

Supporting Information

Design, Synthesis and Characterization of Sulfamide and Sulfamate Nucleotidomimetic Inhibitors of hHint1

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General Methods and Materials. Guanosine was purchased from Acros Organics. Chloroacetaldehyde solution (50% wt in water), Triphenylphosphine (cat no: T84409-1004), Methyl triphenoxy phosphonium iodide (MTPI, cat no: 226432-10), Chlorosulfonyl Isocyanate (cat no: 142662-254), Sulfamide (cat no: 277310) was purchased from Sigma-Aldrich. All solvents were purchased from Fischer Scientific and used as received unless otherwise noted. Anhydrous solvents such as DMF, Acetonitrile were used directly from solvent dispensing system (J. C. Meyer) packed with two columns of neutral alumina and dispensed under argon. DMA and Pyridine was purchased in a sure seal bottle from Sigma-Aldrich. Thin-layer chromatography was performed using EMD pre-coated silica gel 60 F-254 plates. All preparative separations were performed using Teledyne Isco combiflash system and using RediSepRf high performance gold silica pre-packed columns. Analytical HPLC for the stability studies were performed on Agilent C18 zorbax SB-Aq column (3.5 μm , 4.6 x 150 mm) using water (solvent A) and acetonitrile (solvent B) with 0.1% TEA as additive. High-resolution mass spectrometry was performed LTQ Orbitrap Velos (Thermo ScientificTM). Samples and compounds during synthesis were freeze-dried with a lyophilizer available from Labonaco. All ¹H- and ¹³C-NMR spectra were collected in d₆-DMSO (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C using AscendTM Bruker spectrometer 500 MHz at the Department of Medicinal Chemistry CCRB NMR facility at the University of Minnesota unless otherwise stated. All NMR chemical shifts were recorded in δ parts per million using d₆-DMSO as internal reference. Thermodynamic measurements for protein-ligand association were performed in 96-well plates (Nunc 260251 U96 DeepWell 96-Well x 1.3 ml from Thermo Scientific) using MicroCal Auto-ITC200 system (GE Healthcare life sciences). Nickel nitrilotriacetic acid (Ni-NTA) was purchased from Qiagen and cobalt column agarose from Thermofisher Scientific. Biological buffers were purchased from Sigma-Aldrich. Protease inhibitor tablets were obtained from Roche.

Protein Expression and Purification

The full-length sequence of hHint1 was expressed from the pMCSG7 vector (N-terminal, tobacco etch virus (TEV) protease cleavable His6 tag) in Rosetta2 pLysS cells. The cells were grown in 2 x 1L LB (Fisher Scientific) media with ampicillin (100 mg/L, Sigma-Aldrich), chloramphenicol (34 mg/L, Sigma-Aldrich), and glucose (0.1% w/v, BD Difco) at 37 °C with shaking at 250 rpm. At OD₆₀₀ = 0.7, cultures were induced to a final concentration of 1 mM IPTG (Denville Scientific Inc) and incubated at 25°C overnight. The cultures were harvested by centrifugation at 7,500 g at 4 °C for 10 min and the pellets were collected, then resuspended in buffer A (50 mM HEPES pH 7.0, 300 mM NaCl, 10% glycerol, 10 mM imidazole), which was then adjusted to 1 mg·mL⁻¹ lysozyme and Benzonase nuclease (20 μl). The resuspended cells were lysed by sonication (eight cycles of 30 s on, 30 s off) at 4 °C. The cell debris was removed from the lysate by centrifugation at 16,000 g at 4 °C for 45 min. The supernatant was loaded onto a nickel affinity column, washed with buffer A, and then eluted with an imidazole gradient using buffer B (50 mM HEPES pH 7.0, 300 mM NaCl, 10% glycerol, 500 mM imidazole). Fractions containing desired protein was combined and to it was added N-terminally His-tagged TEV protease 2% (w/w). The resulting solutions was transferred to a dialysis tubing (molecular weight cut-off of 6000-7000 Da) and dialyzed against 2 L of TEV cleavage buffer (50 mM HEPES pH 7.0, 300 mM NaCl, 10% glycerol, 0.5 mM EDTA and 1 mM DTT) overnight at 4 °C. The dialyzed protein was then buffer exchanged into buffer A and passed through cobalt affinity chromatography to remove TEV protease. The flow through obtained was concentrated to 5 mL and further purified using size exclusion chromatography (SEC buffer, 20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol). Pure fractions were collected and concentrated. The protein concentration was then determined using A₂₈₀ absorbance in nanodrop using calculated extinction coefficient of 8480 M⁻¹ cm⁻¹ and molecular weight of 14000 Da. The final protein was stored at -80 °C until in use.

Protein Crystallography

Crystals were grown via hanging drop vapor diffusion, with drops comprised of 2 μL of protein (A₂₈₀ = 6-10, in 50mM HEPES, 250 mM NaCl, 10% glycerol v/v, pH 7.5 buffer) and 2 μL of well solution. Well solutions contained 25-35% PEG 8K, and 100 mM MES at pH 6.1-6.5. Crystals formed after 3 days of incubation at 20 °C. Co-crystals with inhibitors were prepared by soaking pre-formed crystals in mother liquor containing 2.5 mM 7 or 5 mM BioAMS for 15-60 minutes. After soaking, crystals were cryoprotected using 20% PEG 400 and flash vitrified with liquid nitrogen. Diffraction data were collected at 100K at beamline 17-ID (IMCA-CAT) using a Dectris Pilatus 6M Pixel Array Dectector at the Advanced Photon Source of Argonne National Laboratories in Argonne, IL. Molecular replacement was conducted with hHint1 coordinates (PDB ID 3TW2) using Phaser1 within PHENIX.2 Modeling and molecular visualization were performed in Coot.3 Ligand restraints were calculated using JLigand,4 and refinement was performed using PHENIX. Data processing and refinement statistics are presented in Table 2.

Isothermal Titration Calorimetry (ITC)

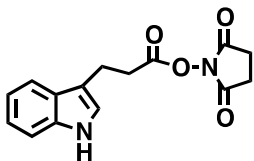
ITC experiments were conducted on a MicroCal Auto-ITC200 system (GE Healthcare life sciences). All titration experiments were performed at 20 °C in ITC buffer (10 mM Tris, 150 mM NaCl, pH 7.5). hHint1 was exchanged into ITC buffer using Micro biospin6 columns (BioRad, USA) and final protein concentrations were determined as described above. To determine the dissociation constant of stock concentration (300-400 μM) of inhibitors was titrated with 15-20 μM of Hint1. Twenty injections of ligand were injected (injection volume 2 μl) into the protein cell. The resulting change in enthalpy was measured and the background heat of dilution was subtracted by performing similar experiments in the absence of inhibitors. The background heat of dilution was subtracted from the resulting data and was fitted into one-site binding model using the ITC200 microcal software. The resulting association constant obtained by fitting the curve was converted into K_d using K_a = 1/K_d relationship.

