Identifying OXA-48 Carbapenemase Inhibitors Using DNA-Encoded Chemical Libraries: Supporting Information

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A) DNA encoded library synthesis

I. General Information

Materials and procedures for the synthesis of this DECL were adapted from those we previously reported.[1]

Materials for the synthesis of DNA-encoded libraries. All DNA materials, including amino-PEG modified **S1** and the 5'phosphorylated encoding oligonucleotides, were obtained from LGC Biosearch Technologies. T4 DNA ligase was obtained from Enzymatics (Qiagen). Chemical building blocks were sourced from a variety of vendors and stored in acetonitrile solutions in Tracetraq barcoded tubes (Biosero). All buffers used were prepared in house using bio-grade reagents and high-purity solvents: HEPES 10X ligation buffer (300 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid, 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM adenosine triphosphate, pH 7.8) and basic borate buffer (250 mM sodium borate/boric acid, pH 9.5).

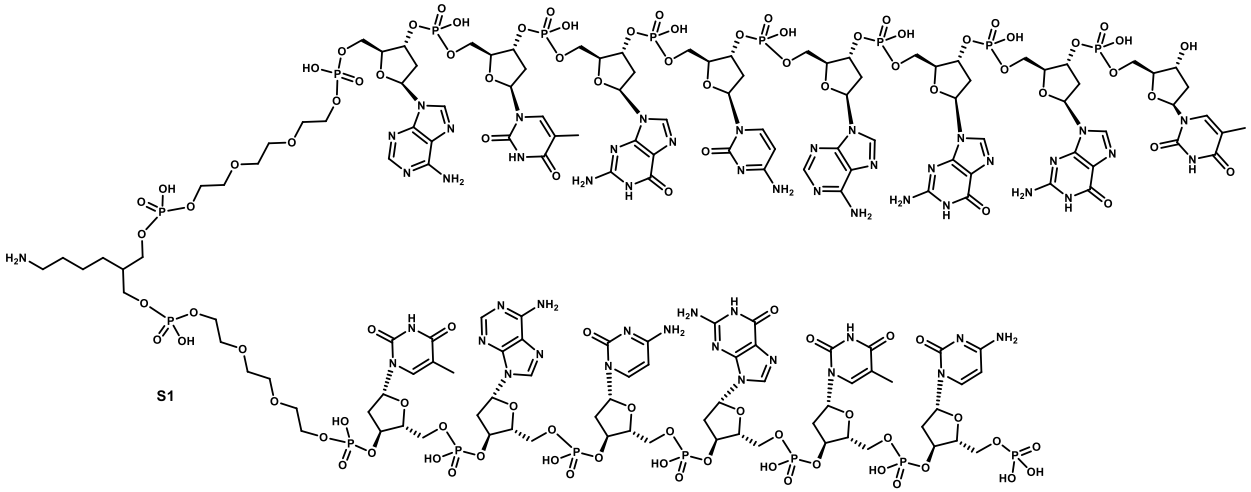


Figure S1. Structure of amino-PEG modified DEL starting material **S1** (5'-Phos-CTGCAT-Spacer 9-Amino C7-Spacer 9 ATGCAGGT 3').

Representative analytical procedure for the analysis of DNA oligonucleotide compositions. Samples of DEL materials and reactions were run on a Vanquish UHPLC system with an LTQ XL ion trap mass spectrometer (ThermoFisher Scientific). Sample injection amounts were typically 5–10 µL and contained 50–200 pmol DNA analyte. An ion-pair mobile phase of 15 mM TEA/100 mM HFIP in water/methanol was used with a Thermo DNAPac RP column (2.1 x 50 mm, 4µm). Mass spectra were acquired in the full scan negative-ion mode over the mass range 500–2000 and m/z patterns were deconvoluted using ProMass software.

Representative procedure for the isolation of DNA by ethanol precipitation. DNA solutions were diluted with a small amount of conc. aq. NaCl (5 M) to contain ~250 mM NaCl. Then absolute ethanol (3X total volume) was added, and the solution was stored at -20 °C overnight. After centrifugation (10,000 × G for 1 h) and decantation of the solution, 70% aq. ethanol (1X

volume) was added. After centrifugation (10,000 × G for 1 h), the solution was decanted and the DNA pellets were reconstituted to 1 mM in water.

Representative procedure for the ligation of DNA oligonucleotides.
An encoding duplex of oligonucleotides (1.1 equiv, 1 mM in water) was added to a DNA substrate (1 equiv, ~1 mM in water), followed by a ligation master mix to provide a final solution concentration of 0.24 mM. The ligation master mix contained HEPES 10X ligation buffer (added to be $1/10^{th}$ of the final volume), ligase (typically $1/100^{th}$ of the final volume) and water to enable final dilution to 0.24 mM. After incubation at room temperature overnight, the ligation completion was assessed by UPLC-MS and by running the ligation on a denaturing TBE-Urea gel. If not complete, additional duplex or master mix was added. After completion of ligation, the DNA was precipitated by the general procedure.

General architecture of the DECL. The DECL is a three-cycle library (three encoded chemistry steps). After extending **S1** with an amino-terminating PEG linker and ligation of a duplexed pair of oligonucleotides (forward primer unit and overhangs), the DEL will be extended by three variable 11-bp regions ("codons"; with 2-bp overhangs to enable annealing/ligation) used to encode chemical transformations within each cycle. The DNA architecture is shown in **Figure S2.**

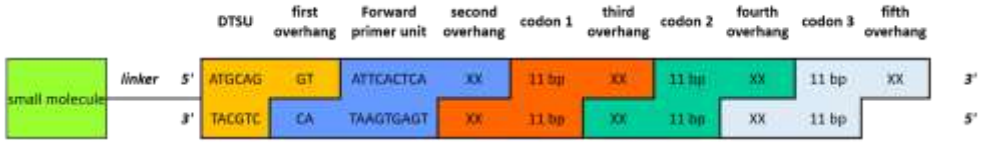


Figure S2. Sequence architecture of the DECL.

II. Synthesis of the triazine DECL

Overall synthetic sequence of the triazine DECL

The triazine DECL was constructed in three cycles around a triazine scaffold. First, DTSU **S1** was modified with a PEG-based linker, followed by the large-scale ligation of an 11-bp DNA duplex needed for a primer region to form the library starting material **S2**. Cycle 1 consisted of acylation of *N*-Boc amino acid building blocks, *N*-Boc deprotection and codon 1 ligation. Cycle 2 consisted of ligation of codon 2, nucleophilic substitution of the cycle 1 amines with cyanuric chloride and subsequent nucleophilic substitution of amine building blocks. Cycle 3 consisted of nucleophilic substitution of amine building blocks and codon 3 ligation. The synthetic sequence is shown in **Figure S3**.

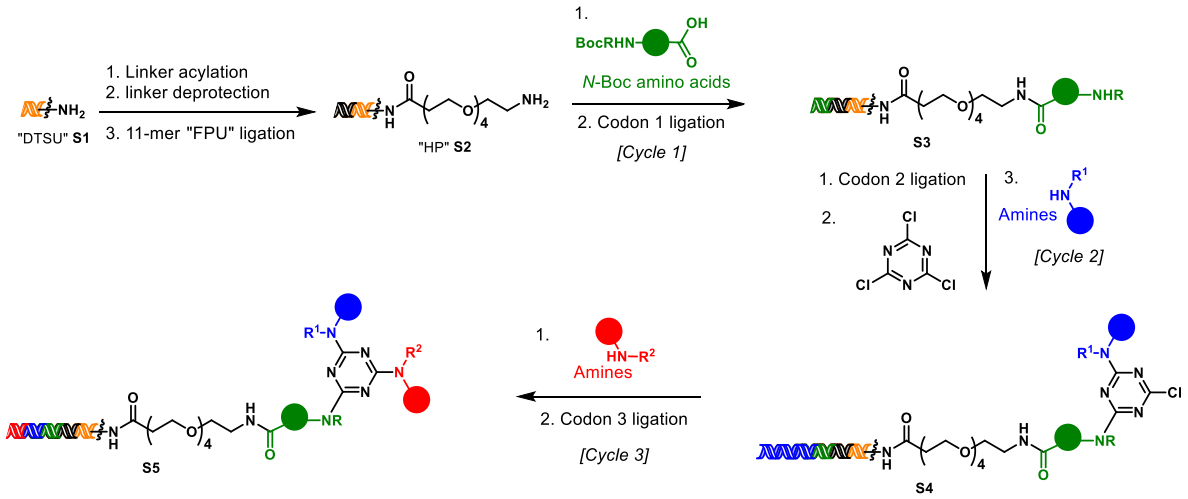


Figure S3. Synthetic sequence of the DECL.

Representative preparation of "HP" S2.

