Supporting Information for

Discovery, SAR, and X-Ray Binding Mode Study of BCATm Inhibitors from a Novel DNA-Encoded Library

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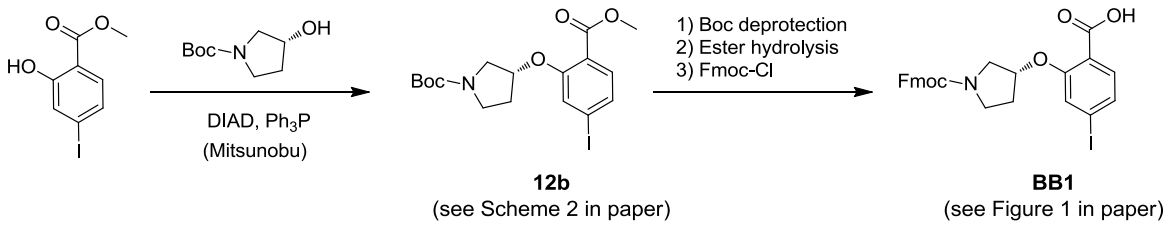
Contents:

Synthesis of DNA encoded library (the CIA Library)

Materials. Cycle 1 scaffolds were synthesized in house or via contract synthesis; the route used to synthesize **BB1** is summarized below (Scheme 1). All cycle 2 and 3 building blocks were obtained from commercial sources or were already available via the GlaxoSmithKline inventory.



Scheme 1. Synthetic Route for BB1.

Stock solutions of all the chemical building blocks were prepared prior to library synthesis in one of the following solvents: DMF, MeCN, 50:50 MeCN:water or DMA depending on solubility and optimized reaction conditions. DNA headpiece (5′-/5Phos/GAGTCA/iSp9/iUniAmM/iSp9/TGACTCCC-3′, Figure 1) and the various DNA tags were obtained from Biosearch Technologies, Novato, CA. T4 DNA Ligase was obtained from Fermentas (30 U/μL). The 10× ligation buffer stock used in ligation reactions was composed as follows: 500 mM Tris pH 7.5, 500 mM NaCl, 100 mM $MgCl_2$, 100 mM DTT, 25 mM ATP.

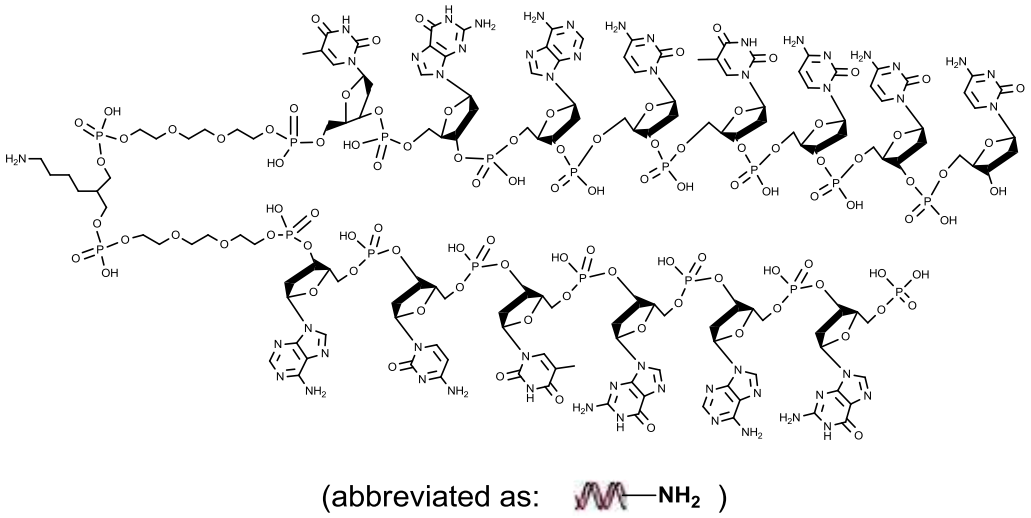
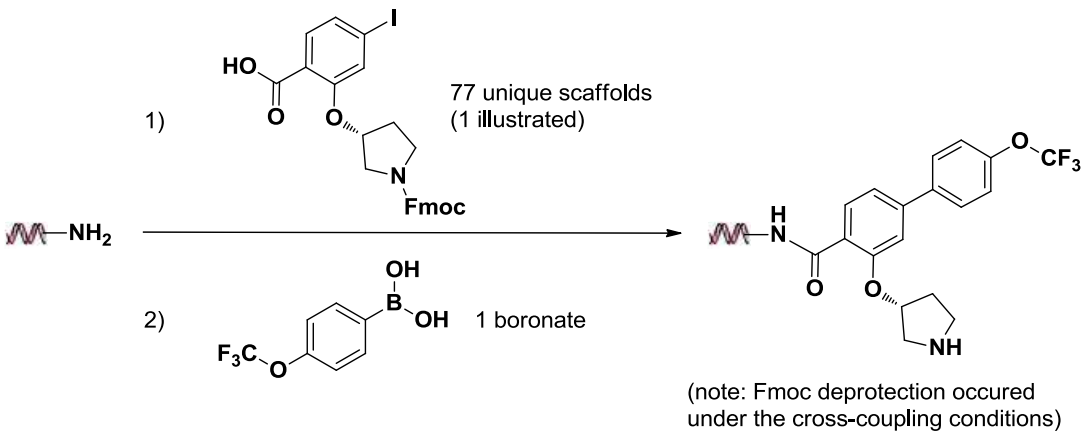


Figure 1. Headpiece, MW = 4937

Instrumentation. Analysis was performed using HPLC/ESI-MS; routine conditions provided herein. After a reaction or enzymatic ligation, an aliquot of the solution was diluted (typically a 1 μL aliquot diluted with 40 μL of water), injected onto a reverse-phase chromatography column (Targa C18, 5μ, 2.1 x 40 mm) and eluted (typically 15 – 70% or 10 – 90% solvent B in solvent A over 5 minutes, 0.5 mL/min flow rate; Solvent A: 0.75% v/v hexafluoroisopropanol (HFIP)/0.038% v/v triethylamine /5 μM EDTA in deionized water; Solvent B: 0.75% v/v HFIP/0.038% triethylamine /5 μM EDTA in 90/10 methanol/deionized water) with detection at UV 260 nm. Effluent was analyzed on an electrospray mass spectrometer in negative ion mode (Thermo Fisher Scientific LCQ Advantage or Bruker uTOF). When necessary, mass deconvolution was achieved using ProMass (Novatia) or MaxEnt (Bruker) software. Purification of library material was performed on a Gilson preparative HPLC system with a Phenomenex Gemini 5μ C18 100 × 21.20 mm 100A column. Typical elution system was 20-70% solvent B in solvent A over 22 minutes (Solvent A: 50 mM triethylammonium acetate, pH 7.5; Solvent B: 1% MeCN in water). Detection was at UV 260 nm.

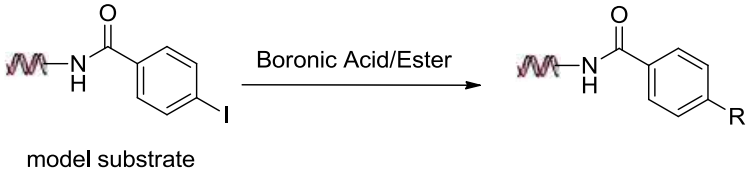
General Procedures. The standard procedure for ethanol precipitations following enzymatic tag ligation or small molecule reactions involved addition of 10% by volume 5M NaCl (*aq*), 200-250% by volume cold ethanol, and then cooling to -78 °C for ≥ 30 minutes or -20 °C for at least one hour; the precipitated material was then isolated as a pellet by centrifugation and subsequent removal of the supernatant. The concentration of all stock solutions containing headpiece were determined by measuring OD absorbance at 260 nm. For quality control of tag ligations, every well was analyzed via gel electrophoresis on 4% agarose gel plates (48 ligation wells per gel plus four extra wells per gel for molecular weight marker and starting material from previous cycle); in addition, two wells per plate (typically A1 and B2) were also analyzed by HPLC/ESI-MS. For quality control of chemical reactions, two wells per plate (typically A1 and B2) were analyzed by HPLC/ESI-MS and mass deconvolution was achieved using ProMass (Novatia) or MaxEnt (Bruker) software when necessary.

Building Block Selection. Building blocks were obtained as previously described and validated against model substrates prior to library production. Validations were conducted using library-like conditions (as described **Cycle 1**, **Cycle 2**, and **Cycle 3** sections below) with analysis via LCMS as described above. Cycle 1 scaffolds were validated in a 2-step protocol to test both the acylation protocol and Suzuki protocol (See Cycle 1 and Cycle 2 sections for conditions). Of the 77 scaffolds tested, 44 yielded an overall purity of 70% desired product and were selected for the library.



Scheme 2. Synthetic Scheme for Scaffold Validation.

Cycle 2 boronate building blocks were validated using the synthetic scheme outlined in Scheme 3 (using conditions outlined in Cycle 2 section). Boronates that gave 60% or better in desired product were selected for use in the library.



Scheme 3. Synthetic Scheme for Boronate Validation.

Cycle 3 building blocks were validated with their respective chemistries using the following model substrates (figure 2) and those passing at greater than 50-70% desired product were selected for use in the library.

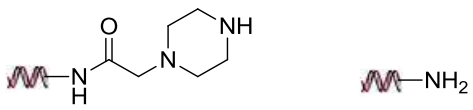


Figure 2. Model substrates used in Cycle 3 BB validations.