Analytical HPLC studies to determine the stability of the Inhibitors

Analytical studies were performed on a Beckman coulter system gold operated by Karat software, with an Agilent C18 Zorbax SBAq column (4.6 x 150 mm, 3.5 μ m). Stock solutions (10 mM) of the inhibitors were prepared in a Tris buffer (10 mM Tris, 200 mM NaCl, pH 7.4). For stability studies, the stock solutions were diluted to a concentration of 50-100 μ M using Phosphate Buffer Saline buffer (PBS) and incubated at 37 $^{\circ}$ C. At indicated time points 200 μ l aliquots of the sample volume were withdrawn and injected into the HPLC system for monitoring the stability and degradation of the compounds. The samples were eluted using the gradient of solvent A (Water) and B (CH₃CN) with a 0.1% triethylamine additive (0-4 min: gradient 0% B, 4-14 min: gradient 20% B, 14-29 min: gradient 80% B, flow rate 0.5 ml/min) with detection at 168-400 nm.

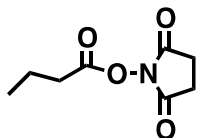
General procedure for Acid-NHS ester preparations:

N-Hydroxysuccinimide (0.62 g, 0.0053 mol, 1.0 equiv.) followed by EDC (0.00795 mmol, 1.5 equiv.) was added to a stirred solution of the respective acid (0.0053 mol, 1.0 equiv) in anhydrous THF (13 mL). The solution was stirred for 21 h at room temperature and evaporated under vacuum to dryness. The resulting crude residue was dissolved in ethylacetate (80 mL). The organic phase was washed with saturated NaHCO₃ (2x20 mL) and NaCl solution (2x20 mL), dried with Na₂SO₄, and filtered. The organic solvent was removed under vacuum to give crude NHS ester. The crude product was recrystallized with ethylacetate/petroleum ether to obtain the desired NHS ester. The esters were used for coupling without any further purification. ¹H and ¹³C NMR indicated relatively clean esters (see below).



Synthesis of 2,5-dioxopyrrolidin-1-yl-3-(1H-indol-3-yl) propanoate (22):

Above NHS ester was prepared using general procedure above. The resulting compound was obtained in 46% yield. ¹H NMR spectrum was (DMSO-d₆): 2.82 (s, 4H), 3.05 (t, 4 H), 6.99 (s, 1H), 7.09 (t, 1H), 7.22 (s, 1H), 7.36 (d, 1H), 7.56 (d, 1H) and 10.88 (s, 1H). ¹³C- DMSO-d₆: 170.31, 168.68, 136.27, 126.68, 122.82, 121.07, 118.38, 118.37, 112.06, 111.48, 31.35, 25.62 and 19.89.



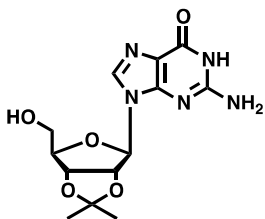
Synthesis of 2,5-dioxopyrrolidin-1-yl-pentanoate (23):

Above NHS ester was prepared using general procedure above. The resulting compound was obtained in 47% yield. ¹H NMR spectrum was (DMSO-d₆): 1.06 (t, 3H), 1.79 (q, 2H), 2.63 (s, 2H) and 2.82 (s, 4 H). ¹³C- DMSO-d₆: 172.00, 170.22, 33.47, 26.57, 19.24 and 13.68.

Synthesis of Sulfamoyl Chloride:

To a 20 mL round-bottom flask charged with chlorosulfonyl isocyanate (600 μ L, 6.85 mmol) under N₂ on ice bath, was added formic acid (285.5 μ L, 6.85 mmol) dropwise over 5 mins under vigorous stirring. After 10 min, the reaction was brought to the room temperature. A generation of white fog was detected in the flask during room temperature. After stirring for an additional hour, the reaction mixture slowly turned into a white solid, which was used directly in the next step without any purification.

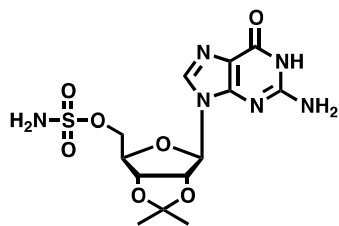
Synthetic Procedure for the Preparation of Inhibitor 4



2', 3'-O-isopropylidene Guanosine (8):

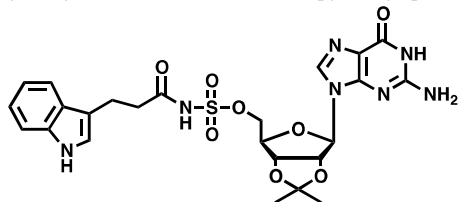
To a cold stirred suspension of guanosine (5.01g, 17.7 mmol) in acetone (300 ml) was added catalytic amount of perchloric acid (1.25 ml) drop-wise. The suspension became gradually clear and the reaction was monitored using TLC (20:80:0.1 MeOH/CHCl₃/TEA solvent). At the end of 2 hours, ammonium hydroxide (2 equivalent to perchloric acid, 2.75 ml) was added drop-wise to neutralize the reaction mixture under an ice bath. The resulting product precipitated out from the solution upon

neutralization. The reaction mixture was then evaporated under rotary evaporator to complete dryness. The crude reaction mixture was then triturated with ice-cold water (200 ml) overnight. The insoluble material was filtered and washed with cold diethyl ether to collect the desired product (3.99 g, 12.39 mmol) in 70 % yield. The ^1H NMR spectrum was (DMSO- d_6): 0.00 (s, TMS internal standard), 1.32 (s, 3H), 1.52 (s, 3H), 3.50-3.56 (m, 2H), 4.10-4.13 (t, 1H), 4.97 (d, 1H), 5.04 (t, 1H), 5.18 (d, 1H), 5.93 (d, 1H), 6.5 (s, 2H), 7.91 (s, 1H) and 10.66 (s, 1H). ^{13}C - DMSO- d_6 : 157.16, 154.15, 151.20, 136.30, 117.21, 113.51, 88.87, 87.09, 84.04, 81.64, 62.07, 27.53 and 25.71 ppm. HRMS (ESI+) calcd for $\text{C}_{13}\text{H}_{18}\text{N}_5\text{O}_5$ [(M+H)+] 324.1308 found 324.1304



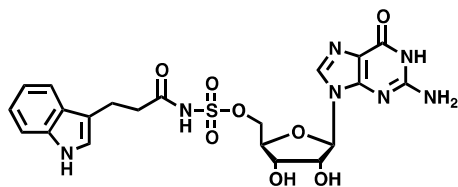
2',3'-O-isopropylidene-5'-O-(sulfamoyl)guanosine (9):