Procedure is as previously described.[1] To four 250-mL fluorinated ethylene propylene centrifuge bottles each charged with DTSU S**1** (25 µmol in 6.43 mL water, 1 equiv, 3.89 mM), aqueous sodium borate buffer (6250 µmol, 25 mL of a 250 mM aq. soln, 250 equiv, pH 9.5), CH_3CN (6 mL) and a solution of Fmoc-15-amino-4,7,10,13-tetraoxapentadecanoic acid (1250 µmol, 2.5 mL of a 500 mM soln in CH_3CN, 50 equiv) was added. After brief mixing, 4-4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride ("DMTMM", 1250 µmol, 2.5 mL of a 500 mM soln in H2O, 50 equiv) was added and the soln was incubated at 25 °C for 2 h. With verification of reaction completion by LC/MS (by formation of expected product, MW = 5461), the DNA was isolated by the general procedure. To the reconstituted DNA solutions (~1 mM), an aq. soln of piperidine (5 mL, 10% piperidine v/v) was added. After incubation at 25 °C for 3 h, the full deprotection of *N*-Fmoc was verified by LC/MS (formation of expected product, MW = 5240). After DNA isolation by the general procedure, the pellets were reconstituted in H_2O (10 mL) and the solutions combined (total volume of 49.8 mL after extra transfer washes). Optical density measurements of this soln indicated an approximate concentration of 1.82 mM (90.6 µmol, 90.6% yield). This material was then ligated with two 5'Phos 11-mer DNA oligomers ("forward primer unit") and isolated by the listed general procedures to provide the fully elongated HP **S2** in near quantitative yield from the linker intermediate. This material was used without further purification.

Procedure for Cycle 1

N-Boc amino acid building blocks (233 BBs, ran as 4 duplicates, 25 µL, 5000 nmol, 200 mM 1:1 pH 9.5 Borate:CH_3CN, 50 equiv) were added to a total of 932 wells, and the soln were concentrated to dryness. Then CH_3CN (25 µL) was added followed by HOBT (55.3 µL, 5000 nmol, 90 mM in CH_3CN, 50 equiv) and EDC (33.3 µL, 6000 nmol, 180 mM CH_3CN, 60 equiv) was added and the solns were incubated at 25 °C for 1 h. Then a premixed soln of **S2** (64.1 µL, 100 nmol, 1.56 mM, 1 equiv) and pH 9.5 Borate buffer (100 µL, 25,000 nmol, 250 mM, 250

equiv) was added and the solns were incubated at 25 °C overnight. The next day reactions were assessed for completion by the general procedure, and then additional H_2O (150 µL) was added to all wells and the reactions were heated at 80 °C for 48 h. The wells were then precipitated by the general procedure. After reconstitution in H_2O (100 µL), unique codon 1 duplexes were ligated in each well by the general procedure. After assessment and precipitation by the general procedures, the wells were pooled to provide cycle 1 intermediate **S3** (75.2 mL, 75.2 µmol, 1 mM in H_2O, 80% yield). Only a portion of **S3** was advanced to cycle 2.

Procedure for Cycle 2

Amino-terminating **S3** was portioned into 1196 wells (20.7 µL, 20.7 nmol, 1 mM, 1 equiv) and unique codon 2 duplexes were ligated by the general procedure. After precipitation by the general procedure, the wells were reconstituted with pH 9.5 Borate buffer (20.7 µL, 5170 nmol, 250 mM, 250 equiv) and cyanuric chloride (5.17 µL, 207 nmol, 40 mM in CH_3CN, 10 equiv) was then added. After incubation at 25 °C for 3 h, amino building blocks (5.2 µL, 1034 nmol, 200 mM in CH_3CN, 50 equiv) were added to each well and left overnight at RT. Several wells were reserved for encoded controls. After precipitation and pooling by the general procedure, cycle 2 intermediate **S4** (24.8 mL, 18.6 µmol, 0.75 mM, 74% yield) was quickly isolated.

Procedure for Cycle 3

Electrophilic-intermediate **S4** was portioned into 1196 wells (20.4 µL, 15.34 nmol, 0.75 mM, 1 equiv) and pH 9.5 Borate Buffer (15.3 µL, 3835 nmol, 250 mM, 250 equiv) and CH_3CN (10 µL) were added. Then amino building blocks (4 µL, 800 equiv, 200 mM, 53 equiv) were added and the soln was heated at 80 °C for 6 h. Several wells were reserved for encoded controls. After cooling, the DNA was precipitated by the general procedure. After reconstitution in H_2O (30 µL), unique codon 3 duplexes were ligated by the general procedure. After precipitation and pooling by the general procedure, the cycle 3 intermediate **S5** (28.8 mL, 16 µmol, 0.56 mM, 86% yield) was isolated.

Preparation of amplifiable triazine DECL samples ("shots") for selection experiments

Procedure is as previously described.[1] On small scale (1–20 nmol of completed library), the triazine DECL material was ligated with two DNA oligonucleotides containing a DNA segment encoding the library design, a segment encoding the experimental usage, a degenerate segment serving as the UMI region, a segment increasing sequencing diversity and a terminal primer segment to allow PCR amplification. After ethanol precipitation and reconstitution, the amount of amplifiable library material within prepared shots was subsequently quantified by qPCR and shots were used without further purification. Alternatively, portions of the library were ligated on large scale (1–5 µmol) with a duplexed 12-bp DNA codon to encode the library design followed by small scale ligation (1–20 nmol) of the remaining regions needed for the amplifiable shot.

III. Quality Control of the DECL

Prior to library construction, focused substrate scope studies were undertaken to optimize the chemical reaction conditions for a broad range of building blocks. Although it is not possible to test every three-cycle combination, building blocks were validated for use in the library by coupling to a test substrate under the optimized library conditions (generally >30% conversion). During the library build, every well in cycle 1 was assessed by LC-MS for successful reaction completion. As cycles 2 and 3 occur after an initial pooling, LC-MS traces are complex. However, we incorporate two controls to evaluate successful post-pool library steps. First a series of parallel single-substrate reactions are set up in corner wells of library production plates to ensure reagents and buffers are added correctly to library wells. Second, a cholesterol-tagged DNA oligomer which bears a reaction-relevant substrate is spiked into select wells. As this oligomer chromatographically separates from library DNA materials on LC-MS, it enables direct observation of successful chemistry within the library well itself. In addition, structure–activity relationships are often observed within DEL hit matter, which generally suggests successful implementation of library chemistry over a range of building blocks to provide the hit series, rather than impurities from a single building block source.