Installation of chemical spacer (Synthesis of AOP-Headpiece, Figure 3). A solution of headpiece DNA (90 μmol in 90 mL of 250 mM pH 9.4 sodium borate buffer) was treated with 40 equivalents of Fmoc-15-amino-4,7,10,13- tetraoxapentadecanoic acid ("AOP") (18 mL of a 200 mM DMA solution) followed by 40 equivalents of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, Acros) (18 mL of a 200 mM aqueous solution). The acylation reaction was allowed to proceed for 16 hours at room temperature. After completion, Fmoc deprotection was attained by addition of 10% v/v of neat piperidine, allowing the reaction to proceed for 30 minutes at room temperature. The deprotected product was precipitated by addition of 5M NaCl (10% by volume) and 3 volumes of cold ethanol, followed by centrifugation. A second precipitation was conducted by diluting the material with 10 mL water, 1 mL 5M NaCl and 64 mL cold ethanol. The isolated pellet was reconstituted in 100 mL water and subjected to centrifugation. The supernatant was collected and lyophilized. The lyophilized pellet was then dissolved in 86.9 mL water to form a 1.0 mM solution of AOP-headpiece (86.9 μmol; 96% yield, concentration determined by OD) and carried on to ligation without any further purification.

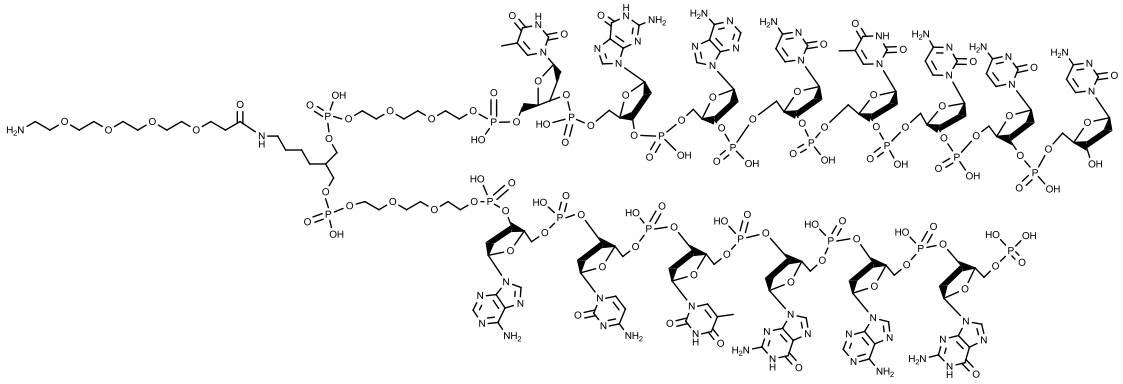


Figure 3. AOP-Headpiece, MW = 5184.48.

Annealing Primer Tags. To a tube containing 50 mL of the top strand primer (100 μmol, 2 mM in water) was added 50 mL of the bottom strand primer (1 equiv., 100 μmol, 2.0 mM in water). The solution was heated to 90 °C for 5 min, then cooled to room temperature.

Sequence of primer tags:

5’ AAATCGATGTG 3′

3′ GGTTTAGCTAC 5′

Tags. The DNA tags contained a 9 bp coding region, flanked by two 2-base 3′ overhangs: All 5′-ends were phosphorylated.

Cycle 1	Cycle 2
5′ XXXXXXXXXAG	XXXXXXXXXGT
3′ ACXXXXXXXXX	TCXXXXXXXXX

Cycle 3	Cycle 4
5′ XXXXXXXXXGA	XXXXXXXXXTT
3′ CAXXXXXXXXX	CTXXXXXXXXX

Cycle 1. To a 1 mM solution of the AOP headpiece (86.8 μmol, 86.8 mL water) was added 34.7 mL of 10× ligation buffer, 3.47 mL T4 DNA ligase, and 48.6 mL water. The resulting solution was equally split into 44 tubes. To each tube was added 2.17 mL of 1 mM annealed primer tag and an aliquot of 1 of 44 unique cycle 1 tag solutions (2.17 mL of 1 mM stock solutions in water). Ligation allowed to proceed at room temperature for 16 h. Gel analysis of each tube was

done to confirm successful ligation (data not shown) in addition to LCMS. After ligation, 5 M NaCl (10% by volume, 832 µL) and 3 volumes of cold ethanol (30.8 mL) were added to each tube to precipitate the DNA. Following centrifugation, each pellet was isolated and reconstituted in 1.97 mL 250 mM pH 9.4 sodium borate buffer. A stock solution of cycle 1 acid scaffold (740 uL of 200 mM DMA stock solution) was mixed with 740 uL of HOAt/DIC (prepared 1:1 by volume from 400 mM DMA stock solutions). The resulting reaction mixture was allowed to sit at RT for 5 minutes and then transferred to the solubilized DNA pellet. LCMS data suggested 37 of the 44 acylation reactions proceeded to at least 70% conversion to desired product. The remaining 7 were subjected to ethanol precipitation and the acylation reactions were repeated. Final cycle 1 material was pooled and ethanol precipitated. The lyophilized pellet was redissolved in 30 mL of water and subjected to centrifugation to pelletize residual small molecule contaminants. Supernatant was then filtered through a 0.45 µm syringe filter to remove residual sediment. The final material was diluted to 50 mL total volume and then subjected to Millipore Amicon Ultra-15 Centrifugal Filter Units (3000-MW cutoff). Recovered concentrated material from filter and subjected to ethanol precipitation. The lyophilized pellet was then deprotected by exposure to 15 mL of 10% piperidine in water for ~1h. The deprotected product was precipitated and centrifuged as previously described. The pellet was repeatedly reconstituted in water and re-lyophilized to remove trace piperidine, yielding ~ 63.6 µmols as determined by OD. Final material diluted with 20 mL water and subjected to centrifugation to remove residual precipitate. Recovered supernatant diluted to 1 mM in water.

Cycle 2. Cycle 2 incorporated 267 boronates and 1 encoded “null” (only solvents/reagents added, no building block) but 2 boronates were later removed due to ligation failure resulting in

265 unique boronates. Due to volume constraints, the cycle 1 product (1 mM in water, 63.6 μmols) was dispensed to into a total of 6 plates (96-well plates, 536 total wells, 125 uL/well). Chemistry was carried out on 3 of the 6 plates and then repeated for the remaining 3 plates. To the well containing oligo was added 40 equiv. of the appropriate boronate (8.33 uL of a 600 mM DMA stock solution), 80 equiv. of Na_2CO_3 (16.67 μL of a 600 mM aqueous stock solution), and 0.5 equiv. $Pd(PPh_3)_4$ (20.83 μL of a 3 mM MeCN stock solution, degassed by sonication). The reactions were heated at 80 °C for 5 h. Reaction material was then transferred to larger volume capacity plates (rinsing source plate with 2×100 uL water). Resulting solutions were ethanol precipitated twice as described previously. Resulting pellets were reconstituted in 125 uL water. To this was added 50 uL of 10× ligation buffer, 5 uL T4 DNA ligase, and 70 uL of water. An aliquot of the 1 of 536 tag solutions (150 uL of 1.0 mM stock solutions in water, 1.2 equivs) was added to each well, and ligation was allowed to proceed at room temperature overnight. Ligations were deemed complete for majority of wells by gel analysis of each well (data not shown) in addition to spot checking by LCMS. Two wells failed ligation and were removed. All other wells were pooled resulting in total volume of 497 mL. To this was added 23 mL neat piperidine (4.8% v/v), mixed, then added 400 mg of solid diethyldithiocarbamate scavenger (563.25 μmols, 8.4 equiv.). Contents were mixed and then heated to 75 °C for 1h. Resulting material was ethanol precipitated twice as previously described. The resulting pellet was dissolved in 50 mL water. Precipitate was filtered and resultant filtrate was subjected to spin filtration (Millipore Amicon Ultra-15 Centrifugal Filter Units, 3000-MW cutoff). Due to the clogging of the filters, material was subjected to organic separation (2×60 mL EtOAc) and saving the aqueous layer. Organic layers washed with 3×60 mL water. Combined aqueous

layers subjected to ethanol precipitation followed by spin filtration using Millipore Amicon Ultra-15 Centrifugal Filter Units, 10,000-MW cutoff.

Cycle 3. Cycle 3 chemistry involved the incorporation of 2977 amine-capping building blocks (acylation with 1887 carboxylic acids, reductive alkylation with 636 aldehydes, sulfonylation with 173 sulfonyl chlorides, urea formation with 53 isocyanates, and S_NAr with 228 heteroaromatic chlorides). For 3 of the reaction sets, an encoded "null" was included (addition of reagents but no addition of building block), leading to the total split size of 2980. The cycle 2 product (41.28 μmols) was dissolved in 41.28 mL water and split into 3077 wells (13 μL/well) using 96-well plates. To each well was then added 7.8 μL of prepared ligation buffer/T4 ligase solution (prepared by mixing 16 mL 10× ligation buffer, 1.6 mL T4 DNA Ligase, and 6.4 mL water). To this was added 15.6 μL of cycle 3 tag and 15.6 uL of cycle 4 tag (combined C3-C4 tag creates a unique DNA tag). The ligations were allowed to proceed at room temperature for 16 h. Ligations analyzed by gel analysis and several wells spot-checked by LCMS. The DNA was ethanol precipitated as previously described. Two ligation pellets were lost during precipitation process. The DNA pellets recovered were then carried into cycle 3 amine-capping chemistry as described below. (Note: all 3077 wells were used for ligation, but only 2980 were used for subsequent chemistry; the 97 wells that were not used for chemistry were discarded prior to pooling.) 636 wells were set aside to undergo reductive alkylation. Each pellet was reconstituted in 13 μL of 250 mM pH 5.5 sodium phosphate buffer to which was added 80 equiv. of the appropriate aldehyde (5.2 μL of a 200 mM DMA stock solution) and 80 equiv. of $NaCNBH_3$ (2.6 μL of a 400 mM DMF stock solution). The reactions were sealed and heated to 60 °C for 16 h. Individual wells were spot-checked by LCMS to assess reaction yields which suggested that the reactions had proceeded. DNA was then ethanol precipitated and recovered

