150 mL) was added DMAP (0.41 g, 3.36 mmol), followed by di-*tert*-butyl dicarbonate (8.07 g, 37.0 mmol). The reaction mixture was stirred at room temperature for 2 hours and then quenched with a saturated aqueous NH₄Cl solution (about 50 mL). The aqueous layer was extracted with dichloromethane (300 mL X 2). The combined organic layers were dried over sodium sulfate, filtered, and then concentrated under reduced pressure. The product was purified by silica gel chromatography (EtOAc/hexanes = 1/2) to give the title compound (9.0 g, 29.2 mmol, 87 % yield) as white solid. ¹H NMR (400 MHz, CDCl₃) δ 9.48 (s, 1H), 7.06 (d, *J* = 7.6 Hz, 1H), 6.87 (t, *J* = 7.8 Hz, 1H), 6.71 (d, *J* = 7.5 Hz, 1H), 4.99 (dd, *J* = 17.5, 14.1 Hz, 2H), 4.48 (d, *J* = 6.5 Hz, 2H), 3.40 (s, 2H), 3.26 (s, 1H), 1.35 (s, 9H). MS (ESI⁺) 235.1 [M+H]⁺

Step 6: (*R*)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one & (*S*)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one

tert-Butyl 2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4] oxazin-8-yl)ethylcarbamate (2.0 g, 6.49 mmol) was dissolved in HCl/dioxane (2 M, 10 mL, 20 mmol), and the mixture was stirred at room temperature for 12 hours. The solvent was then removed by evaporation. The residual material was treated with EtOAc (10 mL) and ether (70 mL), and the formed slurry was stirred for 30 minutes. The solid was collected by filtration, washed with ether, and then dried under reduced pressure at 40°C to give 8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (1.30 g, 5.31 mmol, 82 % yield). After chiral separation, (*R*)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one and (*S*)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one were obtained, ee % > 99% for each. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.80 (s, 1H), 8.14 (s, 3H), 7.10 (d, *J* = 6.8 Hz, 1H), 6.98 (t, *J* = 7.7 Hz, 1H), 6.88 (dd, *J* = 7.7, 1.3 Hz, 1H), 5.98 (s, 1H), 5.07 (dd, *J* = 9.5, 2.3 Hz, 1H), 4.61 (q, *J* = 15.1 Hz, 2H), 2.99 (d, *J* = 6.6 Hz, 1H), 2.83–2.62 (m, 1H). MS (ESI⁺) 209.1 [M+H]⁺

(S)-N-cyclohexyl-3-(3,4-dichlorophenethylamino)-N-(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)propanamide (23b)



Step 1: (S)-8-(2-amino-1-(trimethylsilyloxy)ethyl)-2H-benzo[b][1,4] oxazin-3(4H)-one.

To a solution of (S)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one (3.5 g, 14.30 mmol) in DMF (40 mL) was added di-isopropylethylamine (5.50 ml, 31.5 mmol) and trimethylsilylchloride (1.828 ml, 14.30 mmol) dropwise at 0 °C. The reaction was stirred at this temperature for 2 hours. The progress of the reaction was monitored by LC/MS. Ice-water was added (50 mL) and the mixture was extracted twice with ethyl acetate (100 mL x 2). The combined organic phases were washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure to give the residue (S)-8-(2-amino-1-((trimethylsilyl)oxy)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one (3.0 g, 10.70 mmol, 74.8 % yield) which was used without further purification. MS (ESI+) 281 [M+H]⁺;

Step 2: (S)-benzyl 3-(cyclohexyl(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H -benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)amino)-3-oxopropyl(3,4-dichlorophenethyl)carbamate.