A solution of 8 (0.5 g, 1.54 mmol) in dimethyl acetamide (5 mL) was stirred for 30 min at 0 °C. Next, sulfamoyl chloride (1.69 mmol, 194.2 mg) was added to the reaction mixture after which reaction was brought to the room temperature and stirred for an additional one hour. An excess of TEA (1.5 mL, excess) was added and stirring was continued for an additional 10 min. The reaction mixture was finally quenched with MeOH (5 ml) under ice bath. The reaction mixture was evaporated to dryness and the crude reaction mixture was dissolved in ethyl acetate and washed with saturated NaHCO_3 and Brine. The organic layer was collected dried over Na_2SO_4 , filtered and evaporated to dryness. Purification by flash chromatography (20:80:1 MeOH/ CH_2Cl_2 /TEA) afforded the title compound (600 mg, 1.49 mmol) in 97% yield (with 1.5 equivalent of TEA). ^1H NMR spectrum was (DMSO- d_6): 1.18 (t, 13.45 H), 1.33 (s, 3H), 1.54 (s, 3H), 3.03 (m, 8.86 H), 4.13-4.24 (m, 2H), 4.33 (m, 1H), 5.16 (dd, 1H), 5.25 (d, 1H), 5.33 (s, 1H), 6.05 (d, 1H), 6.66 (s, 2H), 7.61 (s, 2H), 7.86 (s, 1H) and 10.84 (s, 1H). ^{13}C - DMSO- d_6 : 156.68, 153.74, 150.47, 136.17, 116.75, 113.26, 88.49, 84.02, 83.47, 81.07, 68.30, 51.94, 45.21, 26.84, 25.21, 8.62 and 7.20 ppm. HRMS (ESI+) calcd for $\text{C}_{13}\text{H}_{19}\text{N}_6\text{O}_7\text{S}$ [(M+H)+] 403.1036 found 403.10262



5'-O-(N-(3-Indole propionic acid) sulfamoyl)-2', 3'-O-isopropylidene guanosine triethylammonium salt (10):

To an ice cold stirred solution of 9 (200 mg, 0.5 mmol) and 22 (214 mg, 0.75 mmol) in DMF (2 mL) was added DBU (1.1 equiv, 82 μl , 0.55 mmol). After stirring for an additional 10 min the reaction mixture was brought to room temperature and stirred overnight. Next, the volatiles were evaporated under reduced pressure and the mixture was directly loaded onto the C18 column. The purification was achieved using a gradient reverse chromatography (A-ACN, B-1% TEA in water, washed with 2% B and eluted with a gradient of 2-90 % of solvent A). Fractions containing the product were pooled and concentrated. The concentrate was frozen and lyophilized to obtain 150 mg (0.26 mmol, 51 % yield) of the title product as TEA salt (1.0 equivalent of TEA as determined by NMR). ^1H NMR spectrum was (DMSO- d_6): 1.16 (t, 8.61 H), 1.30 (s, 3H), 1.51 (s, 3H), 2.37 (t, 2H), 2.85 (t, 2H), 3.17 (m, 6 H), 3.93 (m, 1H), 4.09 (m, 1H), 4.27-4.31 (m, 2H), 5.10 (d, 1H), 5.21 (d, 1H), 5.76 (s, 1H), 5.96 (d, 1H), 6.61 (s, 2H), 6.93 (t, 1H), 7.05 (t, 1H), 7.07 (s, 1H), 7.30 (d, 1H), 7.46 (d, 1H), 7.93 (s, 1H), 10.64 (s, 1H) and 10.64 (s, 1H). ^{13}C - DMSO- d_6 : 157.18, 154.19, 151.07, 136.67, 127.64, 122.36, 121.17, 118.68, 118.49, 117.25, 113.40, 111.68, 89.51, 84.43, 83.95, 82.05, 79.65, 55.38, 49.06, 46.23, 27.52, 25.65 and 9.20 ppm. HRMS (ESI+) calcd for $\text{C}_{24}\text{H}_{28}\text{N}_7\text{O}_8\text{S}$ [(M+H)+] 574.1720 found 574.1716

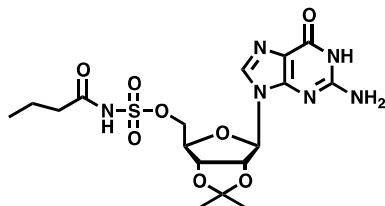


5'-O-[N-(3-Indole propionic acid)sulfamoyl] guanosine triethylammonium salt (4):

A solution of 10 (25 mg, 0.044 mmol) in 80% aqueous TFA (2 ml) was stirred for 30 min after which the reaction mixture was evaporated to dryness (co-evaporated 1% TEA/ethanol for removing TFA). The reaction mixture was loaded onto a celite packed cartridge and purified by using reverse phase chromatography (A-ACN, B-Water + 0.1% TEA). The peak eluted at 20% of ACN contained the final product. Fractions containing the product were combined and lyophilized to obtain the desired final product in quantitative yields (with 1 equivalent of TEA). ^1H NMR spectrum was (DMSO- d_6): 1.13 (t, 10 H), 2.35 (t, 2H), 2.84 (t, 2H), 3.00 (m, 6 H), 3.98 (m, 2H), 4.53 (m, 1H), 5.20 (s, 1H), 5.38 (s, 1H), 5.70 (d, 1H), 6.50 (s, 2H), 6.94 (t, 1H), 7.02 (t, 1H), 7.06 (s, 1H), 7.28 (d, 1H), 7.46 (d, 1H), 7.96 (s, 1H), 10.58 (s, 1H) and 10.51 (s, 1H). ^{13}C -DMSO- d_6 : 157.22, 154.09, 151.95,

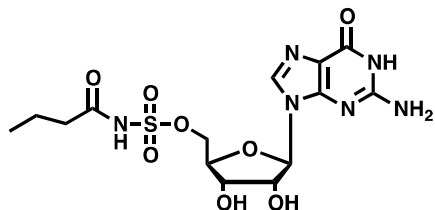
136.66, 136.22, 127.68, 122.39, 121.14, 118.75, 118.47, 117.09, 115.28, 111.66, 86.75, 83.27, 73.79, 71.47, 67.66, 21.94 and 9.47 ppm. HRMS (ESI+) calcd for $C_{21}H_{24}N_7O_8S$ [(M+H)+] 534.1407 found 534.1400. The final purity of the compound was $\geq 99\%$ as indicated by HPLC.