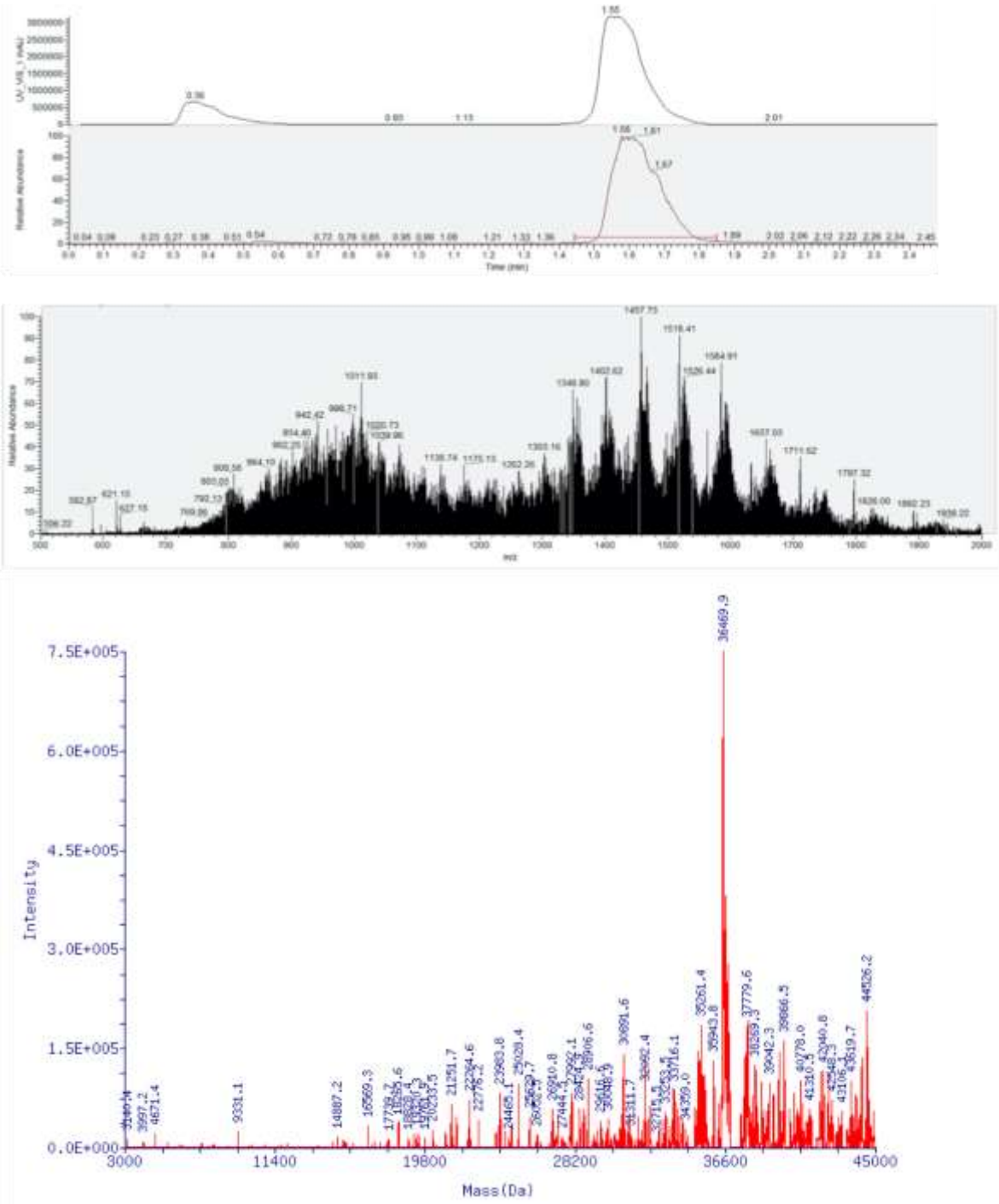


Figure S4. LC-MS analysis of the final DECL library after pooling of all 3 synthesis cycles. Shown are the UV trace, total ion count (TIC), the m/z graph and the deconvoluted mass spectrum of the pooled DECL library (with an average mass of ~36500 Da which suggests successful codon ligations).

B. X-ray Crystallography of CDD-97/OXA-48 Complex

I. Crystallography statistics

Table S1. X-ray crystallography statistics CDD-97/OXA-48 structure.

Statistics for CDD97-OXA-48 Structure (PDB entry 6UVK)	
Data Collection	
Wavelength (Å)	1.00001
Resolution range (Å)	34.69 - 2.2 (2.278 - 2.2)
Space group	$P6_5$
Unit cell dimension	
a, b, c (Å)	123.054, 123.054, 161.329
α, β, γ (°)	90, 90, 120
Unique reflections	65732 (4181)
Multiplicity	8.9 (8.3)
Completeness (%)	93.81 (59.81)
I/sigma	24.76 (2.04)
Wilson B-factor ($Å^2$)	29.47
R-merge	0.091
R-meas	0.096
R-pim	0.032
Reflections used in refinement	65708 (4181)
Reflections used for R-free	3313 (206)
Refinement	
R-work	0.1840 (0.2515)
R-free	0.2232 (0.3189)
Number of non-hydrogen atoms	8453
macromolecules	7907
ligands	137
solvent	409
Protein residues	968
RMS (bonds)	0.003
RMS (angles)	0.71
Ramachandran favored (%)	97.47
Ramachandran allowed (%)	2.53
Ramachandran outliers (%)	0
Rotamer outliers (%)	0
Clashscore	3.74
Average B-factor ($Å^2$)	46.01
macromolecules	46.07
ligands	47.35
solvent	44.35

Table S2. X-ray crystallography statistics of CDD-97 fit to experimental data.

Statistics for CDD-97 (Ligand: QHY) Fit (PDB entry 6UVK)					
Chain	Atoms[1]	RSCC[2]	RSR[3]	B-factors (Å^2)[4]	Q < 0.9[5]
A	30/30	0.97	0.11	36,47,56,60	0
B	30/30	0.96	0.11	41,49,63,66	0
C	30/30	0.94	0.13	31,43,52,53	0
D	30/30	0.94	0.10	24,41,49,57	0

[1] Number of QHY atoms modelled over the total number of QHY atoms
[2] Real space correlation coefficient
[3] Real space R-value
[4] B-factors listed as the minimum, median and 95th percentile and maximum B-factors for all the QHY atoms
[5] The number of QHY atoms with an occupancy less than 0.9

II. Additional Structural Insight

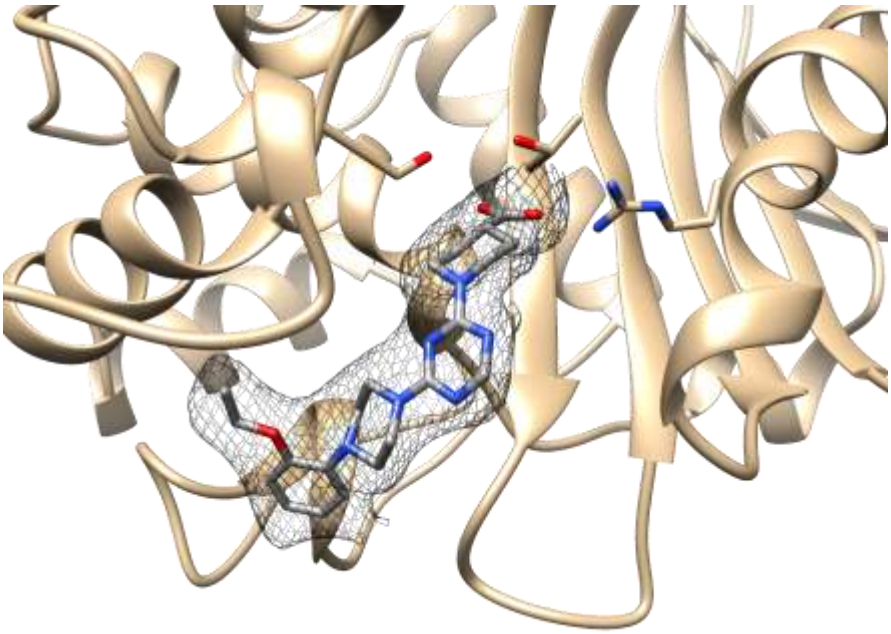


Figure S5. Structure of CDD-97 bound to OXA-48 with the mFo-Fc OMIT map, shown as mesh around the ligand contoured at 3σ level. The OXA-48 molecule with the highest CDD-97 occupancy of 89% (Chain D) was used to represent the electron density shown by the OMIT map.

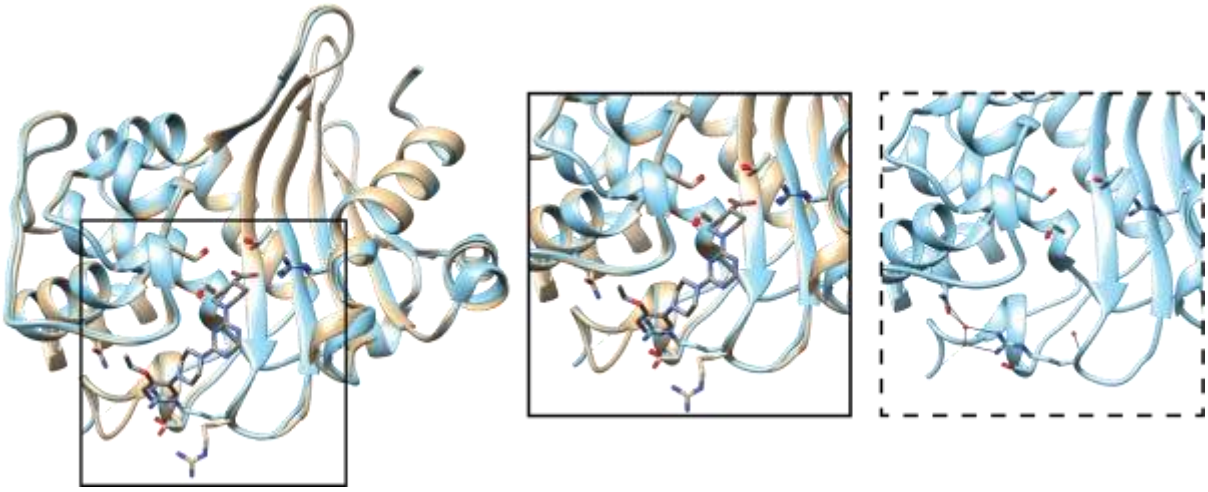
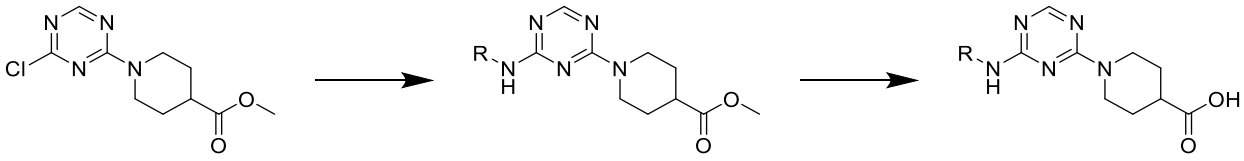


Figure S6. Alignment of CDD-97/OXA-48 complex structure (PDB: 6UVK) shown in tan and apo OXA-48 structure (PDB: 3HBR) shown in blue. The dotted structure shows the apo structure alone and highlights the interactions Arg214 typically makes with Asp159 and water molecules, one of which also coordinates with Gln124. The overlap shows that when CDD-97 binds OXA-48 it displaces Arg214 at the base of the active site and these interactions are lost.