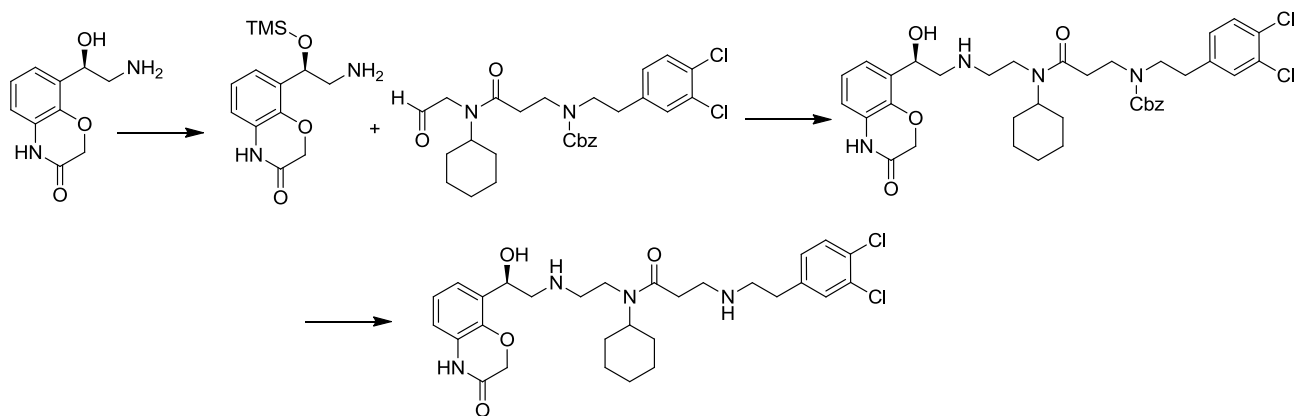
A mixture of (S)-8-(2-amino-1-(trimethylsilyloxy)ethyl)-2H-benzo[b][1,4] oxazin-3(4H)-one (3.45 g, 12.32 mmol), benzyl (3-(cyclohexyl(2-oxoethyl)amino)-3-oxopropyl)(3,4-dichlorophenethyl)carbamate (3.2 g, 6.16 mmol) and magnesium sulfate (1.43 g, 12.32 mmol) in dichloromethane (50 mL) and acetic acid (3.53 ml, 61.6 mmol) was stirred at ambient temperature for 5 hours. To this was added sodium triacetoxyborohydride (2.61 g, 12.32 mmol) at 0 °C slowly, then the reaction was stirred at room temperature for 18 hours. The progress of

the reaction was monitored by LC/MS. Ice-water (10 mL) was added, and the mixture was extracted three times with dichloromethane (100 mL X 3). The combined organic phases were washed with brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography using a gradient of dichloromethane/methanol from 99/1 to 10/1 ($R_f = 0.2$, DCM/MeOH = 10/1) to yield the title compound as a yellow solid, which was stirred in EtAOc (20 mL) for 20 minutes, then filtered to give (*S*)-benzyl 3-(cyclohexyl(2-((2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)amino)ethyl)amino)-3-oxopropyl(3,4-dichlorophenethyl)carbamate (1.6 g, 2.248 mmol, 36.5 % yield) as a white solid. $^1\text{H NMR}$ (400 MHz, MeOD): δ 7.31-7.21 (m, 7H), 7.11-6.88 (m, 3H), 6.78-6.76 (m, 1H), 5.04-4.92 (m, 3H), 4.49 (d, $J = 7.2$ Hz, 2H), 3.48-3.38 (m, 7H), 2.95-2.49 (m, 8H), 1.74-1.18 (m, 10H). MS (ESI+) 713 [M+H] $^+$

Step 3: (*S*)-*N*-cyclohexyl-3-(3,4-dichlorophenethylamino)-*N*-(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)propanamide (23b)

To a solution of (*S*)-benzyl 3-(cyclohexyl(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)amino)-3-oxopropyl(3,4-dichlorophenethyl)carbamate (1.5g, 2.108 mmol) in dichloromethane (100 ml) was added tri-bromoborane (12.65 ml, 12.65 mmol) at 0 °C dropwise. The reaction was stirred for 2 hours at this temperature. The reaction was allowed to warm to room temperature and stirred for an additional 2 hours. Ice-water (30 mL) was then carefully added, and the mixture was stirred for 1 hour. This was extracted twice with dichloromethane (100 mL X 2) and the combined organic phases were washed with brine, dried over MgSO_4 , filtered, and concentrated under reduced pressure to give the crude product, which was purified by HPLC to furnish the title compound as a white solid (905 mg, 1.296 mmol, 61.5 % yield). $^1\text{H NMR}$ (400 MHz, MeOD): δ 7.42-7.38 (m, 2H), 7.16-7.11 (m, 2H), 6.93 (t, $J = 8.0$ Hz, 1H), 6.81-6.78 (dd, $J_1 = 8.0$ Hz, $J_2 = 1.2$ Hz, 1H), 5.20-5.16 (dd, $J_1 = 10.8$ Hz, $J_2 = 3.6$ Hz, 1H), 4.53 (t, $J = 20.8$ Hz, 2H), 3.56-3.27 (m, 3H), 3.27-3.20 (m, 4H), 3.13-2.82 (m, 7H), 1.78-1.19 (m, 11H). MS (ESI+) 579 [M+H] $^+$

(R)-N-cyclohexyl-3-((3,4-dichlorophenethyl)amino)-N-(2-((2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethyl)amino)ethyl)propanamide (23a)



Step 1: (R)-8-(2-amino-1-(trimethylsilyloxy)ethyl)-2H-benzo[b][1,4] oxazin-3(4H)-one