Synthetic Procedure for the Preparation of Inhibitor 5



5'-O-(N-(3-butyrac acid) sulfamoyl)-2', 3'-O-isopropylidene guanosine triethylammonium salt:

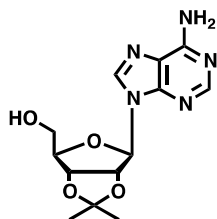
The procedure is similar as described for 4 above using NHS ester (23). The resulting compound was obtained in 60% yield as TEA salt (1.2 equivalent of TEA as determined by NMR). 1H NMR spectrum was (DMSO- d_6): 0.83 (t, 3H), 1.18 (t, 13 H, TEA), 1.30 (s, 3H), 1.46 (m, 2H), 1.51 (s, 3H), 2.0 (t, 2H), 2.8 (broad, 7H), 3.89 (m, 1H), 4.19 (m, 1H), 4.29 (m, 1H), 5.07 (dd, 1H), 5.19 (dd, 1H), 5.95 (d, 1H), 6.58 (s, 2H), 7.91 (s, 1H) and 10.63 (s, 1H). ^{13}C - DMSO- d_6 : 177.93, 156.64, 153.84, 150.78, 136.251, 116.88, 118.49, 113.05, 89.09, 84.12, 83.61, 81.70, 66.78, 41.30, 27.15, 25.27, 19.37 and 14.15 ppm. HRMS (ESI+) calcd for $C_{17}H_{25}N_6O_8S$ [(M+H)+] 473.1455 found 473.1450.



5'-O-[N-(3-Butyric acid)sulfamoyl] guanosine triethylammonium salt (5):

The procedure is similar as described for 4 above. The resulting compound was obtained in 71% yield as TEA salt (1.3 equivalent of TEA as determined by NMR). The final product is highly hygroscopic in nature. 1H NMR spectrum was (DMSO- d_6): 0.83 (t, 3H), 1.18 (t, 12 H), 1.46 (m, 2H), 2.0 (t, 2H), 3.0 (broad, 8H), 4.10-4.15 (m, 2H), 4.48 (m, 1H), 5.24 (dd, 1H), 5.41 (dd, 1H), 5.70 (d, 1H), 6.48 (s, 2H), 7.91 (s, 1H) and 10.61 (s, 1H). ^{13}C - DMSO- d_6 : 177.93, 156.64, 153.84, 150.78, 136.251, 116.88, 118.49, 113.05, 89.09, 84.12, 83.61, 81.70, 66.78, 41.30, 27.15, 25.27, 19.37 and 14.15 ppm. HRMS (ESI+) calcd for $C_{14}H_{21}N_6O_8S$ [(M+H)+] 433.1142 found 433.1134.

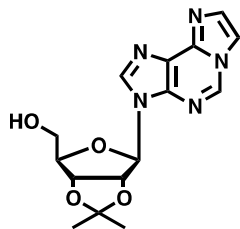
Synthetic Procedure for the Preparation of Inhibitor 7



2', 3'-O-isopropylidene Adenosine (11):

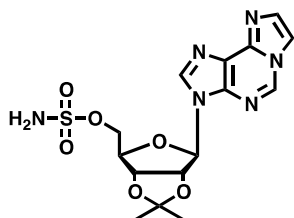
To a cold stirred solution of adenosine (2.02g, 7.56 mmol) in acetone (150 ml) was added catalytic amount of perchloric acid (0.91 ml) in a drop-wise manner. The milky white suspension turned clear after 2 h of stirring. The solution was then neutralized using ammonium hydroxide (2 equivalents to perchloric acid) under ice-bath. The reaction mixture was then evaporated to complete dryness and purified using flash silica gel chromatography (gradient: 0% for 4 min, 0-15% for 4-10 min and eluted at 15% MeOH:CH₂Cl₂). Fractions containing the product were evaporated to obtain the desired product (2.3 g, 3.58 mmol) in 99 % yield.

The ^1H NMR spectrum was (DMSO- d_6): 1.33 (s, 3H), 1.55 (s, 3H), 3.54-3.56 (m, 2H), 4.22 (m, 1H), 4.97 (dd, 1H), 5.23 (t, 1H), 5.35 (d, 1H), 6.12 (d, 1H), 7.34 (s, 2H), 8.17 (s, 1H) and 8.35 (s, 1H). ^{13}C - DMSO- d_6 : 156.60, 153.09, 149.28, 140.16, 119.57, 113.51, 90.07, 86.82, 83.68, 81.82, 62.05, 27.55 and 25.66 ppm. HRMS (ESI+) calcd for $\text{C}_{13}\text{H}_{18}\text{N}_5\text{O}_4$ [(M+H)+] 308.1359 found 308.1351.



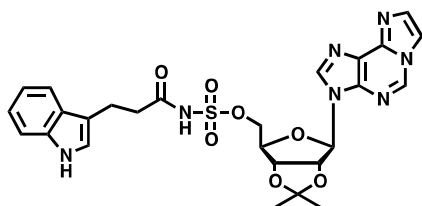
2', 3'-O-isopropylidene EthenoAdenosine (**12**):

To a stirred solution of **11** (1.1 g, 3.58 mmol) in sodium acetate buffer (100 ml, 0.1 M pH 6.5) was added 25 ml of chloroacetaldehyde solution (50% wt), heated to 40 °C and stirred overnight. Next day, the reaction mixture was brought to the room temperature and extracted with EtOAc (2 x 100 ml). The organic layer was then washed with saturated NaHCO_3 and Brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness under reduced pressure. Purification by flash silica gel chromatography (gradient: 0% for 4 min, 0-15% for 4-10 min and eluted at 15:75 MeOH/ CH_2Cl_2) afforded the title compound in 33% (400 mg) yield. The ^1H NMR spectrum was (DMSO- d_6): 1.35 (s, 3H), 1.57 (s, 3H), 3.55-3.56 (m, 2H), 4.12-4.16 (m, 1H), 4.99 (d, 1H), 5.09 (t, 1H), 5.37 (d, 1H), 6.27 (d, 1H), 7.58 (s, 1H), 8.10 (s, 1H), 8.53 (s, 1H) and 9.31 (s, 1H). ^{13}C - DMSO- d_6 : 140.90, 140.41, 138.39, 137.65, 133.31, 123.66, 113.61, 112.74, 90.37, 87.27, 84.35, 81.81, 61.92, 27.51, and 25.66 ppm. HRMS (ESI+) calcd for $\text{C}_{15}\text{H}_{18}\text{N}_5\text{O}_4$ [(M+H)+] 332.1359 found 332.1350.