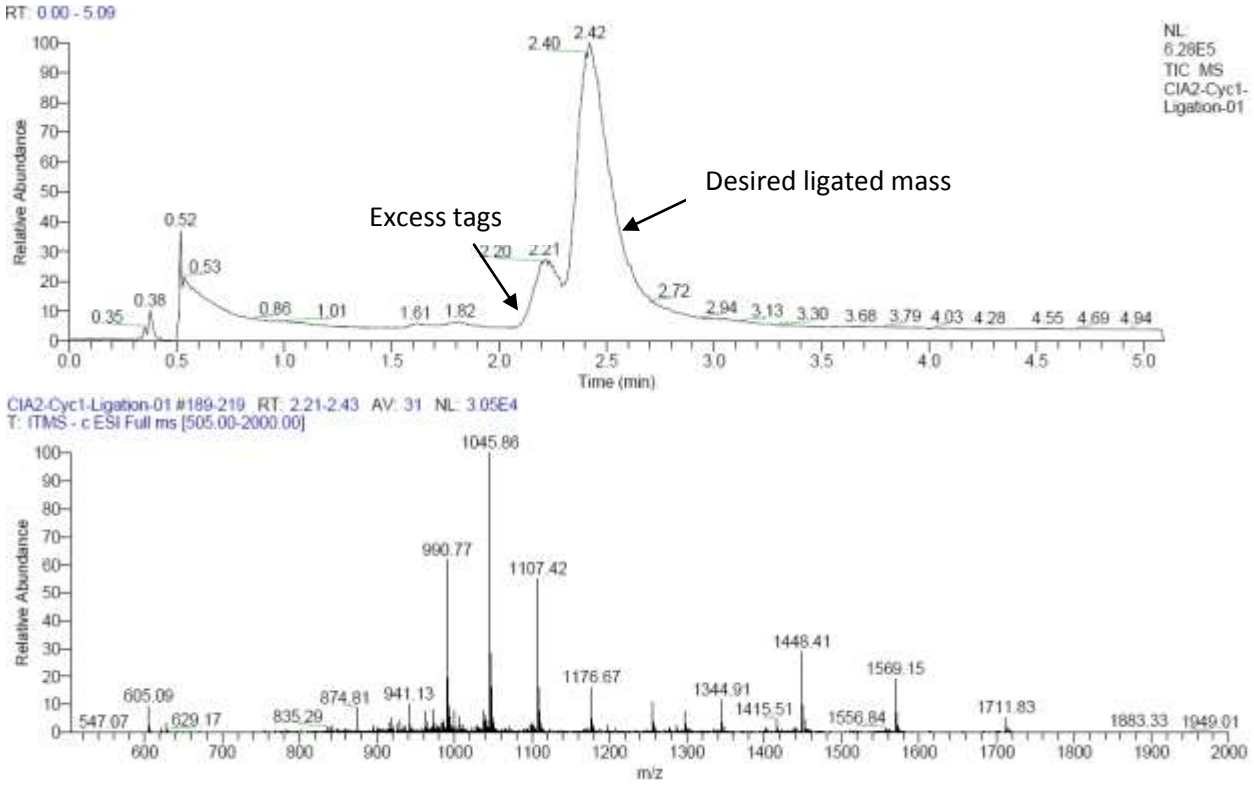
pellets were reconstituted in 20 μL water. Material was then placed in a -20 °C freezer for storage prior to final pooling step. 229 wells were set aside to undergo S_NAr with heteroaromatic chlorides (including one null). Each pellet was reconstituted in 13 μL of 250 mM pH 9.4 sodium borate buffer to which was added 50 equiv. of the appropriate heteroaromatic chloride (3.25 μL of a 200 mM DMA stock solution). The reactions were sealed and heated to 90 °C for 18 h. Individual wells were spot-checked by LCMS to assess reaction yields which suggested that the reactions had proceeded. DNA was then ethanol precipitated and recovered pellets were reconstituted in 20 μL water. Material was then placed in a -20 °C freezer for storage prior to the final pooling step. 226 wells were set aside to undergo sulfonamide formation and urea formation (173 sulfonyl chlorides and 53 isocyanates). Each pellet was reconstituted in 13 μL of 250 mM pH 9.4 sodium borate buffer to which was added 40 equiv. of the appropriate sulfonyl chloride or isocyanate (1.04 μLs of a 500 mM acetonitrile stock solution). The reactions were mixed by pipette and allowed to sit at room temperature overnight. Individual wells were spot-checked by LCMS to assess reaction yields which suggested that the reactions had proceeded. DNA was then ethanol precipitated and recovered pellets were reconstituted in 20 μL water. Material was then placed in a -20 °C freezer for storage prior to final pooling step. 992 wells were set aside to undergo amide formation via DMTMM activating agent. Each pellet was reconstituted in 13 μL of 250 mM pH 9.4 sodium borate buffer to which was added 40 equiv. of the appropriate carboxylic acid (2.6 μLs of a 200 mM DMA stock solution) and 40 equiv. of DMTMM (2.6 μLs of a 200 mM aqueous stock solution). The reactions were mixed by pipette and allowed to sit at room temperature for 19 h. A second addition of carboxylic acid and DMTMM was then done and the reaction was allowed to sit at room temperature for an additional 6 h. Individual wells were spot-checked by LCMS to assess reaction yields which

suggested that the reactions had proceeded. DNA was then ethanol precipitated and recovered pellets were reconstituted in 20 μL water. Material was then placed in a -20 °C freezer for storage prior to final pooling step. 658 wells were set aside to undergo amide formation via HOAt/DIC activating agent (including one "null"). Each pellet was reconstituted in 13 μL of 250 mM pH 9.4 sodium borate buffer. A stock solution of carboxylic acid (5 μL of 200 mM DMA stock solution) was mixed with 5 μL of HOAt/DIC (prepared 1:1 by volume from 400 mM DMA stock solutions). Resulting mixture was allowed to sit for 5 minutes and then 5.2 μL of this "premix" stock was delivered to the solubilized DNA pellet. A second addition of the carboxylic acid (from a freshly prepared "premix" stock) was repeated after 2 h. The reactions were mixed by pipette and allowed to sit at room temperature overnight. Individual wells were spot-checked by LCMS to assess reaction yields which suggested that the reactions had not proceeded. DNA was then ethanol precipitated and recovered pellets were reconstituted in 13 μL of 250 mM pH 9.4 sodium borate buffer. The reaction was then repeated as previously described. Wells were rechecked by LCMS which indicated some progression to desired product. DNA was then ethanol precipitated and recovered pellets were reconstituted in 20 μL water. Material was then placed in a -20 °C freezer for storage prior to final pooling step. 238 wells were set aside to undergo amide formation via HOAt/DIC activating agent followed by a BOC-deprotection (including one "null"). Each pellet was reconstituted in 13 μL of 250 mM pH 9.4 sodium borate buffer. A stock solution of Boc-protected amino acid (5 μL of 200 mM DMA stock solution) was mixed with 5 uL of HOAt/DIC (prepared 1:1 by volume from 400 mM DMA stock solutions). The resulting mixture was allowed to sit for 5 minutes and then 5.2 μL of this "premix" stock was delivered to the solubilized DNA pellet. A second addition of the carboxylic acid (from a freshly prepared "premix" stock) was repeated after 2 hours. The reactions were

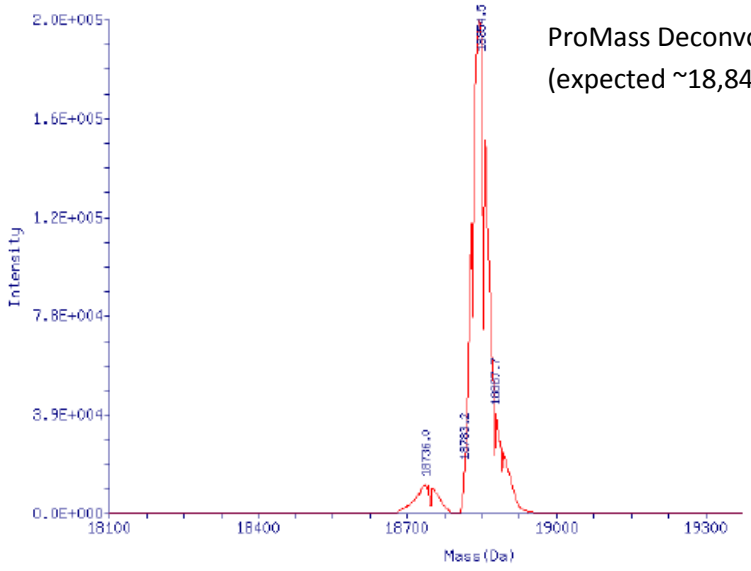
mixed by pipette and allowed to sit at room temperature overnight. Individual wells were spot-checked by LCMS to assess reaction yields which suggested that the reactions had proceeded. DNA was then ethanol precipitated and recovered pellets were reconstituted in 55 μL 250 mM pH 9.4 sodium borate buffer. Boc deprotection was afforded by heating to 90 °C for 18 hours. DNA was then cooled to room temperature and pooled.

All library samples were thawed and pooled together. Samples were then subjected to ethanol precipitation. Final material was LC-purified, yielding 14.3 μmols as determined by OD, and was then ligated with closing primer as indicated below before affinity-based selections.

Characterization of Library Synthesis (Chemistry and DNA Tag Ligation). Analysis of the library was conducted as previously described using both LCMS and GEL techniques. Data below is shown as a representative data set for each cycle. Discrete masses are observed for cycle 1 only prior to pooling. Further cycles contain a range of masses due to pooling of previous cycle material. In Cycles 2 and 3, analysis is done by looking for a bulk mass shift rather than discrete expected mass.