The title compound was prepared via the same procedure as (S)-8-(2-amino-1-(trimethylsilyloxy)ethyl)-2H-benzo[b][1,4] oxazin-3(4H)-one using instead (R)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one. MS (ESI+) 281 [M+H]⁺

Step 2: (R)-benzyl 3-(cyclohexyl(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H -benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)amino)-3-oxopropyl(3,4-dichlorophenethyl)carbamate

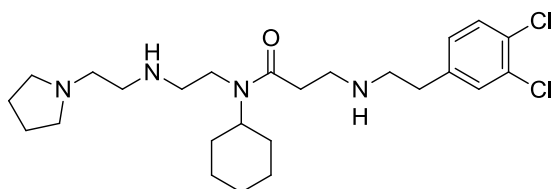
The title compound was prepared according to the procedure described for (S)-benzyl 3-(cyclohexyl(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)amino)-3-oxopropyl(3,4-dichlorophenethyl)carbamate using instead (R)-8-(2-amino-1-(trimethylsilyloxy)ethyl)-2H-benzo[b][1,4] oxazin-3(4H)-one. ¹H NMR (400 MHz, MeOD): δ 7.31-7.18 (m, 7H), 7.11-6.88 (m, 3H), 6.78-6.76 (m, 1H), 5.04-4.92 (m, 3H), 4.49 (d, *J* = 7.2 Hz, 2H), 3.47-3.38 (m, 6H), 2.95-2.48 (m, 8H), 1.71-1.19 (m, 9H). MS (ESI+) 713

Step 3: (R)-N-cyclohexyl-3-(3,4-dichlorophenethylamino)-N-(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)propanamide (23a)

The title compound was prepared according to the procedure described for (S)-N-cyclohexyl-3-(3,4-dichlorophenethylamino)-N-(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)propanamide using instead (R)-benzyl 3-(cyclohexyl(2-(2-hydroxy-2-(3-

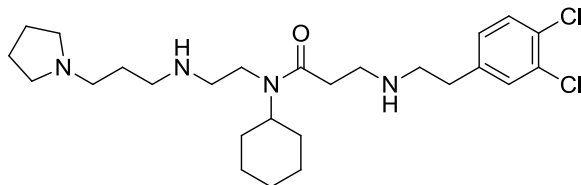
oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)amino)-3-oxopropyl(3,4-dichlorophenethyl)carbamate. ^1H NMR (400 MHz, MeOD): δ 7.34-7.31 (m, 2H), 7.09-7.06 (t, J = 8.0 Hz, 2H), 6.88 (t, J = 8.0 Hz, 1H), 6.75-6.72 (dd, J_1 = 8.0 Hz, J_2 = 1.2 Hz, 1H), 5.05-5.02 (dd, J_1 = 8.8 Hz, J_2 = 4.0 Hz, 1H), 4.54-4.44 (m, 2H), 3.56-3.50 (m, 1H), 3.31-3.20 (m, 2H), 2.82-2.66 (m, 11H), 2.55-2.51 (m, 2H), 1.80-1.10 (m, 11H). MS (ESI+) 579 $[\text{M}+\text{H}]^+$

***N*-cyclohexyl-3-((3,4-dichlorophenethyl)amino)-*N*-(2-((2-(pyrrolidin-1-yl)ethyl)amino)ethyl)propanamide (17)**



The title compound was prepared by a procedure analogous to that reported for (*S*)-*N*-cyclohexyl-3-(3,4-dichlorophenethylamino)-*N*-(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)propanamide, substituting 2-(pyrrolidin-1-yl)ethanamine for (*S*)-8-(2-amino-1-hydroxyethyl)-2Hbenzo[b][1,4]oxazin-3(4H)-one. ^1H NMR (400 MHz, DMSO- d_6) δ 9.13 – 8.50 (m, 3H), 7.60 (dd, J = 6.4, 3.2 Hz, 1H), 7.39 (d, J = 3.5 Hz, 1H), 3.83 – 3.47 (m, 2H), 3.26 – 2.71 (m, 13H), 2.38 – 1.18 (m, 13H), 1.16 – 1.01 (m, 1H). MS (ESI+) 483.2 $[\text{M}+\text{H}]^+$.