2', 3'-O-isopropylidene-5'-O-(sulfamoyl)EthenoAdenosine (**13**):

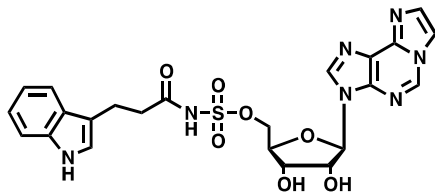
In a 10 ml round-bottom flask containing **12** (100 mg, 0.30 mmol, 1 eq.) was dissolved in anhydrous DMF (1 mL). To the cold stirred solution was added sulfamoyl chloride (103.4 mg, 0.90 mmol, 3 eq.) followed by the slow addition of triethylamine (40.4 μL , 0.30 mmol, 1.0 eq.). The reaction solution was stirred for an additional 1 h at room temperature. DMF was evaporated under vacuum and the crude mixture was then purified using reverse phase chromatography to obtain (0.27 mmoles, 110 mg) desired product in 90% yield. The ^1H NMR spectrum was (DMSO- d_6): 1.37 (s, 3H), 1.59 (s, 3H), 4.16-4.23 (m, 2H), 4.47 (m, 1H), 5.15 (d, 1H), 5.48 (d, 1H), 6.39 (d, 1H), 7.60 (s, 3H, broad peak overlaid with 1H), 8.13 (s, 1H), 8.50 (s, 1H) and 9.31 (s, 1H). ^{13}C - DMSO- d_6 : 140.91, 140.70, 138.18, 137.82, 133.32, 123.90, 114.15, 112.82, 90.08, 84.15, 81.53, 68.44, 46.18, 27.36, and 25.78 ppm. HRMS (ESI+) calcd for $\text{C}_{15}\text{H}_{19}\text{N}_6\text{O}_6\text{S}$ [(M+H)+] 411.1087 found 411.1076.



2',3'-O-isopropylidene-5'-O-[N-(3-Indolepropionic acid)sulfamoyl]EthenoAdenosine Triethylammonium salt (**13a**):

The procedure is similar as described for **4** above using NHS ester (**22**). The resulting compound was obtained in 55% yield as TEA salt (1.1 equivalent of TEA as determined by NMR). ^1H NMR spectrum was (DMSO- d_6): 1.15 (t, 9 H), 1.33 (s, 3H), 1.57 (s, 3H), 2.35 (t, 2H), 2.84 (t, 2H), 3.06 (broad, 6H), 4.04 (d, 2H), 4.45 (m, 1H), 5.07 (m, 1H), 5.39 (m, 1H), 6.30 (d, 1H), 6.95 (m, 1H), 7.02 (m, 1H), 7.06 (s, 1H), 7.29 (d, 1H), 7.46 (d, 1H), 7.56 (d, 1H), 8.08 (s, 1H), 8.59 (s, 1H), 9.30 (s, 1H) and 10.67 (s, 1H). ^{13}C - DMSO- d_6 : 140.93, 140.44, 138.47, 137.67, 136.68, 133.29, 127.66, 123.47, 122.36, 121.15, 118.71, 118.45, 113.63, 112.71,

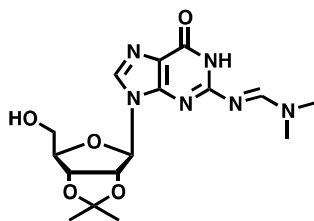
111.67, 90.37, 84.57, 84.34, 82.10, 27.52, 25.62, 21.93 and 9.28 ppm. HRMS (ESI+) calcd for C₂₆H₂₈N₇O₇S [(M+H)⁺] 582.1771 found 582.1764.



5'-O-[N-(3-Indole propionic acid)sulfamoyl]EthenoAdenosine Triethylammonium salt (**7**):

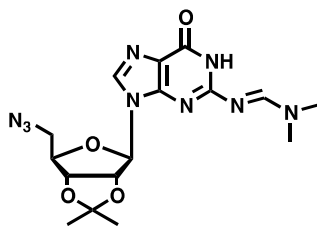
The procedure is similar as described for **4** above. The resulting compound was obtained in 74% yield as TEA salt (1.3 equivalent of TEA as determined by NMR). The final product is highly hygroscopic in nature. ¹H NMR spectrum was (DMSO-d₆): 1.10 (t, 13 H), 2.37 (t, 2H), 2.87 (t, 2H), 2.91 (broad, 6H), 4.04 (d, 2H), 4.67 (m, 1H), 5.37 (m, 1H), 5.53 (m, 1H), 6.07 (d, 1H), 6.94 (m, 1H), 7.03 (m, 1H), 7.08 (s, 1H), 7.29 (d, 1H), 7.48 (d, 1H), 7.56 (d, 1H), 8.07 (s, 1H), 8.62 (s, 1H), 9.29 (s, 1H) and 10.67 (s, 1H). ¹³C-DMSO-d₆: 141.01, 140.37, 139.11, 137.51, 136.66, 133.21, 127.68, 122.39, 121.14, 118.75, 118.46, 115.30, 112.62, 111.65, 87.86, 83.66, 74.61, 71.48, 46.22 and 21.93 ppm. HRMS (ESI+) calcd for C₂₃H₂₄N₇O₇S [(M+H)⁺] 542.1458 found 542.1457. The final purity of the compound was ≥99 % as indicated by HPLC.

Synthetic Procedure for the Preparation of Inhibitor **6**



N,N-Dimethylaminomethylene-2',3'-O-isopropylidene-5'-deoxy-5'-azido-2'-deoxyadenosine (**14**):

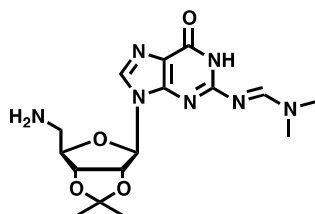
To a suspension of **8** (0.575 g, 1.78 mmol) in DMF (6 mL), N,N-dimethylformamide dimethyl acetal (0.891 mL, 6.7 mmol) was added under argon to yield an orange-brown solution. The reaction mixture was stirred at 50 °C for 4 h. The solvent was removed under reduced pressure and at elevated temperatures; the white residue was then removed by filtration. The filtrate was dried under reduced pressure, dissolved in MeOH (2.5 mL) and precipitated with 5 mL of EtOAc. After storage overnight at 4 °C, the precipitate was removed by filtration and washed thoroughly with EtOAc. The precipitate was dried overnight under reduced pressure and the product was obtained as a white powder in 80% yield (0.538 g, 1.42 mmol). The ¹H NMR spectrum was (DMSO-d₆): 1.33 (s, 3H), 1.55 (s, 3H), 3.04 (s, 3H), 3.16 (s, 3H), 3.51-3.55 (m, 2H), 4.12-4.15 (m, 1H), 4.97 (dd, 1H), 5.05 (t, 1H), 5.28 (d, 1H), 6.04 (d, 1H), 8.02 (s, 1H), 8.57 (s, 1H) and 11.37 (s, 1H). ¹³C-DMSO-d₆: 158.7, 158.03, 157.86, 149.92, 137.64, 120.24, 113.58, 88.97, 86.74, 83.93, 81.57, 61.87, 41.22, 35.12, 27.54 and 25.71 ppm. HRMS (ESI+) calcd for C₁₆H₂₃N₆O₅ [(M+H)⁺] 379.1730 found 379.1738.