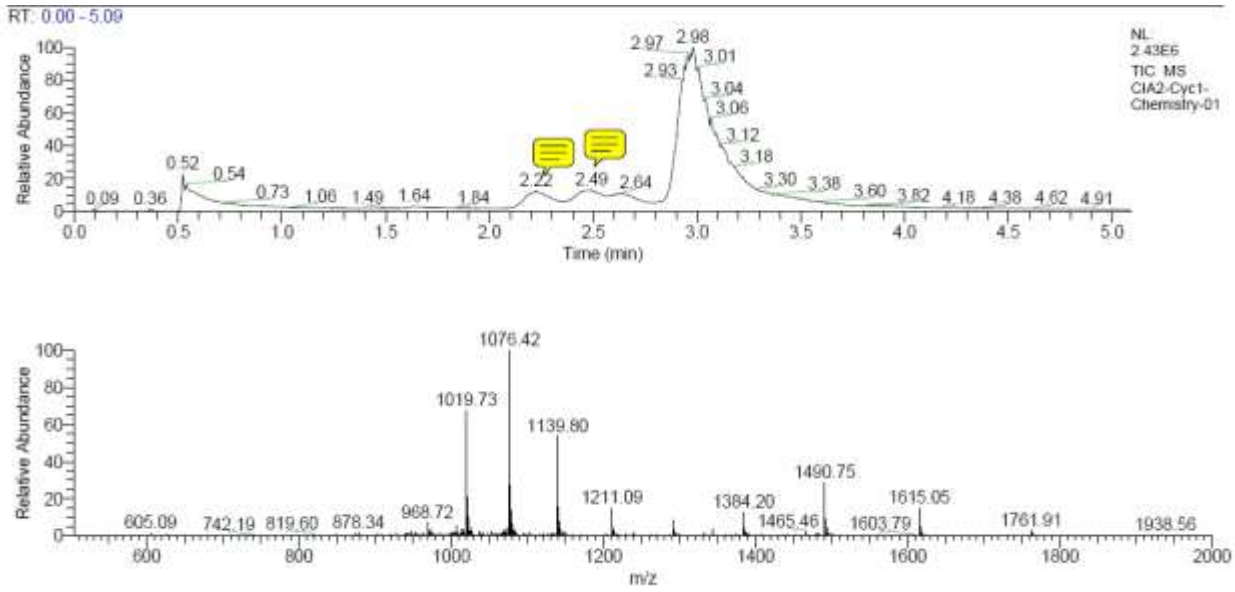


Zoom Display Deconvoluted Mass Spectrum:

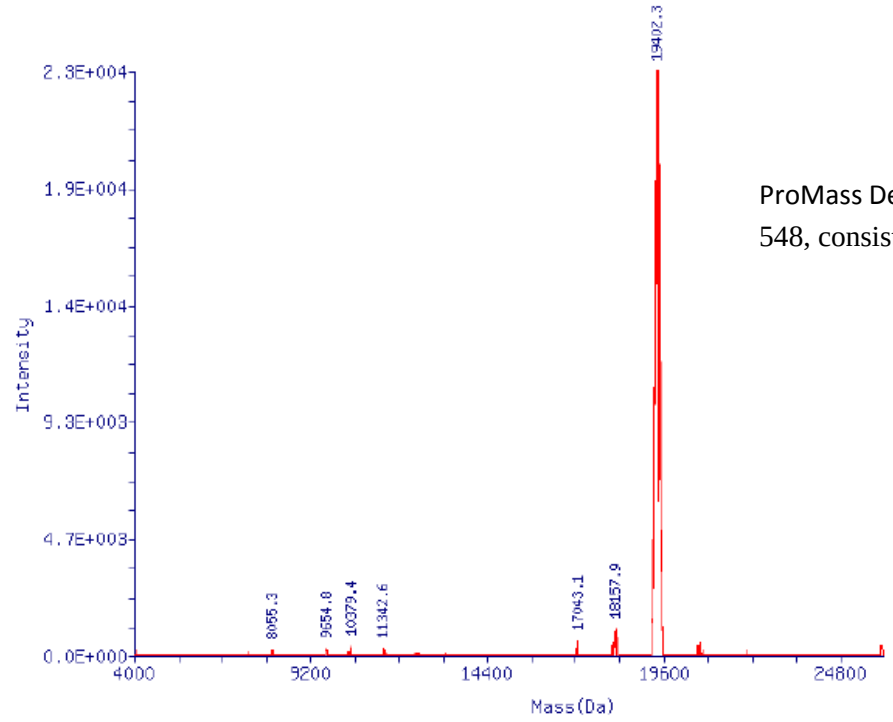


ProMass Deconvolution = 18,854 Da
(expected ~18,841)

Figure 4. LCMS analysis and deconvolution of ligation step for cycle 1-tube 1. Starting material MW = 5184. Expected ligated product MW = 18,841.



Deconvoluted Mass Spectrum:



ProMass Deconvolution = 19,402 Da (Δ = 548, consistent with scaffold mass)

Figure 5. LCMS analysis and deconvolution of chemistry step for cycle 1-tube 1. Expected mass shift observed for BB-1.

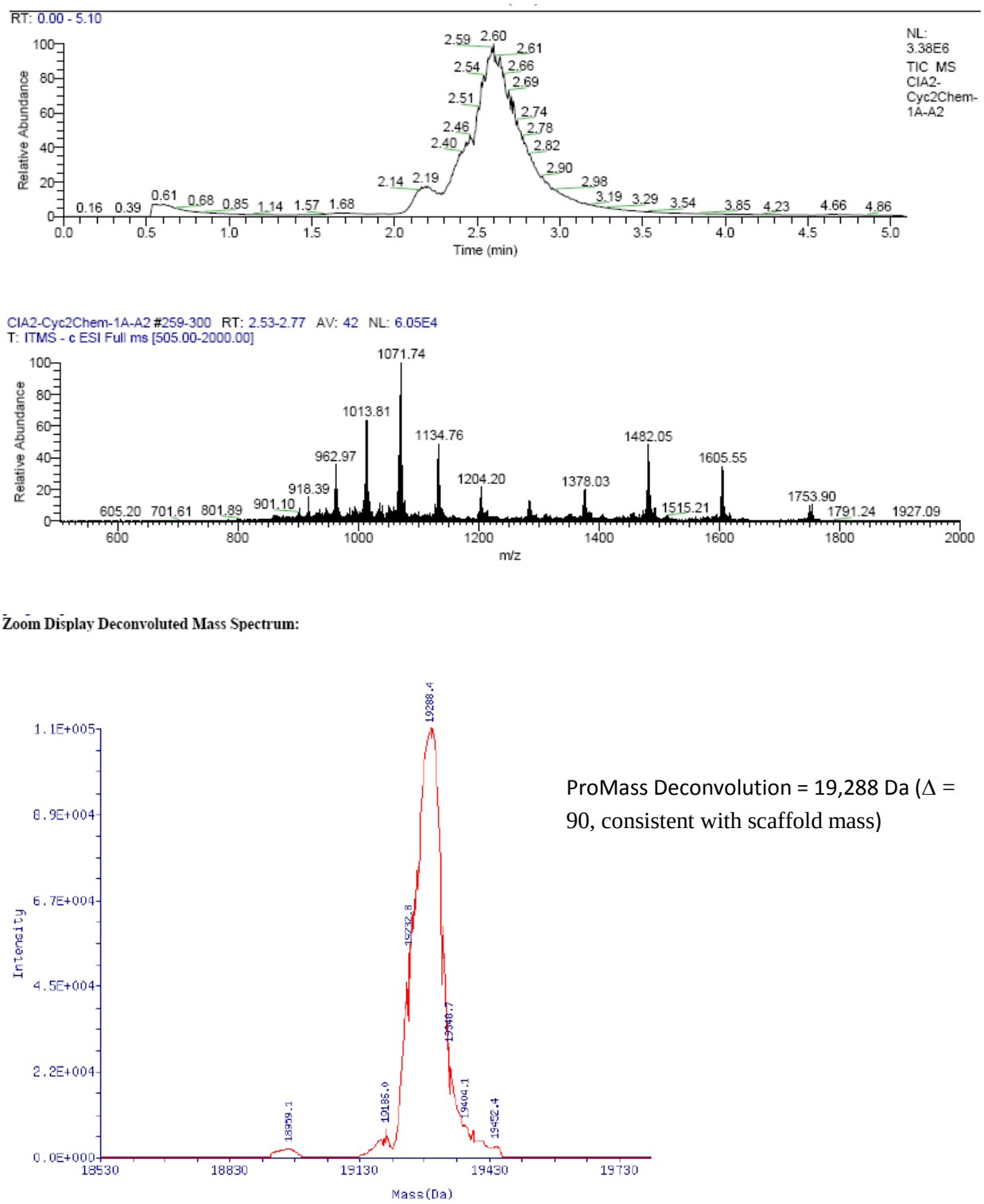


Figure 6. LCMS analysis and deconvolution of chemistry step for cycle 2 (well 01-A1). Expected mass shift observed for BB-2.