C. Chemical synthesis of CDD-97 derivatives

I. Synthetic Route A



Methyl 1-(4-chloro-1,3,5-triazin-2-yl)piperidine-4-carboxylate

150 mg 2,4-dichlorotriazine was added to 5 mL dichloromethane, followed by incubation in ice bath. 170 µL triethylamine was added dropwise to this mixture, resulting in significant clearing of solution. 150 µL Methyl isonipecotate was added dropwise and the reaction allowed to stir until completion observed by TLC and/or LC-MS monitoring. Upon completion, reaction mixture diluted with ethyl acetate and washed with 0.1 M hydrochloric acid, saturated sodium bicarbonate, and brine. Organic layer was dried with sodium sulfate, followed by drying *in vacuo*. Crude product was dissolved in dichloromethane and purified by silica gel chromatography using a hexanes/ethyl acetate gradient. Product fractions dried *in vacuo*, yielding 105 mg of desired product as white solid, 41% yield.

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.37 (s, 1H), 4.65-4.56 (m, 2H), 3.74 (s, 3H), 3.25-3.17 (m, 2H), 2.70-2.63 (m, 1H), 2.07-2.01 (m, 2H), 1.80-1.72 (m, 2H).

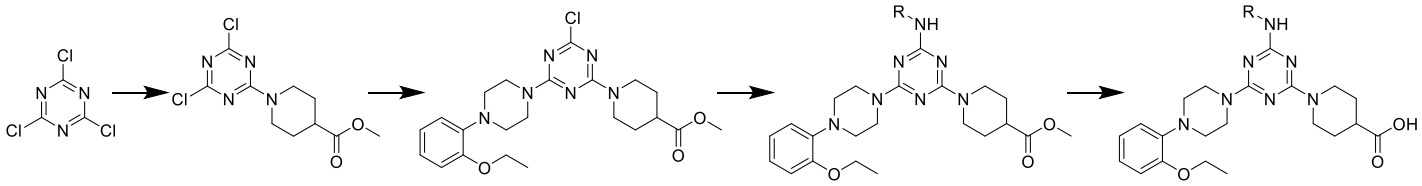
General Procedure A

1 eq. Methyl 1-(4-chloro-1,3,5-triazin-2-yl)piperidine-4-carboxylate was dissolved in 5 mL ethanol, followed by addition of 1.2 eq. triethylamine. 1.1 eq. of amine was then added and

reaction mixture heated to 60°C and monitored by TLC and/or LC-MS. Upon completion, reaction mixture diluted with ethyl acetate and washed with 0.1 M hydrochloric acid, saturated sodium bicarbonate, and brine. Organic layer was dried with sodium sulfate, followed by drying *in vacuo*. Crude product was dissolved in dichloromethane and purified by silica gel chromatography using a hexanes/ethyl acetate gradient. Product fractions dried *in vacuo*, yielding intermediate product.

Preceding intermediate product was dissolved in 3:1 methanol:water and treated with 2 eq. lithium hydroxide hydrate. Reaction mixture was stirred until completion by LC-MS monitoring, followed by acidification to pH ~7 with 0.1M hydrochloric acid and purification by 12g C18 column on a Biotage Isolera One. Product fractions dried *in vacuo*, yielding product as a white solid followed by characterization by LC-MS and 1HNMR.

II. Synthetic Route B



Methyl 1-(4,6-dichloro-1,3,5-triazin-2-yl)piperidine-4-carboxylate

184 mg cyanuric chloride was added to 5 mL dichloromethane, followed by incubation in ice bath. 297 µL methyl isonipecotate was added dropwise and the reaction allowed to stir until completion observed by TLC and/or LC-MS monitoring. Upon completion, reaction mixture diluted with ethyl acetate and washed with 0.1 M hydrochloric acid, saturated sodium bicarbonate, and brine. Organic layer was dried with sodium sulfate, followed by drying *in vacuo*. Crude product was dissolved in dichloromethane and purified by silica gel chromatography using a hexanes/ethyl acetate gradient. Product fractions dried *in vacuo*, yielding 200 mg of desired product as white solid, 69% yield.

^{1}H NMR (600 MHz, $CDCl_3$) δ 4.55 (td, J = 4.0, 13.7 Hz, 2H), 3.72 (s, 3H), 3.27-3.21 (m, 2H), 2.68-2.62 (m, 1H), 2.07-2.00 (m, 2H), 1.80-1.72 (m, 2H).

Methyl 1-(4-chloro-6-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylate

198 mg methyl 1-(4,6-dichloro-1,3,5-triazin-2-yl)piperidine-4-carboxylate was dissolved in 7 mL ethanol, followed by addition of 1.1 mL triethylamine. 149 mg 1-(2-ethoxyphenyl)piperazine was then added and reaction mixture heated to 60°C and monitored by TLC and/or LC-MS. Upon completion, reaction mixture diluted with ethyl acetate and washed with 0.1 M hydrochloric acid, saturated sodium bicarbonate, and brine. Organic layer was dried with sodium sulfate, followed by drying *in vacuo*. Crude product was dissolved in dichloromethane and purified by silica gel chromatography using a hexanes/ethyl acetate gradient. Product fractions dried *in vacuo*, yielding 138 mg product as a white solid, 44% yield.

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.02-6.98 (m, 1H), 6.94-6.86 (m, 3H), 4.59 (dt, J = 13.4, 3.6 Hz, 2H), 4.09 (q, J = 7.0 Hz, 2H), 4.01-3.93 (br s, 4H), 3.70 (s, 3H), 3.13-3.02 (m, 6H), 2.61-2.55 (m, 1H), 1.97 (dd, J = 13.5, 3.1 Hz, 2H), 1.76-1.65 (br s, 2H), 1.47 (t, 7.0 Hz, 3H).

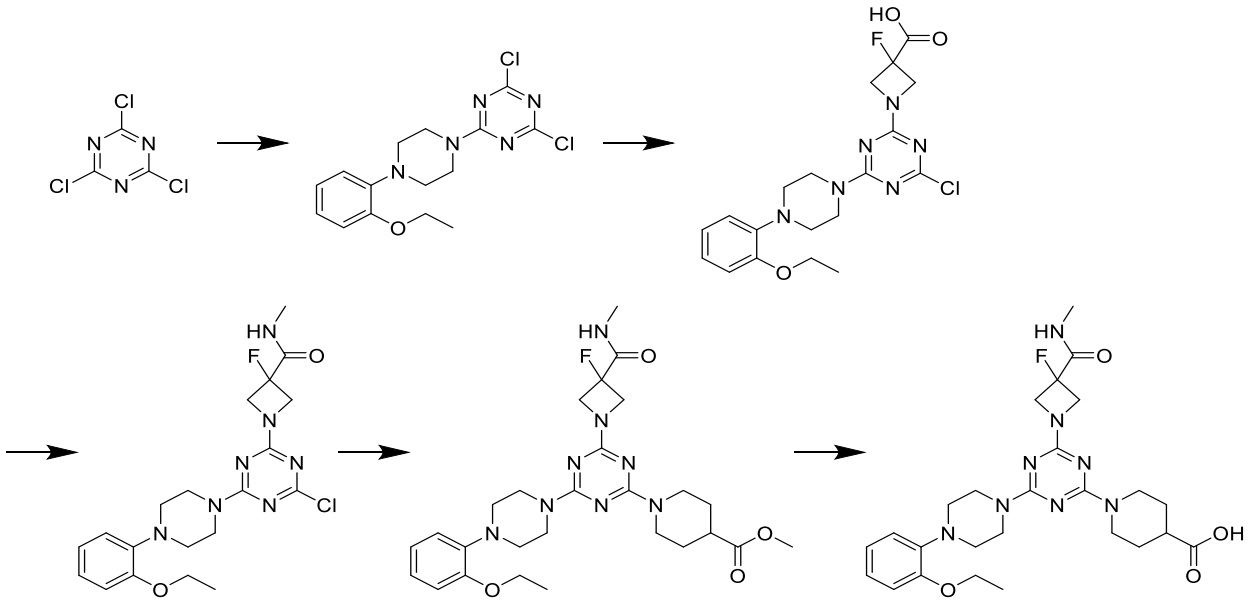
General Procedure B

1 eq. Methyl 1-(4-chloro-6-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylate was dissolved to a 0.2 M solution in ethanol, followed by addition of 2.1 eq. amine to solution. Reaction mixture was heated to 100°C for 1 hour, followed by drying *in vacuo*. Purification by 12g C18 column using Biotage Isolera One followed by drying product fractions yielded intermediate product.