N,N-Dimethylaminomethylene-2',3'-O-isopropylidene-5'-deoxy-5'-azido-2'-deoxyadenosine (**15**):

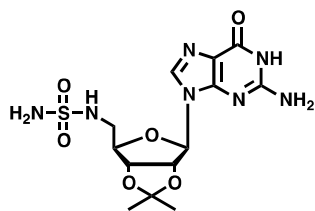
(Preparation of N,N-Dimethylaminomethylene-2',3'-O-isopropylidene-5'-deoxy-5'-Iodo (**14a**) as described previously)⁵ A stirred suspension of **14** (0.440g, 1.162 mmol) in anhyd THF (22 mL) under argon was cooled to -70 °C. To this solution was added Methyltriphenoxyphosphonium iodide (0.788 g, 1.742 mmol; 1.5 equiv). Due to the light sensitivity of the reactant and the product all subsequent steps were carried out under the exclusion of light. After 30 min of stirring the reaction mixture was brought to the room temperature and stirred for another 4 h. The reaction was stopped by the addition of MeOH (10 mL) and evaporated to dryness under reduced pressure to obtain an oily dark residue. The residue was dissolved in MeOH/CHCl₃ (1:4; 2.5 mL) and subjected to silica gel normal chromatography (Combiflash: CHCl₂/MeOH, 9:1). After purification the desired product was obtained as a yellow-orange solid in 92% yield (0.522 g, 1.15 mmol). The ¹H NMR spectrum was (DMSO-d₆): 1.35 (s, 3H), 1.56 (s, 3H), 3.06 (s, 3H), 3.19 (s, 3H), 3.54-3.58 (m, 2H), 4.264 (m, 1H), 5.0 (dd, 1H), 5.43 (dd, 1H), 5.05 (t, 1H), 6.12 (d, 1H), 8.04 (s, 1H), 8.61 (s, 1H) and 11.43 (s, 1H). ¹³C-DMSO-d₆: 158.59, 158.02, 157.85, 149.84, 137.82, 120.41, 114.04, 88.78, 84.78, 83.67,

81.70, 60.23, 41.68, 35.14, 27.43 and 25.70 ppm. HRMS (ESI+) calcd for $C_{16}H_{22}IN_6O_4$ [(M+H)+] 489.0747 found 489.0736. To a stirred solution of (14a) (400 mg, 0.8 mmol) in dry DMF (5ml) was added sodium azide (260 mg, 8 mmol). The reaction mixture was stirred under argon at RT overnight. Next day, the precipitate in the reaction mixture was filtered and washed with cold methanol. The filtrate was evaporated to dryness and the crude product was purified using flash chromatography. The desired peak was eluted at 10 % MeOH/ $CHCl_3$ mixture, which was combined and evaporated to obtain 166 mg of the final product in 55% yield. The 1H NMR spectrum was (DMSO- d_6): 1.36 (s, 3H), 1.55 (s, 3H), 3.06 (s, 3H), 3.19 (s, 3H), 3.55-3.58 (m, 2H), 4.26 (m, 1H), 4.99 (dd, 1H), 5.42 (d, 1H), 6.16 (d, 1H), 8.04 (s, 1H), 8.60 (s, 1H) and 11.37 (s, 1H). ^{13}C - DMSO- d_6 : 158.55, 157.97, 157.78, 149.52, 138.21, 120.42, 113.81, 89.70, 86.68, 84.30, 84.23, 60.23, 41.68, 35.21, 27.31, and 25.64 ppm. HRMS (ESI+) calcd for $C_{16}H_{23}N_6O_5$ [(M+H)+] 404.1795 found 404.1786.



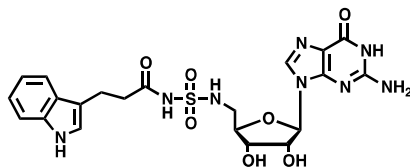
N,N-Dimethylaminomethylene-2',3'-O-isopropylidene-5'-deoxy-5'-amino (16):

To a stirred solution of 15 (160 mg, 0.392 mmol) in dioxane (12 mL) was added H_2O (1.6 mL), TEA (65.6 μ L, 0.464 mmol) and triphenylphosphine (0.312 g, 1.18 mmol). The reaction was then stirred for 3 h at 50 $^{\circ}C$. The reaction was brought to the room temperature, concentrated, and the residue was purified using flash silica column chromatography. Then product was eluted using a gradient of 20% $CH_3OH/CHCl_3$ (containing 0.5% TEA) over a period of 20 mins. The product eluted in a broad peak, which was concentrated to get the desired product (80 mg, 0.212 mmol) in 54% yield. The 1H NMR spectrum was (DMSO- d_6): 0.95 (s, 1H), 1.33 (s, 3H), 1.54 (s, 3H), 2.75 (m, 2H), 3.04 (s, 3H), 3.19 (s, 3H), 4.08 (m, 1H), 4.98 (dd, 1H), 5.34 (d, 1H), 5.99 (d, 1H), 8.03 (s, 1H) and 8.56 (s, 1H). ^{13}C - DMSO- d_6 : 158.63, 158.04, 157.81, 149.95, 137.98, 120.46, 113.66, 89.94, 86.68, 83.38, 81.85, 46.18, 43.82, 35.14, 27.56, 25.78 and 11.91 ppm. HRMS (ESI+) calcd for $C_{16}H_{24}N_7O_4$ [(M+H)+] 378.1890 found 378.1885.



N,N-Dimethylaminomethylene-2',3'-O-isopropylidene-5'-deoxy-5'-N-sulfamoyl (17):

To a stirred solution of 16 (0.150 g, 0.31 mmol) in 1,4-dioxane (8 ml) was added sulfamide (0.090 g, 0.93 mmol) and the reaction mixture was refluxed for 2 hours. The reaction mixture was then evaporated and redissolved in CH_2Cl_2 (15 ml) and water (15 ml). The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The crude product (17) was dissolved in MeOH/4N NaOH (5 ml, 1:1) solution and heated at 60 $^{\circ}C$ for 2 h and 20 mins. At the end of the reaction, 1N HCl was added under ice the reaction mixture. Methanol from the reaction mixture was evaporated and the aqueous solution was then lyophilized to obtain crude white product. The crude product was then purified using reverse phase chromatography to obtain desired product (0.030 g, 0.074 mmol) in 24 % yield. 1H NMR spectrum was (DMSO- d_6): 0.941 (t, 1H), 1.36 (s, 3H), 1.54 (s, 3H), 3.12-3.33 (m, 2 H), 4.28 (m, 1H), 5.02 (dd, 1H), 5.22 (dd, 1H), 5.93 (d, 1H), 6.60 (s, 2H), 6.72 (s, 2H), 6.96 (s, 1H), 7.86 (s, 1H) and 10.76 (s, 1H). ^{13}C - DMSO- d_6 : 157.40, 154.41, 150.71, 137.09, 117.90, 113.70, 89.65, 84.38, 83.05, 82.19, 46.18, 43.82, 27.56, 25.76 and 12.24 ppm. HRMS (ESI+) calcd for $C_{13}H_{20}N_7O_6S$ [(M+H)+] 402.1196 found 402.1193.