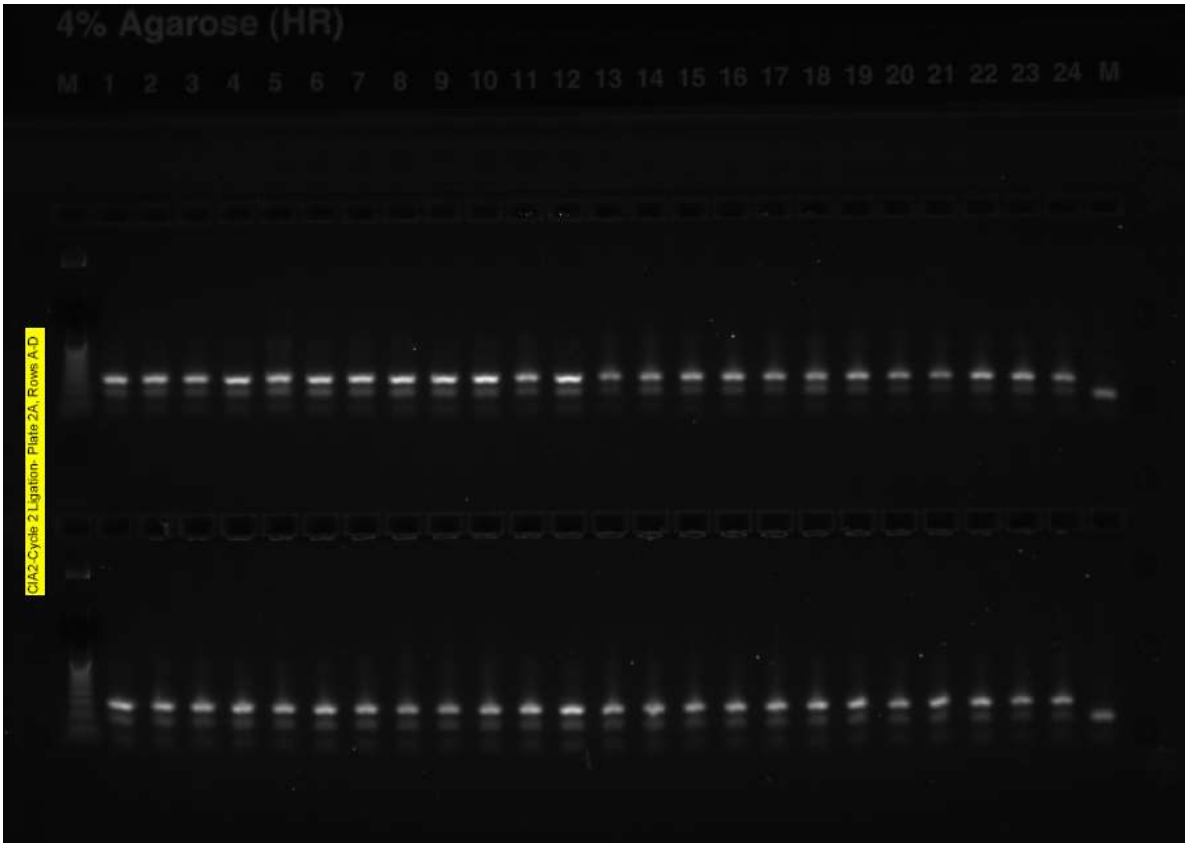


Figure 7. Gel electrophoresis of plate 2, rows A-D, cycle 2 ligation. Far left column contains MW marker and starting material in the far right lane for comparative purposes.

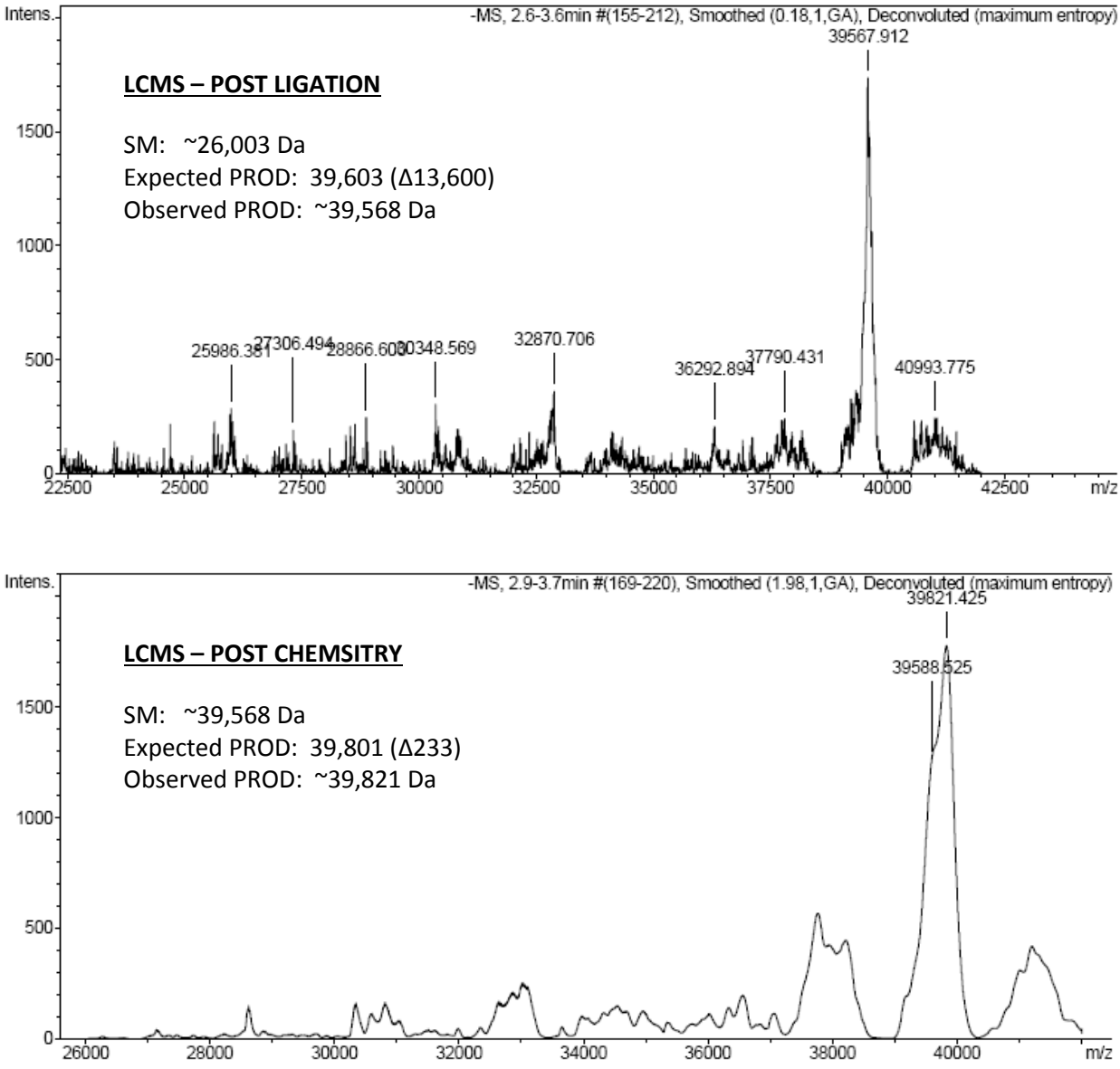


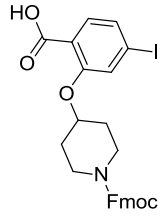
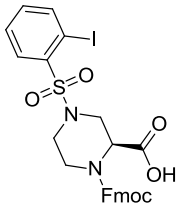
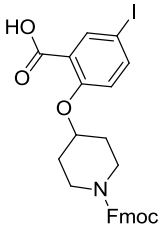
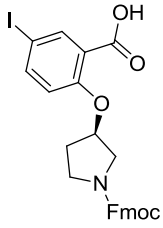
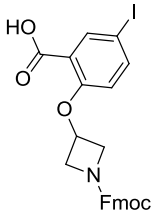
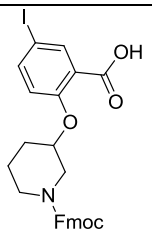
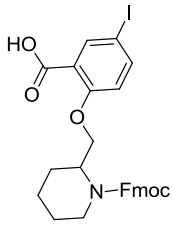
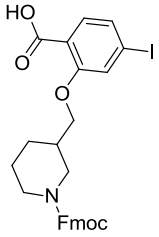
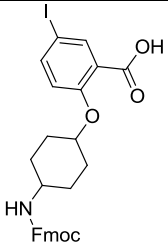
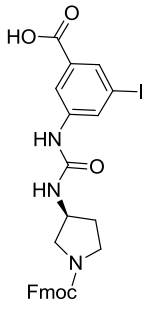
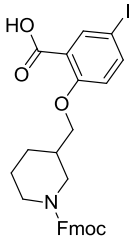
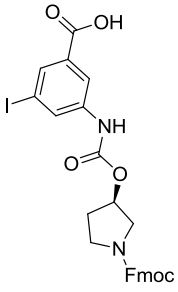
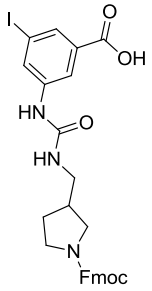
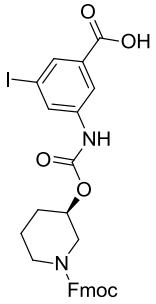
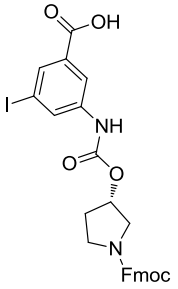
Figure 8. Deconvoluted LCMS of cycle 3 ligation and chemistry for plate 2 (Boc protected amino acid set), well B2 (top = ligation; bottom = chemistry). Observed mass shift from ligation QC to chemistry QC = 253 Da; expected shift of 233.