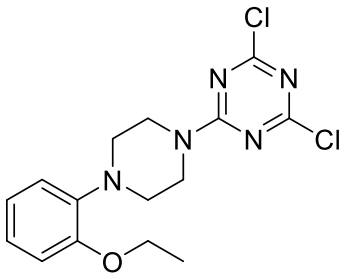
Preceding intermediate product was dissolved in 3:1 methanol:water and treated with 2 eq. lithium hydroxide hydrate. Reaction mixture was stirred until completion by LC-MS monitoring, followed by acidification to pH ~7 with 0.1M hydrochloric acid and purification by 12g C18 column on a Biotage Isolera One. Product fractions dried *in vacuo*, yielding product as a white solid followed by characterization by LC-MS and 1HNMR.

III. Compound Syntheses and Characterization

Synthetic Scheme of Compound **1** (CDD-000163)



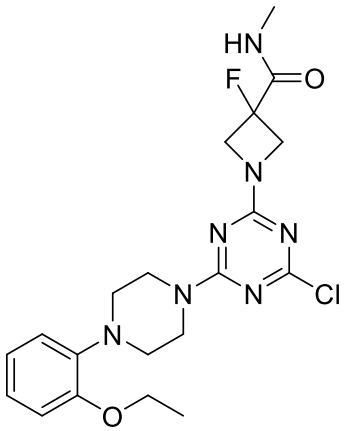
2,4-dichloro-6-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazine



119 mg cyanuric chloride was added to 5 mL dichloromethane, followed by incubation in ice bath. 90 µL triethylamine was added dropwise to this mixture. 267 mg 1-(2-ethoxyphenyl) piperazine was added dropwise and the reaction allowed to stir until completion observed by TLC and/or LC-MS monitoring. Upon completion, reaction mixture diluted with ethyl acetate and washed with 0.1 M hydrochloric acid, saturated sodium bicarbonate, and brine. Organic layer was dried with sodium sulfate, followed by drying *in vacuo*. Crude product was dissolved in dichloromethane and purified by silica gel chromatography using a hexanes/ethyl acetate gradient. Product fractions dried *in vacuo*, yielding 170 mg of desired product, 74% yield.

^{1}HNMR (600 MHz, $CDCl_3$) δ 7.03 (t, J = 7.7 Hz, 1H), 6.93 (t, J = 7.7 Hz, 1H), 6.91-6.87 (m, 2H), 4.10 (q, J = 7.0 Hz, 2H), 4.07 (t, J = 5.0 Hz, 4H), 3.15 (t, J = 5.0 Hz, 4H), 1.47 (t, J = 7.0 Hz, 3H).

1-(4-chloro-6-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)-3-fluoro-N-methylazetidine-3-carboxamide

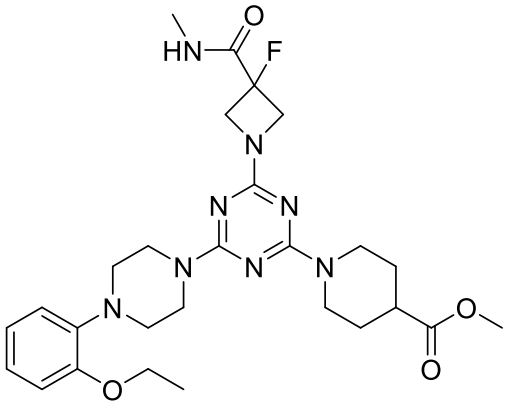


170 mg of 2,4-dichloro-6-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazine dissolved in 1 mL ethanol, followed by addition of 60.1 mg 3-fluoroazetidine-3-carboxylic acid and 0.2 mL triethylamine. Reaction mixture heated at 60 °C; stirring continued until no starting material remained by LC-MS and only one peak observed by UV and MS traces. Reaction mixture dried in vacuo and used for next step without further purification.

Crude intermediate was dissolved in 1 mL DMF, followed by addition of 222 mg methylamine hydrochloride, 250 µL triethylamine, and 266 mg HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate). Reaction mixture stirred for 1 hour, confirmed complete by LC-MS, then diluted with ethyl acetate and washed with 0.1 M hydrochloric acid, saturated sodium bicarbonate, and brine. Organic layer was dried with sodium sulfate, followed by drying *in vacuo*. Crude product was dissolved in dichloromethane and purified by silica gel chromatography using a hexanes/ethyl acetate gradient. Product fractions dried *in vacuo*, yielding 45.0 mg of desired product as white solid, 21% overall yield.

^{1}HNMR (600 MHz, $CDCl_3$) δ 7.00 (t, J = 7.0 Hz, 1H), 6.94-6.86 (m, 3H), 6.37 (br s, 1H), 4.68-4.59 (m, 2H), 4.38-4.22 (m, 2H), 4.09 (q, J = 6.9 Hz, 2H), 4.09-3.90 (m, 4H), 3.15-3.03 (m, 4H), 2.92 (d, J= 4.9 Hz, 3H), 1.47 (t, J = 7.0 Hz, 3H).

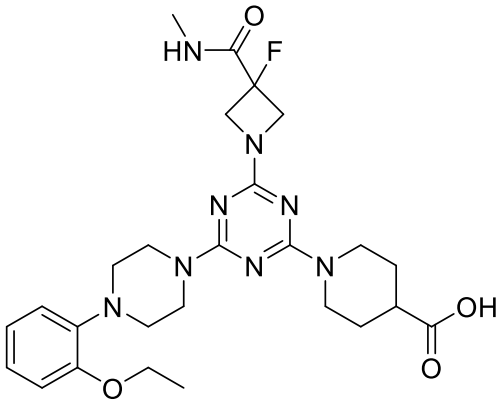
Methyl 1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-6-(3-fluoro-3-(methylcarbamoyl)azetidin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylate



45.0 mg 1-(4-chloro-6-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)-3-fluoro-N-methylazetidine-3-carboxamide dissolved in 0.5 mL DMF, followed by addition of 21 µL TEA and 20 µL methyl isonipecotate. Reaction mixture was heated at 100°C for 1 hour by microwave. Reaction mixture diluted with ethyl acetate and washed with 0.1 M hydrochloric acid, saturated sodium bicarbonate, and brine. Organic layer was dried with sodium sulfate, followed by drying *in vacuo*. Crude product was dissolved in dichloromethane and purified by silica gel chromatography using a hexanes/ethyl acetate gradient. Product fractions dried *in vacuo*, yielding 26.6 mg of desired product as white solid, 48% yield.

^{1}HNMR (600 MHz, $CDCl_3$) δ 7.01-6.96 (m, 1H), 6.94-6.90 (m, 2H), 6.87 (d, J = 7.9 Hz, 1H), 6.36 (t, J = 4.6 Hz, 1H), 4.63 (d, J = 13.4 Hz, 2H), 4.55 (dd, J = 10.5 Hz, 2H), 4.21 (dd, J = 10.4 Hz, 2H), 4.09 (q, J = 6.9 Hz, 2H), 4.35-4.29 (m, 4H), 3.69 (s, 3H), 3.10-3.04 (m, 4H), 2.96-2.89 (m, 5H), 2.57-250 (m, 1H), 1.92 (dd, J = 13.3, 3.0 Hz, 2H), 1.70-1.62 (m, 2H), 1.48 (t, J = 7.0 Hz, 3H).

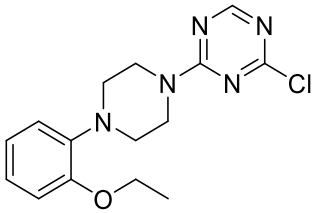
1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-6-(3-fluoro-3-(methylcarbamoyl)azetidin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **1** (CDD-000163)



26.5 mg methyl 1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-6-(3-fluoro-3-(methylcarbamoyl)azetidin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylate was dissolved in 3:1 methanol:water, followed by addition of 3.8 mg lithium hydroxide hydrate. Reaction mixture was stirred until completion by LC-MS monitoring, followed by acidification to pH ~7 with 0.1M hydrochloric acid and purification by 12g C18 column on a Biotage Isolera One. Product fractions dried *in vacuo*, yielding 4.5 mg product as a white solid, 17% yield.

^{1}H NMR (600 MHz, d_4-MeOD) δ 7.03 – 6.88 (m, 4H), 4.70 (d, J = 13.1 Hz, 2H), 4.48 (dd, J = 20.5, 10.5 Hz, 2H), 4.20 (dd, J = 22.2, 10.5 Hz, 2H), 4.12 (q, J = 7.0 Hz, 2H), 3.99 – 3.84 (m, 4H), 3.09 – 3.01 (m, 4H), 2.92 – 2.85 (m, 2H), 2.83 (s, 3H), 2.46 – 2.33 (m, 1H), 1.97 – 1.82 (m, 2H), 1.60 (qd, J = 12.5, 4.0 Hz, 2H), 1.47 (t, J = 7.0 Hz, 3H).
Calc.'d m/z [M+H] ($C_{26}H_{36}FN_8O_4$+): 543.28381; Obs.'d m/z: 543.28537

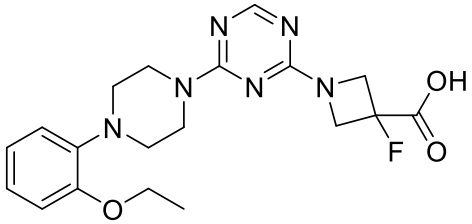
2-chloro-4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazine



68 mg of 1-(2-ethoxyphenyl)piperazine and 50 µL triethylamine was dissolved in 1 mL acetonitrile then added dropwise to a stirred solution of 50 mg 2,4-dichloro-1,3,5-triazine, followed by stirring for 1 hour. RM was then dried *in vacuo* and purified by normal phase chromatography using a hexanes/ethyl acetate gradient. Product fractions dried *in vacuo*, yielding 47.7 mg of 2-chloro-4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazine as a clear oil.