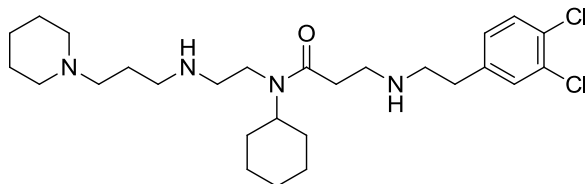
***N*-cyclohexyl-3-((3,4-dichlorophenethyl)amino)-*N*-(2-((3-(pyrrolidin-1-yl)propyl)amino)ethyl)propanamide (18)**



The title compound was prepared by a procedure analogous to that reported for (*S*)-*N*-cyclohexyl-3-(3,4-dichlorophenethylamino)-*N*-(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)ethylamino)ethyl)propanamide, substituting 3-(pyrrolidin-1-yl)propan-1-amine for (*S*)-8-(2-amino-1-hydroxyethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one. ^1H NMR (400 MHz,

DMSO- d_6) δ 8.79 – 8.64 (m, 3H), 7.65 – 7.55 (m, 1H), 7.43 – 7.33 (m, 2H), 3.28 – 2.90 (m, 15H), 2.92 – 2.72 (m, 2H), 2.09 – 1.22 (m, 14H). MS (ESI+) 497.1 [M+H]⁺.

***N*-cyclohexyl-3-((3,4-dichlorophenethyl)amino)-*N*-(2-((3-(piperidin-1-yl)propyl)amino)ethyl)propanamide (19)**



The title compound was prepared by a procedure analogous to that reported for (*S*)-*N*-cyclohexyl-3-(3,4-dichlorophenethylamino)-*N*-(2-(2-hydroxy-2-(3-oxo-3,4-dihydro-2H-benzo[*b*][1,4]oxazin-8-yl)ethylamino)ethyl)propanamide, substituting 3-(piperidin-1-yl)propan-1-amine for (*S*)-8-(2-amino-1-hydroxyethyl)-2Hbenzo[*b*][1,4]oxazin-3(4H)-one. ¹H NMR (400 MHz, DMSO- d_6) δ 8.65 – 8.56 (m, 2H), 7.62 – 7.56 (m, 1H), 7.37 (d, *J* = 2.3 Hz, 1H), 5.73 (s, 1H), 3.25 – 2.73 (m, 20H), 2.12 – 1.38 (m, 15H), 1.39 – 1.24 (m, 2H). MS (ESI+) 511.2 [M+H]⁺.

X-RAY CRYSTALLOGRAPHY

SMYD2 protein was purified as previously described¹ and concentrated to 3mg/mL in a final buffer containing 0.02M Tris pH 8.0, 100mM NaCl, 1mM TCEP. The protein ligand complex was made using a slight molar excess of *S*-adenosylmethionine (SAM) and A-893 from concentrated DMSO stocks. Co-crystals were grown using the hanging drop format with a 1:1 v/v drop composition of the protein complex: well solution (20% w/v PEG 10,000, 0.1 HEPES pH 7.5) at 17°C. The crystals were quickly transferred through the cryoprotectant consisting of 17%(v/v) propylene glycol and well solution prior to flash freezing in liquid nitrogen for data collection. A number of crystals were screened to find one suitable for data collection. Reasonable data were ultimately collected to 2.79Å at the Advanced Photon Source IMCA-CAT beamline (17-ID)² and reduced and scaled with autoPROC³ software with a completeness of 99.9% with Rmerge=0.155. The crystals belong to spacegroup P212121 with unit cell dimensions a=52.80, b=71.30, c=118.93, $\alpha=\beta=\gamma=90^\circ$. The structure was solved by molecular replacement with the program Phaser⁴ and pdbcode 3S7B (protein and SAM only) as the model, and refined using Refmac⁵ and Buster⁶. Data collection and refinement statistics are shown in the table below.

1. Wang, L.; Ling, L.; Zhang, H.; Luo, X.; Dai, J.; Zhou, S.; Gu, J.; Zhu, J.; Atadja, P.; Lu, C.; Li, E.; Zhao, K. Structure of Human SMYD2 Protein Reveals the Basis of p53 Tumor Suppressor Methylation. *J. Biol. Chem.* **2011**, 286, 38725-38737.

2. Use of the IMCA-CAT beamline 17-ID (or 17-BM) at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with Hauptman-Woodward Medical Research Institute. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357.

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Data Collection

PDB ID

4YND

Ligand		A-893
Space Group		P2 ₁ 2 ₁ 2 ₁
Cell dimensions	a, b, c (Å)	52.80, 71.30, 118.93
	α , β , γ (deg)	90.00, 90.00, 90.00
Resolution (Å)		2.79
Completeness (%)		99.9
Redundancy		6.4
I/I(σ)		4.27 (at 2.77Å)

Refinement

Resolution (Å)		2.79
R _{work} /R _{free} (%)		19.8/25.3
Number of atoms (protein/water/other)		3433/131/69
Mean B-factor (Å ²)		33.0
r.m.s.d bonds (Å)		0.007
r.m.s.d angles (deg)		0.93
Ramachandran	Favored (%)	98.0
	Allowed (%)	2.0
	Disallowed (%)	0.0