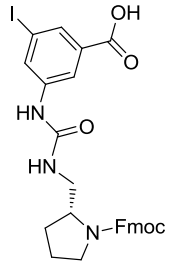
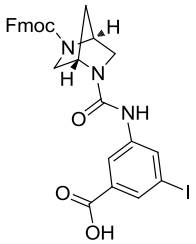
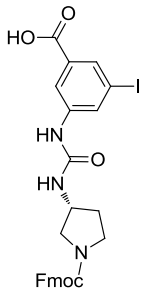
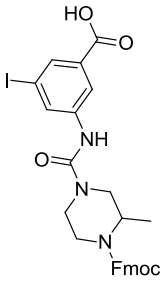
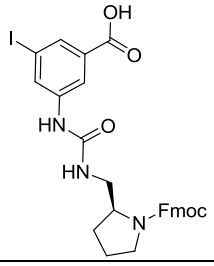
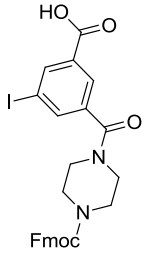
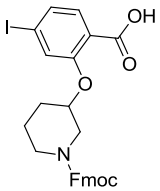
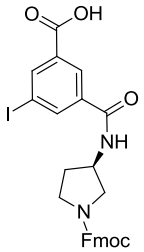
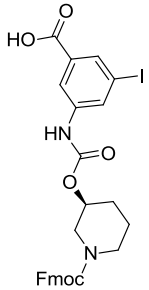
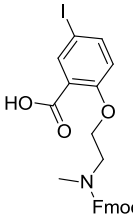
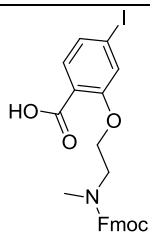
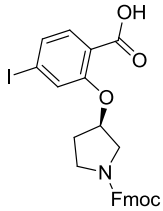
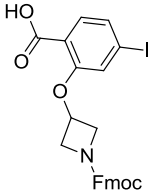
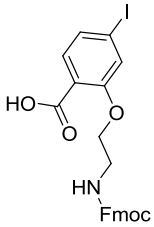
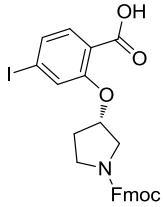
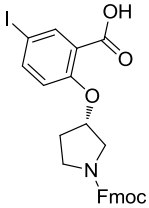
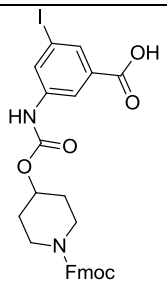
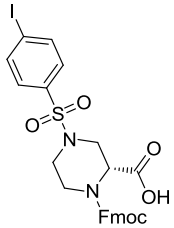
Closing Primer Ligation. Prior to selection, 100 nmol aliquots of the library were ligated with six different closing DNA primers containing a 12-nucleotide duplex and a single stranded degenerate region. "Closing" ligation was done using standard ligation protocol at 16 ^{o}C overnight. The degenerate region was filled in with mixture of dATP, dCTP, dGTP and dTTP (1:1 premixed solution of dNTPs, each at 10 mM in water). For this purpose, the library with closing primer ligated on was incubated at 37 $^{\circ}$C for 30 min in ligation buffer (50 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT, 2 mM ATP) supplemented with 10 mM dNTP (Roche, Catalog # 11 581 295 001) and DNA polymerase I Klenow Fragment (3'-5'exo$^-$; from New England BioLabs, Catalog # M0212S). Reaction completion was confirmed by analysis on 4% agarose gel. After the closing procedure was complete, the library was precipitated with 2.5 volumes of 100% cold ethanol and 1/10 volume of 3M Ammonium Acetate (pH4.5) to remove excess of dNTPs and unligated primers. The precipitated library was reconstituted in water to a final concentration of 0.5 mM, which was ready for affinity selections. The generic sequence of the closing primer was:

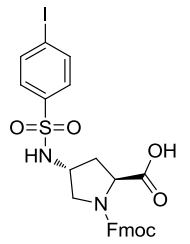
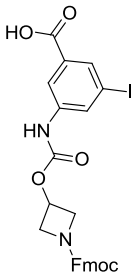
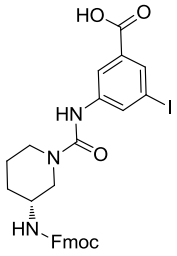
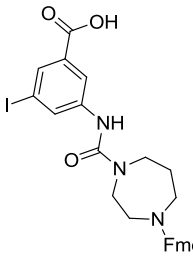
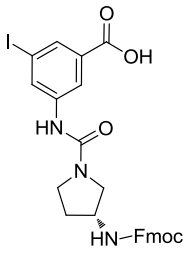
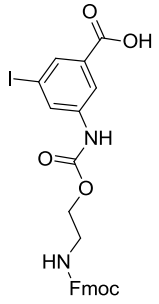
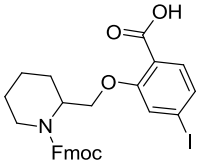
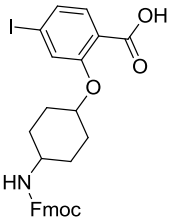
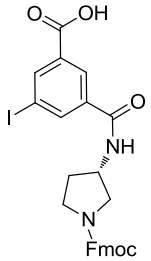
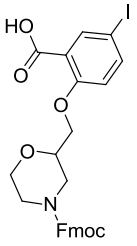
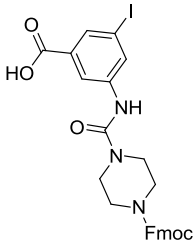
5′ XXXXXXXXXX 3′
3′ AAXXXXXXXXXXNNNNNNNNNNNNGTCTGTTCGAAGTGGACG 5′

The variable region X was unique to each closing primer and served as an identifier for the particular library and selection experiment. The variable N region was a randomized sequence used to identify PCR duplication in the sequence data.

The structures of the 44 scaffolds (BB1) used in the library synthesis:

Cpd #	Structure	Cpd #	Structure	Cpd #	Structure
1		2		3	
4		5		6	
7		8		9	
10		11		12	
13		14		15	

16		17		18	
19		20		21	
22		23		24	
25		26		27	
28		29		30	
31		32		33	

34		35		36	
37		38		39	
40		41		42	
43		44			

Affinity Screening of Encoded Library. Five μg of chemically biotinylated BCATm protein was immobilized on a 200 μL Phynexus affinity column packed with 5 μL agarose streptavidin resin by pipeting 100 μL of 0.05 μg/mL protein solution up and down for 20 minutes. The column was then washed 5× by aspirating and expelling 100 μL of selection buffer (50 mM HEPES, pH 7.5; 50 mM NaCl; 1 mM CHAPS; 1.0 mg/mL sheared salmon sperm DNA (sssDNA, Ambion)) plus 1 mM free biotin to occupy all biotin binding sites. Five nmol of library in selection buffer was incubated on the column for one hour, then the column was

washed 10 times by aspirating and expelling 100 μL of selection buffer to remove non-binders. Bound molecules were eluted by heat denaturing the protein by incubating the column with 100 μL of selection buffer at 80 ºC for 10 minutes. Eluant was then incubated with fresh immobilized protein to start a second round of affinity selection. Three rounds of selection were performed. To exclude molecules bound to the resin matrix, a parallel selection was done in identical fashion on streptavidin resin alone as a no target control (NTC). For DNA sequencing, the final eluant containing 1e9 molecules was amplified by PCR for 17 cycles then sequenced using Illumina high throughput sequencing technology.

Synthesis of the off-DNA compounds

All solvents and reagents were used as obtained. ^{1}H NMR spectra were recorded on a Varian Mercury 400 plus. Chemical shifts are expressed in parts per million (ppm, δ units). Coupling constants (*J*) are in units of hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), t (triplet), q (quartet), dd (double doublet), dt (double triplet), m (multiplet), br (broad). Analytical purity was ≥ 95% unless stated otherwise. The purity of final compounds was checked using an Agilent 1100 HPLC system coupled with a Thermo Finnigan LCQ Mass Spectrometer. All mass spectra were performed by electrospray ionization (ESI). Two different HPLC conditions were used to analyze compound purity: LC-MS method A: 10-98% AcCN-H_2O (0.1%TFA) in 2.7 min, stay at 98% AcCN for 0.38 min, with the flow rate of 0.9 mL/min on a Phenomenex Luna 3μ C8(2) 100A 30 × 3.00 mm 3 column. LC-MS method B: 10-95% AcCN-H_2O (0.1% formic acid) in 3.0 min, with the flow rate of 0.5 mL/min on a Kinetex 2.6 μ C18 100A 30 × 2.10 mm column. High resolution mass

spectrometry (HRMS) was completed on a Waters qTOF Premiere mass spectrometer operating in W mode positive ionization with a resolving power of approximately 15000. Flow injection was completed using a Waters Nanoacquity LC. HRMS acceptable error is 3 mDa or 5 ppm, although most analyses are observed within 0.5 mDa with isotope fits in good agreement with the proposed structures. Purification of final compounds for biological testing was performed on a Gilson GX-281 system with a Phenomenex Luna 5μ C8(2) 100 × 21.20 mm 100A column running gradient of 5-95% MeCN/H_2O (+0.1% TFA or 0.1% formic acid) over 15-20 minutes with flow rate of 22 mL/min.

***tert*-Butyl 3-(5-iodo-2-(methoxycarbonyl)phenoxy)pyrrolidine-1-carboxylate (12a).** To a solution of methyl 2-hydroxy-4-iodobenzoate (**11**, 0.834 g, 3.0 mmol) and *tert*-butyl 3-hydroxypyrrolidine-1-carboxylate (0.562 g, 3.00 mmol) in THF (3 mL) was added at room temperature diisopropyl azodicarboxylate (0.642 mL, 3.30 mmol) and triphenylphosphine (0.866 g, 3.30 mmol). The resulted mixture was stirred for 12 h then concentrated under vacuum. The residue was chromotagraphed with a silica gel column (80 g) eluted with 0-50% EtOAc/hexane gradient over 22 min to afford the title compound in 90% yield (1.21g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 (d, J = 7.8 Hz, 1H), 7.38 (m, 1H), 7.26 (d, J = 1.2 Hz, 1H), 4.92 (br, 1H), 3.85 (s, 3H), 4.64-3.53 (m, 4H), 2.22 (br, 1H), 2.11 (br, 1H).