^{1}H NMR (600 MHz, d_6-acetone) δ 8.39 (s, 1H), 7.06-7.02 (m 1H), 6.96-6.90 (m, 3H), 4.16-4.05 (m, 6H), 3.19-3.15 (m, 4H), 1.50, (t, J = 7.0 Hz, 3H).

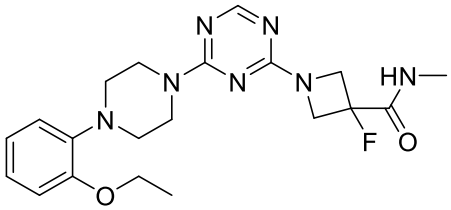
1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)-3-fluoroazetidine-3-carboxylic acid Compound **2** (CDD-000096)



A mixture of 50 mg of 2-chloro-4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazine, 18 mg 3-fluoroazetine-3-carboxylic acid, and 50 µL triethylamine in 1 mL ethanol was heated at 60°C overnight. Reaction mixture was dried *in vacuo* then purified by normal phase chromatography using a dichloromethane/methanol gradient. Product fractions dried *in vacuo* to yield 9.0 mg of **2** as white solid.

^{1}H NMR (600 MHz, d_6-DMSO) δ 8.18 (s, 1H), 6.96 – 8.85 (m, 4H), 4.47 (dd, J = 17.9, 11.0 Hz, 2H), 4.26 (dd, J = 20.3, 11.1 Hz, 2H), 4.04 (q, J = 7.0 Hz, 2H), 3.87 (br s, 4H), 3.01 (br s, 4H), 1.37 (t, J = 6.9 Hz, 3H).
Calc.'d m/z [M+H] ($C_{19}H_{24}FN_6O_3^+$): 403.18884; Obs.'d m/z: 403.18765

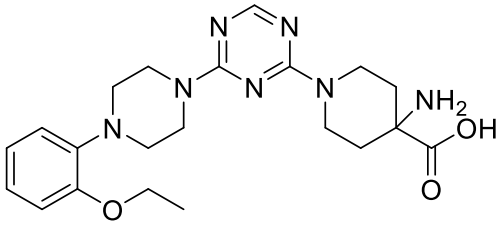
Synthesis of 1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)-3-fluoro-N-methylazetidine-3-carboxamide
Compound **3** (CDD-000147)



A mixture of 9 mg Compound **2** in 0.2 mL DMF was treated with 12 µL DIEA, 56 µL 2M methylamine in THF, and finally 9.4 mg HATU. Reaction mixture stirred overnight, then quenched with water. Reaction mixture dried *in vacuo* followed by normal phase chromatography using a hexanes/ethyl acetate gradient. Product fractions dried in vacuo to yield 1.9 mg of **3** as a white solid.

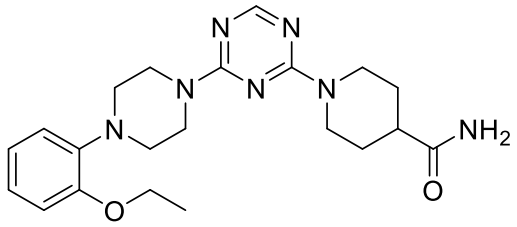
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.20 (s, 1H), 7.02-6.96 (m, 1H), 6.94-6.86 (m, 3H), 6.39 (br s, 1H), 4.62 (qd, J = 10.6, 1.4 Hz, 2H), 4.35-4.20 (m, 2H), 4.10 (q, J = 7.0 Hz, 2H), 4.04-3.90 (m, 4H), 3.10 (t, J = 4.9 Hz, 4H), 2.92 (d, J = 4.9 Hz, 3H), 1.48 (t, J = 7.0 Hz, 3H).
Calc.'d m/z [M+H] ($C_{20}H_{27}FN_7O_2^+$): 416.22048; Obs.'d m/z: 416.21930

Synthesis of 4-amino-1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **15** (CDD-000709)



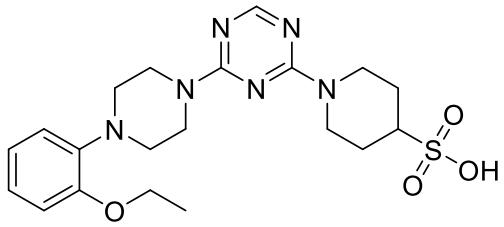
^{1}H NMR (600 MHz, d_4-MeOD) δ 8.12 (s, 1H), 7.03 – 6.89 (m, 4H), 4.12 (q, J = 7.0 Hz, 2H), 3.99 – 3.94 (m, 4H), 3.90 (s, 4H), 3.11 – 3.04 (m, 4H), 2.11 – 2.04 (m, 2H), 1.61 (dt, J = 10.8, 5.2 Hz, 2H), 1.47 (t, J = 7.0 Hz, 3H).
Calc.'d m/z [M+H] ($C_{21}H_{30}N_7O_3^+$): 428.24046; Obs.'d m/z: 428.240011

Synthesis of 1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxamide
Compound **14** (CDD-000553)



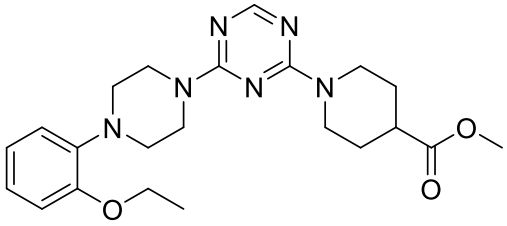
^{1}H NMR (600 MHz, d_4-MeOD) δ 8.12 (s, 1H), 7.03 – 6.89 (m, 4H), 4.78 (s, 2H), 4.12 (q, J = 7.0 Hz, 2H), 4.01 – 3.92 (m, 4H), 3.12 – 3.04 (m, 4H), 2.99 – 2.91 (m, 2H), 2.55 (tt, J = 11.7, 3.7 Hz, 1H), 1.88 (dd, J = 12.9, 2.2 Hz, 2H), 1.63 (qd, J = 12.6, 4.3 Hz, 2H), 1.48 (t, J = 7.0 Hz, 3H).
Calc.'d m/z [M+H] ($C_{21}H_{30}N_7O_2^+$): 412.24555; Obs.'d m/z: 412.24501

Synthesis of 1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-sulfonic acid
Compound **16** (CDD-000862)



^{1}H NMR (600 MHz, d_4-MeOD) δ 8.12 (s, 1H), 7.03 – 6.88 (m, 4H), 4.85 (s, 2H), 4.12 (q, J = 7.0 Hz, 2H), 3.96 (s, 4H), 3.23 (q, J = 7.3 Hz, 1H), 3.12 – 3.05 (m, 4H), 3.00 – 2.87 (m, 2H), 2.20 (d, J = 12.7 Hz, 2H), 1.69 (qd, J = 12.7, 4.3 Hz, 2H), 1.47 (t, J = 7.0 Hz, 3H).
Calc.'d m/z [M+H] ($C_{20}H_{29}N_6O_4S^+$): 449.19655; Obs.'d m/z: 449.19629

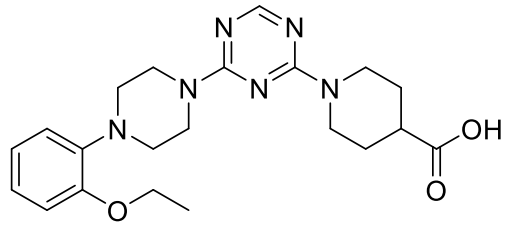
Synthesis of methyl 1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylate
Compound **5** (CDD-000095)



Intermediate product for CDD-000097 via General Procedure A.

^{1}H NMR (600 MHz, d_6-acetone) δ 8.13 (s, 1H), 6.98 – 6.87 (m, 4H), 4.66 (d, J = 13.2 Hz, 2H), 4.11 (q, J = 7.0 Hz, 2H), 3.95 (s, 4H), 3.67 (s, 3H), 3.11 – 3.05 (m, 6H), 2.68 (tt, J = 11.1, 4.0 Hz, 1H), 1.98 – 1.93 (m, 2H), 1.60 (d, J = 9.9 Hz, 2H), 1.44 (t, J = 7.0 Hz, 3H).
Calc.'d m/z [M+H] ($C_{22}H_{31}N_6O_3^+$): 427.24522; Obs.'d m/z: 427.24500

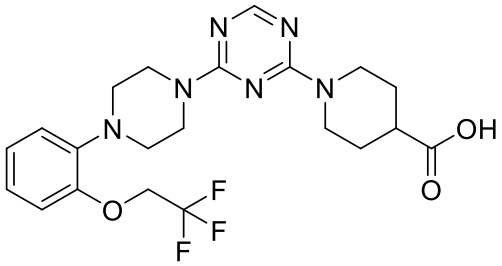
Synthesis of 1-(4-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **4** (CDD-000097)



Prepared via General Procedure A.