5'-N-[N-(3-Indole propionic acid)sulfamoyl] guanosine triethylammonium salt (6):

To a cold stirred solution of 17 (20 mg, 0.05 mmol) and 22 (1.5 equiv, 21.4 mg, 0.075 mmol) in DMF (0.4 mL) was added DBU (1.1 equiv, 8.2 μ L, 0.055 mmol). After 10 min the reaction mixture was brought to the room temperature and stirred overnight. Next, the volatiles were evaporated under reduced pressure and the crude reaction mixture was used for the final step without any

purification. To the crude reaction mixture was added 80% aq TFA (1 ml) and stirred for 30 mins. After which the reaction mixture was evaporated to dryness (co-evaporated 1% TEA/ethanol for removing TFA) under reduce pressure. The reaction mixture was loaded onto the combiflash and purified using reverse phase chromatography (A-ACN, B-Water + 0.1% TEA). The peak eluted at 20% of ACN contained the desired product. Fractions containing the product were pooled, concentrated and lyophilized to obtain the desired final product in quantitative yields (with 1 equivalent of TEA). ^1H NMR spectrum was 700 MHz (DMSO- d_6): 0.95 (t, 9 H), 2.73 (m, 6 H), 2.87-2.97 (m, 4H), 3.87-3.92 (m, 2H), 4.11 (m, 1H), 4.41-4.50 (m, 3H), 5.51 (d, 1H), 5.57 (d, 1H), 6.85-6.95 (m, 4H), 7.39-7.41 (m, 2H), 7.69-7.74 (m, 2H), and 10.72 (d, 2H). ^{13}C -DMSO- d_6 : 157.58, 156.42, 154.12, 151.15, 136.83, 128.76, 128.10, 127.44, 123.16, 121.19, 118.39, 111.64, 91.22, 88.26, 80.68, 78.38, 72.61, 66.19, 53.68, 52.36, 46.27, 21.94 and 10.21 ppm. HRMS (ESI+) calcd for $\text{C}_{21}\text{H}_{25}\text{N}_8\text{O}_7\text{S}$ [(M+H)+] 533.1567 found 533.1567. The final purity of the compound was $\geq 99\%$ as indicated by HPLC.

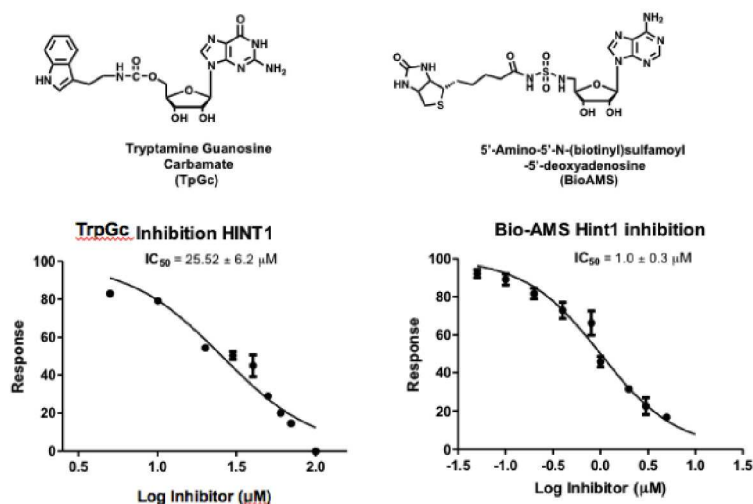


Figure S1: A dose response curve generated by the performing the titration of hHint1 activity in the presence of compound **3** (left, annotated as TrpGc) and BioAMS (right). The hHint1 activity was performed at a saturating concentration of the TrpAMP substrate (10 μM) using fluorescence activity described previously.⁶ The resulting response vs concentration was fitted into one site model using a graph pad prism to determine inhibitory concentration that resulted into 50% of inhibition (IC_{50}).

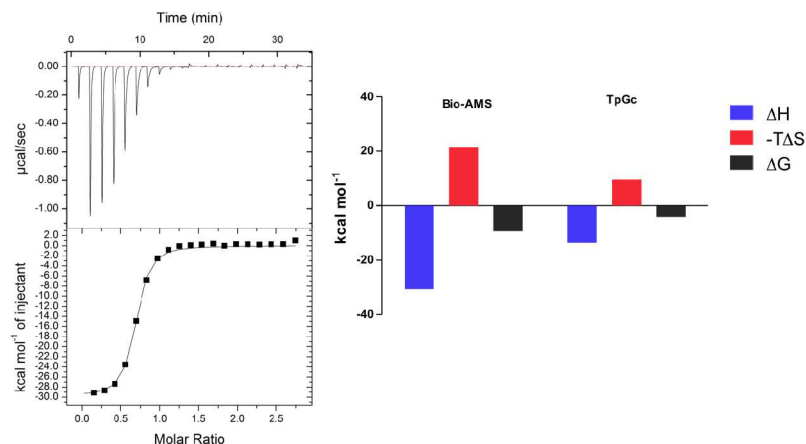


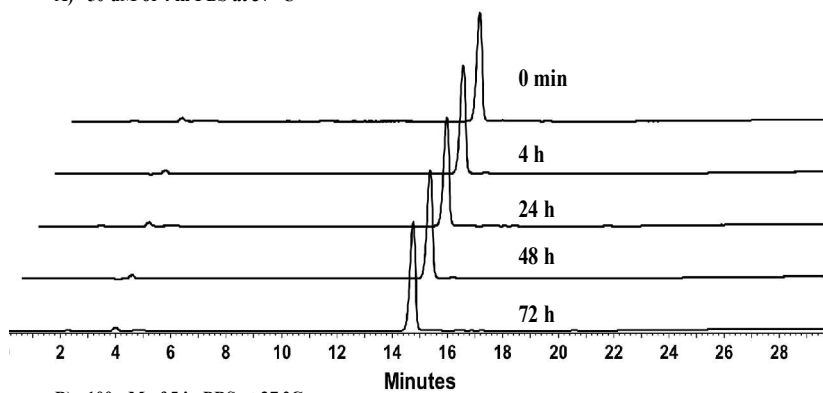
Figure S2: Left: A typical binding isotherm created after plotting integrated heat peaks against the molar ratio of BioAMS (300 μM , 10 mM Tris, 150 mM NaCl, pH 7.5) titrated into the solution of hHint1 (20 μM). Right: A binding signature (free energy, binding enthalpy and entropy) plotted for comparison of binding events resulted from two (compound **3** as TrpGc and BioAMS) different ligands with hHint1.