***(R)-tert*-Butyl 3-(5-iodo-2-(methoxycarbonyl)phenoxy)pyrrolidine-1-carboxylate (12b).** Using (*R*)-tert-butyl 3-hydroxypyrrolidine-1-carboxylate as starting material, the tiltle compound was prepared analogously to **12a** in quantitative yield (1.34 g). LC-MS (ESI) *m/z* $[M+1]^+$ = 447.56; Rt = 2.48 min (LC-MS method A).

***tert*-Butyl 3-(5-iodo-2-(methylcarbamoyl)phenoxy)pyrrolidine-1-carboxylate (13a).** To a solution of **12a** (1.21 g, 2.71 mmol) in THF-methanol (4 mL:4 mL), 2M NaOH solution (3.69

mL, 7.38 mmol) was added. The reaction was stirred at 25 °C for 2 h then was concentrated under vacuum. The residue was treated with methanamine (HCl salt, 256 mg, 3.79 mmol) in acetonitrile (15 mL) followed by HATU (1.237 g, 3.25 mmol) and DIEA (1.131 mL, 6.50 mmol). The reaction was stirred for 2 h then concentrated, the residue was chromotagraphed with a silic gel column eluted with 0-100% EtOAc/hexanes gradient over 20 min to afford the title compound in 36% yield (430 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 446.69, Rt = 2.29 min (LC-MS method A).

(*R*)-*tert*-Butyl 3-(5-iodo-2-(methylcarbamoyl)phenoxy)pyrrolidine-1-carboxylate (13b). Using **12b** as starting material, the title compound was prepared analogously to **13a** in quantitative yield. LC-MS (ESI) *m/z* $[M+1]^+$ = 446.62, Rt = 2.21 min (LC-MS method A).

***tert*-Butyl 3-((3'-(methoxycarbonyl)-4-(methylcarbamoyl)-[1,1'-biphenyl]-3-yl)oxy)pyrrolidine-1-carboxylate (14a, R_1 = 3-COOMe).** The suspension of **13a** (656 mg, 1.470 mmol), (3-(methoxycarbonyl)phenyl)boronic acid (291 mg, 1.617 mmol), cesium carbonate (958 mg, 2.94 mmol), and $Pd(PPh_3)_4$ (85 mg, 0.073 mmol) in THF (5 mL) and water (1 mL) was stirred under nitrogen at 70 °C for 12 h. The reaction was diluted with water and extracted with dichlormethane. The organic layer was dried over Na_2SO_4, concentrated then the residue was chromotagraphed to afford the title compound **14a** in 81% yield (540 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 454.75, Rt = 2.28 min (LC-MS method A).

***tert*-Butyl 3-((4-(methylcarbamoyl)-2'-(methylsulfonamido)-[1,1'-biphenyl]-3-yl)oxy)pyrrolidine-1-carboxylate (14ba, R_1= 2-$NHSO_2Me$).** Using **13a** (89 mg, 0.20 mmol) and N-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methanesulfonamide (65 mg, 0.22 mmol) as starting materials, the title compound was prepared analogously to **14a** in quantitative yield. LC-MS (ESI) *m/z* $[M+1]^+$ = 489.66; Rt = 2.07 min (LC-MS method A).

(*R*)-tert-Butyl 3-((4-(methylcarbamoyl)-2'-(methylsulfonamido)-[1,1'-biphenyl]-3-yl)oxy)pyrrolidine-1-carboxylate (14bb, R_1= 2-$NHSO_2Me$). Using **13b** as starting material, the title compound was prepared analogously to **14ba** in 51% yield (227 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 489.65; Rt = 2.05 min (LC-MS method A).

tert-Butyl 3-((2'-methyl-4-(methylcarbamoyl)-[1,1'-biphenyl]-3-yl)oxy)pyrrolidine-1-carboxylate (14c, R_1=2-Me). Using **13a** (220 mg, 0.49 mmol) and *o*-tolylboronic acid (67.0 mg, 0.49 mmol) as starting material the title compound was prepared analogously to **14a** in 58% yield (118 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 410.73; Rt = 2.39 min (LC-MS method A).

***tert*-Butyl 3-((4-(methylcarbamoyl)-[1,1'-biphenyl]-3-yl)oxy)pyrrolidine-1-carboxylate (14d, R_1 = H).** Using **13a** (220 mg, 0.49 mmol) and phenylboronic acid (60.1 mg, 0.49 mmol) as starting material the title compound was prepared analogously to **14a** in 47% yield (92 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 396.75; Rt = 2.30 min (LC-MS method A).

3'-((1-(5-bromothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-4'-(methylcarbamoyl)-[1,1'-biphenyl]-3-carboxylic acid (15c, R_1= 3-COOH, R_2 = 5-bromothiophene-2-carbonyl). A solution **14a** in dichloromethane (8 mL) was treated with trifluoroacetic acid (1.0 mL) at room temp for 12 h. The reaction was condensed in vacuo and the residue was mixed with a solution of 5-bromothiophene-2-carboxylic acid (148 mg, 0.715 mmol), HATU (272 mg, 0.715 mmol), and DIEA (568 µL, 3.25 mmol) in acetonitrile (5 mL). The reaction was stirred at room temp for 2 h then diluted with water and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, concentrated, and the residue was chromatographed on a silica gel column eluted with ethyl acetate/hexane to afford the intermediate methyl 3'-((1-(5-bromothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-4'-(methylcarbamoyl)-[1,1'-biphenyl]-3-carboxylate in 86% yield (296 mg). A portion of the intermediate (59 mg, 0.107 mmol) was treated with a solution of

methanol/THF/NaOH (3M aqueous solution) (3 mL, 2:1:1, v/v/v) at room temp for 2h. The reaction was concentrated under vacuum and purified by preparative HPLC to afford the desired title compound in 38% yield (21.6 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 528.95, 531.16; Rt = 2.28 min (LC-MS method B); ^{1}H NMR (400 MHz, DMSO-d6) δ 12.45 (br, 1H), 8.23 (s, 1H), 8.00-7.91 (m, 3H), 7.78 (d, *J* = 7.2 Hz, 1H), 7.64 (t, *J* = 7.6 Hz, 1H), 7.51 (s, 1H), 7.28 (d, *J* = 12.8 Hz, 1H), 7.39 (d, *J* = 7.6 Hz, 1H), 7.30 (d, *J* = 12.8 Hz, 1H), 5.48 (d, *J* = 28.4 Hz, 1H), 4.15-3.67 (m, 4H), 2.74 (d, *J* = 4.0 Hz, 3H), 2.28 (d, *J* = 45.2 Hz, 2H). HRMS $(M+H)^+$ calcd for $[C_{24}H_{21}BrN_2O_5S + H]$ 529.0433; found 529.0434.

3'-((1-(5-bromo-2-((tert-butoxycarbonyl)amino)benzoyl)pyrrolidin-3-yl)oxy)-4'-(methylcarbamoyl)-[1,1'-biphenyl]-3-carboxylic acid (15a). Using **14a** and 5-bromo-2-((tert-butoxycarbonyl)amino)benzoic acid as starting material, the title compound was prepared analogously to **15c** in 18% yield (51.8 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 638.00, 640.02; Rt = 2.72 min (method B); ^{1}H NMR (400 MHz, DMSO-d6) δ 12.35 (br, 1H), 9.05 (s, 1H), 8.23 (d, *J* = 17.2 Hz, 1H), 8.05-7.94 (m, 3H), 7.78-7.74 (m, 1H), 7.66-7.61 (m, 1H), 7.57 (s, 1H), 7.49 (d, *J* = 15.2 Hz, 1H), 7.40-7.35 (m, 1H), 7.22 (s, 1H), 6.68 (d, *J* = 8.0 Hz, 1H), 5.43 (d, *J* = 32.8 Hz, 1H), 3.85-3.57 (m, 4H), 2.82 (td, *J* = 4.8 Hz, 3H), 2.20 (m, 2H), 1.42 (s, 9H).

3'-((1-(2-amino-5-bromobenzoyl)pyrrolidin-3-yl)oxy)-4'-(methylcarbamoyl)-[1,1'-biphenyl]-3-carboxylic acid (15b). Compound **15a** (39.2 mg) was treated with 15% trifluoric acid in dichloromethane for 2h. The reaction was condensed in vacuo and the residue was purified by preparative HPLC to afford the title compound in 70% yield (22.9 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 538.24, 540.25; Rt = 2.16 min (method E); ^{1}H NMR (400 MHz, DMSO-d6) δ 13.05 (br, 1H), 11.43 (br, 1H), 8.22(s, 1H), 8.05-7.94 (m, 3H), 7.7.76 (d, *J* = 4.0 Hz, 1H), 7.7.63 (t, *J* = 8.0 Hz, 1H), 7.48-7.45 (m, 1H), 7.37 (d, *J* = 7.2 Hz, 1H), 7.22 (s, 1H), 6.68 (d, *J* =

7.6 Hz, 1H), 5.42 (d, *J* = 13.6 Hz, 1H), 3.80-3.40 (m, 4H), 2.82 (m, 3H), 2.21 (br, 2H). HRMS $(M+H)^+$ calcd for $[C_{26}H_{24}N_3O_5Br + H]$ 538.0978; found 538.0981.

3-((1-(5-bromothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-N-methyl-2'-(methylsulfonamido)-[1,1'-biphenyl]-4-carboxamide (15d). Using **14ba** as starting material, the title compound was prepared analogously to **15c** in 43% yield (23 mg). LC-MS (ESI) *m/z* $[M+1]^+$ =578.18, 580.19; Rt = 2.34 min (method B); ^{1}H NMR (400 MHz, CDCl3) δ 8.22 (d, *J* = 8.0 Hz, 1H), 7.64 (br, 1H), 7.55 (d, *J* = 8 Hz, 1H), 7.46-7.42 (m, 1H), 7.32-7.26 (m, 3H), 7.08 (d, *J* = 8.0 Hz, 3H), 6.44 (br, 1H), 5.27 (br, 1H), 4.23-3.87 (m, 4H), 3.05 (s, 3H), 2.97 (d, *J* = 4.0 Hz, 3H), 2.42-2.36 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{24}H_{24}N_3O_5S_2Br + H]$ 578.0419; found 578.0422.