^{1}H NMR (600 MHz, d_4-MeOD) δ 8.11 (s, 1H), 7.02 – 6.88 (m, 4H), 4.62 (d, J = 9.7 Hz, 2H), 4.12 (q, J = 7.0 Hz, 2H), 3.99 – 3.92 (m, 4H), 3.08 (dd, J = 11.0, 6.2 Hz, 6H), 2.63 (s, 1H), 1.98 (d, J = 10.9 Hz, 2H), 1.63 (d, J = 8.4 Hz, 2H), 1.47 (t, J = 7.0 Hz, 3H).
^{13}C NMR (151 MHz, CDCl3) δ 175.67, 165.49, 163.60, 163.38, 151.20, 141.10, 122.65, 120.84, 118.28, 113.03, 63.28, 49.97, 43.05, 42.63, 42.14, 41.74, 40.29, 27.65, 14.81.
Calc.'d m/z [M+H] ($C_{21}H_{29}N_6O_3^+$): 413.22957; Obs.'d m/z: 413.22858

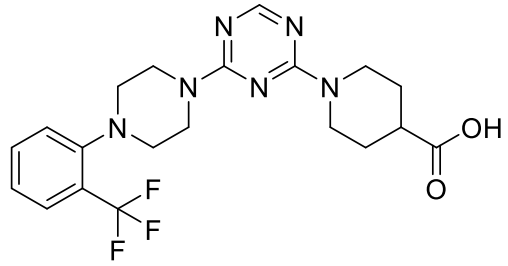
Synthesis of 1-(4-(4-(2-(2,2,2-trifluoroethoxy)phenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **7** (CDD-000178)



Prepared via General Procedure A.

1H NMR (600 MHz, d_4-MeOD) δ 8.09 (s, 1H), 7.05 – 6.98 (m, 4H), 4.72-4.58 (s, 2H), 4.57 (q, J = 8.6 Hz, 2H), 3.97-3.87 (m, 4H), 3.10-2.91 (m, 6H), 2.71-2.62 (m, 1H), 1.98-1.87 (m, 2H), 1.66-154 (m, 2H).
Calc.'d m/z [M+H] ($C_{21}H_{26}F_3N_6O_3^+$): 467.20186; Obs.'d m/z: 467.20032

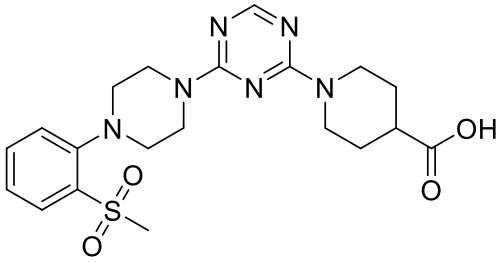
Synthesis of 1-(4-(4-(2-(trifluoromethyl)phenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **8** (CDD-000180)



Prepared via General Procedure A.

1H NMR (600 MHz, d_4-MeOD) δ 8.10 (s, 1H), 7.68 (d, J = 7.6 Hz, 1H), 7.62 (t, J = 7.6 Hz, 1H), 7.52 (d, J = 8.0 Hz, 1H), 7.33 (t, J = 7.6 Hz, 1H), 4.67 (s, 2H), 3.94 (s, 4H), 2.99 (s, 1H), 2.98 – 2.94 (m, 5H), 2.42-2.36 (m, 1H), 1.94-1.88 (m, 2H), 1.65-1.56 (m, 2H).
Calc.'d m/z [M+H] ($C_{20}H_{24}F_3N_6O_2^+$): 437.19074; Obs.'d m/z: 437.18983

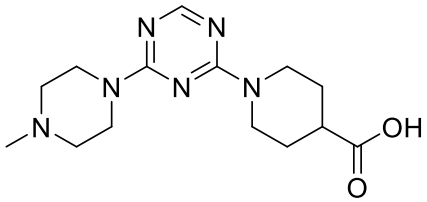
Synthesis of 1-(4-(4-(2-(methylsulfonyl)phenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **9** (CDD-000181)



Prepared via General Procedure A.

1H NMR (600 MHz, d_4-MeOD) δ 8.11 (s, 1H), 8.02 (dd, J = 7.9, 1.5 Hz, 1H), 7.69 (td, J = 7.9, 1.5 Hz, 1H), 7.54 (dd, J = 8.0, 0.8 Hz, 1H), 7.42 (td, J = 8.0, 0.9 Hz, 1H), 4.88 (s, 4H), 4.64-4.51 (m, 2H), 3.40 (s, 3H), 3.15-3.02 (m, 6H), 2.65-2.58 (m, 1H), 2.00-1.93 (m, 2H), 1.65-1.56 (m, 2H).
Calc.'d m/z [M+H] ($C_{20}H_{27}N_6O_4S^+$): 447.18090; Obs.'d m/z: 447.18033

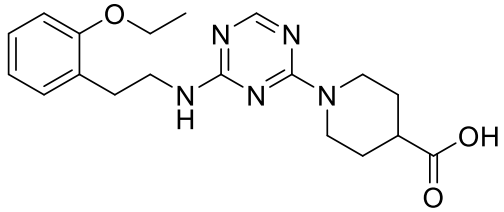
Synthesis of 1-(4-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **6** (CDD-000187)



Prepared via General Procedure A.

1H NMR (600 MHz, d_4-MeOD) δ 8.08 (s, 1H), 4.64 (d, J = 6.7 Hz, 2H), 3.84 (s, 4H), 2.98 (t, J = 11.9 Hz, 2H), 2.53 (t, J = 5.0 Hz, 4H), 2.47-2.41 (m, 1H), 2.37 (s, 3H), 1.96-1.90 (m, 2H), 1.65-1.57 (m, 2H).
Calc.'d m/z [M+H] ($C_{14}H_{23}N_6O_2^+$): 307.18770; Obs.'d m/z: 307.18742

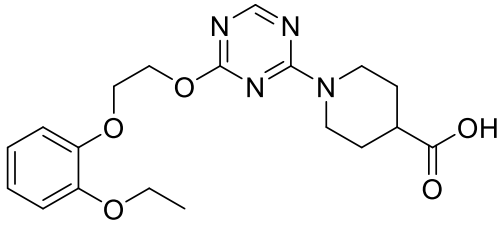
Synthesis of 1-(4-((2-ethoxyphenethyl)amino)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **11** (CDD-000375)



Prepared via General Procedure A.

1H NMR (600 MHz, d_4-MeOD) 7.96 (s, 1H), 7.20-7.10 (m, 2H), 6.94-6.82 (m, 2H), 4.71-4.50 (m, 2H), 4.07 (q, J = 7.0 Hz, 2H), 3.61-3.53 (m, 2H), 3.13-3.01 (m, 2H), 2.95-2.87 (m, 2H), 2.67-2.56 (m, 1H), 2.02-1.91 (m, 2H), 1.68-1.54 (m ,2H), 1.42 (t, J = 6.9 Hz, 3H).
Calc.'d m/z [M+H] ($C_{19}H_{26}N_5O_3^+$): 372.20302; Obs.'d m/z: 372.20212

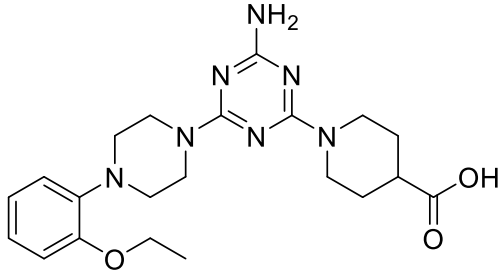
Synthesis of 1-(4-(2-(2-ethoxyphenoxy)ethoxy)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **10** (CDD-000900)



Pre-mixed 2-(2-ethoxyphenoxy)ethan-1-ol and 60% sodium hydride, dispersion in mineral oil, in THF prior to following General Procedure A.