Table 1: Thermodynamic parameters of BioAMS and **3 binding to hHint1**

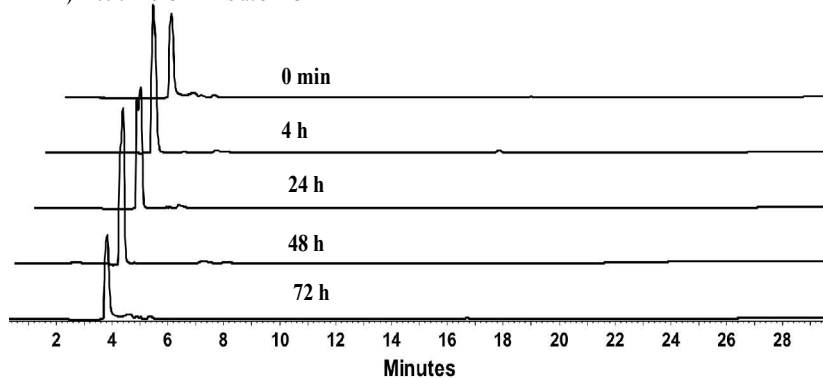
Ligand	K_d (μM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG (kcal/mol)	n
Bio-AMS	0.32 ± 0.1	21.30 ± 2.40	12.68 ± 2.68	-8.7 ± 0.20	1.00 ± 0.1
3 (TrpGc) ^a	3.65 ± 1.00	-13.54 ± 1.00	9.54 ± 4.17	-4.1 ± 2.0	0.98 ± 0.5

^a Data adapted from previously published result by Garzon et al.⁷

A) 50 μ M of 4 in PBS at 37 °C



B) 100 μ M of 5 in PBS at 37 °C



C) 50 μ M of 7 in PBS at 37 °C

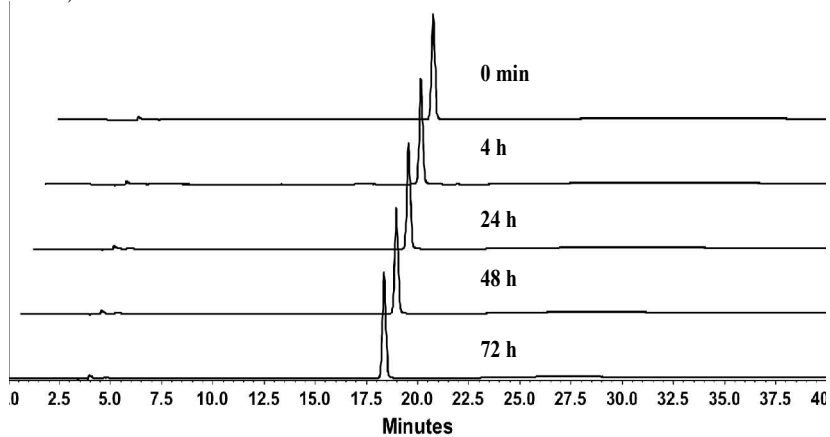
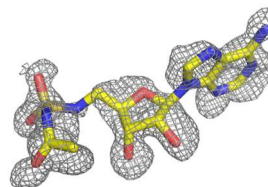
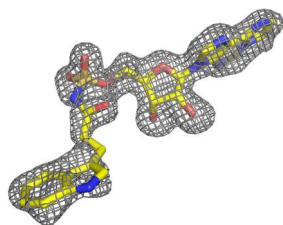


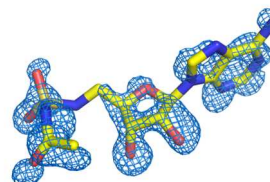
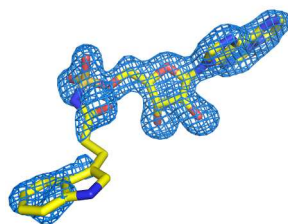
Figure S3: HPLC stability studies performed by monitoring the UV trace at absorbance 168-280 nm. Samples containing 50-100 μ M of the resulting compounds, A) 4 B) 5 and C) 7 were incubated at 37 °C in PBS pH 7.4. At indicated time intervals 200 μ l of the solution were injected on the HPLC and monitored for any appearance of degradation peaks.

Table 2:

Ligand Omit Map
(mFo-DFc)
Contoured at 2σ
(Chain A)



Ligand Omit Map
(mFo-DFc)
Contoured at 3σ
(Chain A)



PDB ID code	5I2E	5I2F
Ligand ID	67D	BS5
Resolution (Å)	1.60	1.25
Space group	C2	C2
a	76.77	77.49
b	46.35	46.31
c	63.93	64.07
Cell axis angle (°)	90.00	90.00
	94.73	94.44
	90.0	90.00
Data Processing		
Resolution range (Å)	39.64 - 1.600	39.724-1.250
(high shell)	(1.605 - 1.600)	(1.254-1.250)
Observations measured	93619	203409
(high shell)	(798)	(2030)
Unique reflections	29254	61412
(high shell)	(281)	(619)
Average multiplicity	3.2	3.3
(high shell)	(2.8)	(3.3)
Completeness (%)	98.5	97.9
(high shell)	(96.9)	(94.9)
R_{merge}	0.043	0.027
(high shell)	(0.368)	(0.133)
Mean $\langle I/\sigma \rangle$	15.0	24.1
(high shell)	(2.8)	(7.6)
Refinement statistics		
Resolution range (Å)	38.264 - 1.600	31.951-1.250
(high shell)	(1.657 - 1.600)	(1.270-1.250)
Working set reflections	27778	58285
(high shell)	(2739)	(2544)
R_{free} reflections	1446	3119
(high shell)	(149)	(151)
R (%)	0.1761	0.1554
(high shell)	(0.2730)	(0.1783)
R_{free} (%)	0.1990	0.1751
(high shell)	(0.2930)	(0.1752)
No. of non-hydrogen atoms	1989	2122
No. of solvent waters	109	260
Mean B-factors (Å²)		
Protein atoms	23.95	13.13
Solvent atoms	32.69	26.22
Ligand atoms	31.02	20.19
RMS deviations From Ideal Geometry		
Bond lengths (Å)	0.005	0.005
Bond angles (°)	1.090	0.947
Ramachandran plot outliers (%)	0.0	0.0
MolProbity score	1.2	0.99

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