1H NMR (600 MHz, d_4-MeOD) δ 7.98 (s, 1H), 7.01 (dd, J=7.7, 1.7 Hz, 1H), 6.97 (dd, J = 7.8, 1.7 Hz, 1H), 6.93 (td, J = 7.6, 1.7 Hz, 1H), 6.89 (J = 7.5, 1.8 Hz, 1H), 4.70-4.57 (br s, 2H), 4.19-4.11 (br s, 2H), 4.08 (q, J = 7.0 Hz, 2H), 3.80-3.69 (br s, 2H), 3.02-2.88 (br s, 2H), 2.43-2.36 (m, 1H), 2.01 (br s, 2H), 1.65-1.56(m, 2H), 1.40 (t, J = 6.9 Hz, 3H).
Calc.'d m/z [M+H] ($C_{19}H_{25}N_4O_5^+$): 389.18195; Obs.'d m/z: 389.18106

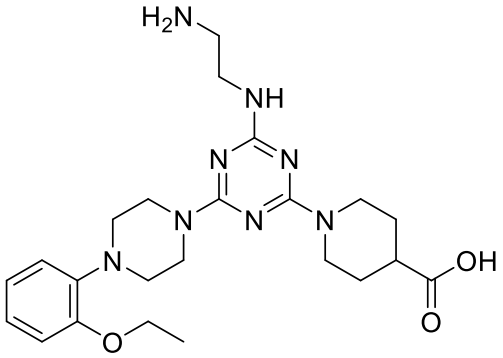
Synthesis of 1-(4-amino-6-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **13** (CDD-000616)



Synthesized using General Procedure B, using 1 mL 7N ammonium hydroxide as solvent/reactant.

^{1}H NMR (600 MHz, d_4-MeOD) δ 7.10 – 6.79 (m, 4H), 4.67 (d, J = 12.7 Hz, 2H), 4.12 (q, J = 7.0 Hz, 2H), 3.94 – 3.84 (m, 4H), 3.08 – 3.01 (m, 4H), 2.88 (td, J = 13.0, 2.6 Hz, 2H), 2.37 (tt, J = 11.5, 3.7 Hz, 1H), 1.89 (dd, J = 13.1, 2.7 Hz, 2H), 1.61 (qd, J = 12.2, 4.1 Hz, 2H), 1.47 (t, J = 7.0 Hz, 3H).
Calc.'d m/z [M+H] ($C_{21}H_{30}N_7O_3^+$): 428.24046 ; Obs.'d m/z: 428.23975

Synthesis of 1-(4-((2-aminoethyl)amino)-6-(4-(2-ethoxyphenyl)piperazin-1-yl)-1,3,5-triazin-2-yl)piperidine-4-carboxylic acid
Compound **12** (CDD-000864)



Synthesized using General Procedure B, using 3 eq. 1,2-ethylenediamine as reactant.

^{1}H NMR (600 MHz, d6-DMSO) δ 6.97-6.83 (m, 4H), 4.42 (br s, 2H), 4.01 (q, 6.8 Hz, 2H), 3.83-3.73 (m, 8H), 3.30-3.24 (m, 2H), 2.94 (br s, 4H), 2.83-2.75 (m, 2H), 2.70-2.64 (m 2H), 2.09-2.03 (m, 1H), 1.70 (br s, 2H), 1.39-1.30 (m, 5H).
Calc.'d m/z [M+H] ($C_{23}H_{35}N_8O_3^+$): 471.28266; Obs.'d m/z: 471.28232

IV. Purity of Synthesized Compounds

All synthesized compounds were confirmed before proceeding with downstream assays. All compounds were >95% pure as judged by 1HNMR and LC-MS/HRMS. As with all the synthesized compounds, the identity and purity of the lead compound (CDD-97) was assessed using 1HNMR and LC-MS/HRMS.

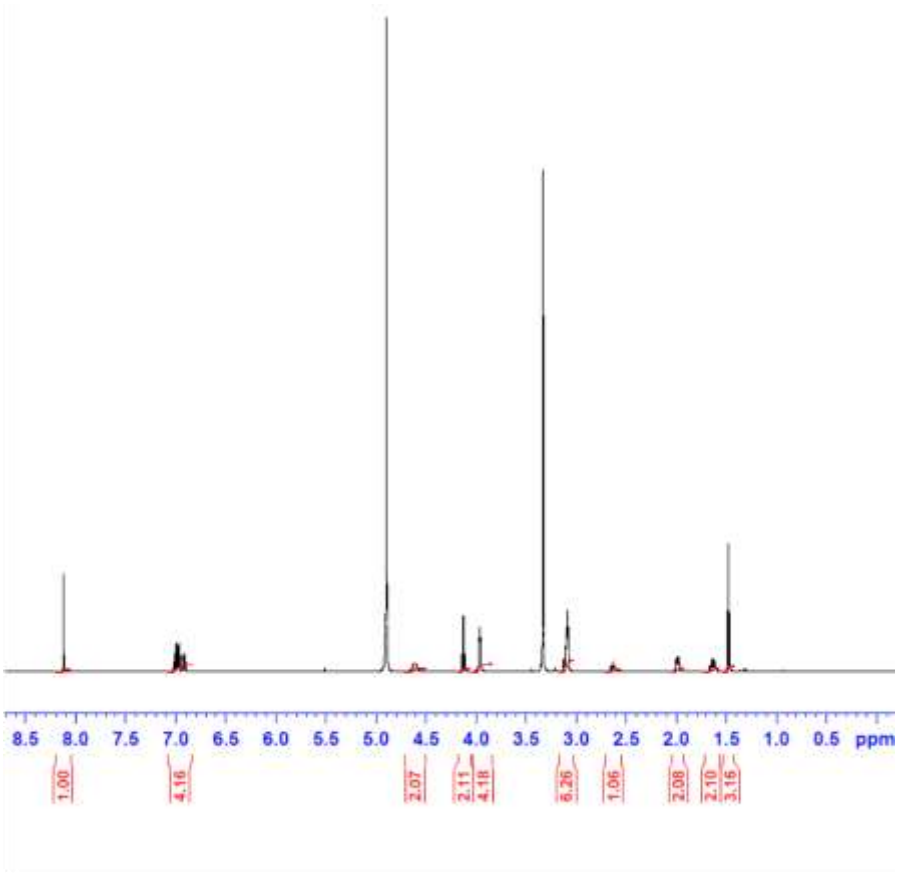


Figure S7. 1HNMR Spectrum of synthesized CDD-97

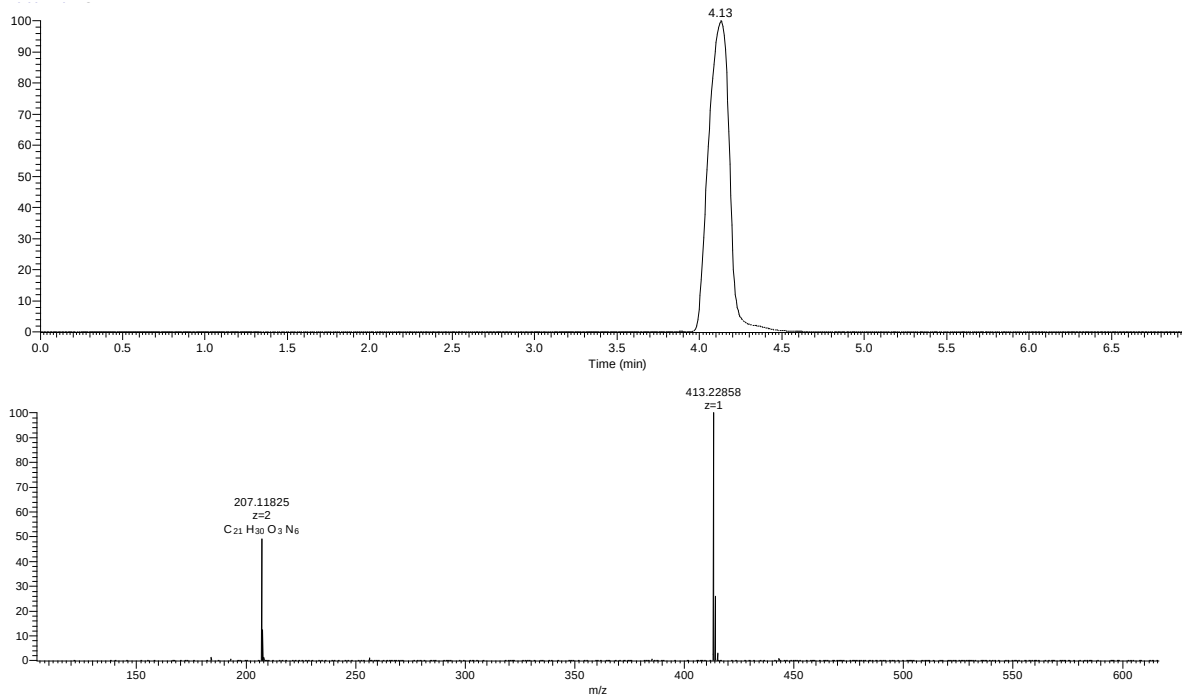


Figure S8. LC-MS/HRMS spectrum of synthesized CDD-97

D. References

1. Faver, J.C.; Riehle, K.; Lancia, D.R.; Milbank, J.B.; Kollmann, C.S.; Simmons, N.; Yu, Z.; Matzuk, M.M. *ACS Comb. Sci.* **2019**, *21*, 2, 75–82.