(*R*)-3-((1-(5-bromothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-N-methyl-2'-(methylsulfonamido)-[1,1'-biphenyl]-4-carboxamide (15e). Using **14bb** as starting material, the title compound was prepared analogously to **15c** in 52% yield (46 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 578.17; 580.17 Rt = 2.33 min (method B); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, *J* = 8.0 Hz, 1H), 7.66 (br s, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.40-7.47 (m ,1H), 7.26-7.36 (m, 2H), 7.13 (br s, 1H), 7.06 (d, *J* = 8.0 Hz, 1H), 6.76-6.80 (m, 1H), 6.35-6.44 (m, 1H), 5.22-5.35 (m, 1H), 3.82-4.21 (m, 4H), 3.00 (br s, 3H), 2.92 (d, *J* = 4.0 Hz, 3H), 2.25-2.50 (m, 2H). Enantiomeric excess (ee) > 99% (Column IC, 4.1x150mm, 40:60 Heptane: EtOH with 0.1% isopropylamine as modifier, UV254, flow rate 1 mL/min, Rt=7.30 min; the other enantiomer has Rt=10.25 min; ΔRt=2.95min). HRMS $(M+H)^+$ calcd for $[C_{24}H_{24}N_3O_5S_2Br + H]$ 578.0419; found 578.0424.

(*R*)-3-((1-(5-chlorothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-N-methyl-2'-(methylsulfonamido)-[1,1'-biphenyl]-4-carboxamide (15f). Using **14bb** and 5-chlorothiophene-2-carboxylic acid as starting material, the title compound was prepared

analogously to **15c** in 51% yield (42 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 534.20, Rt = 2.30 min (method B); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, *J* = 8.0 Hz, 1H), 7.66 (br s, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.39-7.46 (m, 1H), 7.26-7.38 (m, 2H), 7.13 (br s, 1H), 7.06 (d, *J* = 8 Hz, 1H), 6.92 (m, 1H), 6.76 (m, 1H), 6.55 (br s, 1H), 5.21-5.34 (m, 1H), 4.08 -4.20 (m, 4H), 3.00 (br s, 3H), 2.92 (d, *J* = 4.0 Hz, 3H), 2.25-2.50 (m, 2H). ee% assessment**:** the compound was prepared from the same bach of chiral starting material (*R*)-tert-butyl 3-hydroxypyrrolidine-1-carboxylate under the same reaction conditions as those for compound **15e**. Using the same chiral HPLC method as described for **15e** did not observe the presence of the other enantiomer. HRMS $(M+H)^+$ calcd for $[C_{24}H_{24}ClN_3O_5S_2 + H]$ 534.0924; found 534.0927.

(*R*)-3-((1-(5-cyanothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-N-methyl-2'-(methylsulfonamido)-[1,1'-biphenyl]-4-carboxamide (15g). Using **14bb** and 5-cyanothiophene-2-carboxylic acid as starting material, the title compound was prepared analogously to **15c** in 20% yield (16 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 525.22, Rt = 2.11 min (method B); ^{1}H NMR (400 MHz, DMSO-d6) δ 9.07 (d, *J* = 8.8 Hz, 1H), 8.00 (dd, *J* = 4.0, 15.6 Hz, 1H), 7.93 (dd, *J* = 4.8, 24.8 Hz, 1H), 7.75 (dd, *J* = 3.2, 16.4 Hz, 1H), 7.70 (s, 1H), 7.7.48-7.7.37 (m, 4H), 7.39 (s, 1H), 7.12 (t, *J* = 8.8 Hz,1H), 5.33 (d, *J* = 28.0 Hz, 1H), 4.15-3.88 (m, 2H), 3.82-3.64 (m, 2H), 2.82 (s, 3H), 2.75 (s, 3H), 2.40-2.17 (m, 2H). ee% assessment**:** the compound was prepared from the same bach of chiral starting material (*R*)-tert-butyl 3-hydroxypyrrolidine-1-carboxylate under the same reaction conditions as those for compound **15e**. Using the same chiral HPLC method as described for **15e** did not observe the presence of the other enantiomer. HRMS $(M+H)^+$ calcd for $[C_{25}H_{24}N_4O_5S_2 + H]$ 525.1266; found 525.1266.

(*R*)-3-((1-(5-bromothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-N,2'-dimethyl-[1,1'-biphenyl]-4-carboxamide (15h). Using **14c** as starting material, the title compound was

prepared analogously to **15c** in 46% yield (64 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 499.20, 501.20; Rt = 2.82 min (method B); ^{1}H NMR (400 MHz, DMSO-d6) δ 8.17 (d, *J* = 8.0 Hz, 1H), 7.65 (br s, 1H), 7.24-7.36 (m, 3H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.06-7.12 (m, 2H), 6.90 (br s, 1H), 5.16-5.21 (m, 1H), 4.10-4.23 (m, 1H), 3.85-4.03 (m, 3H), 2.96 (d, *J* = 8.0 Hz, 1H), 2.37-2.48 (m, 1H), 2.25-2.35 (m, 1H), 2.26 (s, 3H). ee% assessment**:** the compound was prepared from the same bach of chiral starting material (*R*)-tert-butyl 3-hydroxypyrrolidine-1-carboxylate under the same reaction conditions as those for compound **15e**. Using the same chiral HPLC method as described for **15e** did not observe the presence of the other enantiomer. HRMS $(M+H)^+$ calcd for $[C_{24}H_{23}BrN_2O_3S + H]$ 499.0691; found 499.0692.

(*R*)-3-((1-(5-bromothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-N-methyl-[1,1'-biphenyl]-4-carboxamide (15i). Using **14d** as starting material, the title compound was prepared analogously to **15c** in 35% yield (48 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 485.17, 487.15; Rt = 2.26 min (method B); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, *J* = 8.0 Hz, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 7.48 (t, *J* = 8.0 Hz, 2H), 7.43 (br d, *J* = 8.0 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 1H), 7.24-7.36 (br s, 1H), 7.25-7.26 (br s, 1H), 7.15 (br s, 1H), 7.07 (d, *J* = 4.0 Hz, 1H), 5.25-5.20 (m, 1H), 3.72-4.38 (m, 4H), 3.01-3.28 (m, 2H), 2.95 (d, *J* = 4.0 Hz, 3H), 2.39-2.51 (m, 1H), 2.25-2.38 (m, 1H). ee% assessment**:** the compound was prepared from the same bach of chiral starting material (*R*)-tert-butyl 3-hydroxypyrrolidine-1-carboxylate under the same reaction conditions as those for compound **15e**. Using the same chiral HPLC method as described for **15e** did not observe the presence of the other enantiomer. HRMS $(M+H)^+$ calcd for $[C_{23}H_{21}BrN_2O_3S + H]$ 485.0535; found 485.0535.

2-((1-(5-bromothiophene-2-carbonyl)pyrrolidin-3-yl)oxy)-N-methylbenzamide (16). To a solution of *t*-butyl 3-(5-iodo-2-(methylcarbamoyl)phenoxy)pyrrolidine-1-carboxylate (**13a**) (134

mg, 0.3 mmol) in methanol (6 mL) was added at 25 °C palladium on carbon (64 mg, 0.060 mmol). The reaction was stirred at 25 °C under 1 atm H_2 for 3 h. The reaction was concentrated and the residue was dissolved in dichloromethane (3 mL) followed by treatment with TFA (0.231 mL, 3.00 mmol). The resultant was stirred at 25 °C for 3 h, concentrated, then re-dissolved in acetonitrile (3 mL). To this solution was added 5-bromothiophene-2-carboxylic acid (31.1 mg, 0.150 mmol), HATU (68.4 mg, 0.180 mmol) and DIEA (0.125 mL, 0.720 mmol). The reaction was stirred at 25 °C for 2 h then concentrated, and the residue was purified by preparative HPLC to afford the title compound in 36% yield for 3 steps (44 mg). LC-MS (ESI) *m/z* $[M+1]^+$ = 409.15; 411.15, 487.15; Rt = 2.13 min (method B); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, *J* = 8.0 Hz, 1H), 7.55 (br s, 1H), 7.45 (t, *J* = 8.0 Hz, 1H), 7.31 (br s, 1H), 7.14 (t, *J* = 8.0 Hz, 1H), 7.08 (d, *J* = 4.0 Hz, 1H), 6.98 (d, *J* = 8.0 Hz, 1H), 5.19 (s, 1H), 3.85-4.20 (m, 4H), 2.95 (s, 3H), 2.45-2.2 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{17}H_{17}BrN_2O_3S + H]$ 409.0222; found 409.0224.

BCATm fluorescent assay. All reagents were purchased from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK) unless otherwise stated. Assay buffer was 50 mM HEPES (pH 7.5), 50 mM NaCl and 1 mM CHAPS. Horseradish Peroxidase was initially diluted to 500 units/ml in water. 4-methyl-2-oxovalerate was initially diluted to 10 mM in assay buffer. L-Leucine and α-ketogluterate were both initially diluted to 100 mM in 50 mM HEPES (pH 7.5) with pyridoxal phosphate (PLP) initially diluted to 10 mM in 50 mM HEPES (pH 7.5). Amplex red (Invitrogen, Paisley, UK) was initially diluted to 20 mM in DMSO. BCATm and L-GOx protein were cloned, expressed, and isolated in house (GSK, Stevenage, UK) (references: (1) Arima, J.; Tamura, T.; Kusakabe, H.; Ashiuchi, M.; Yagi, T.; Tanaka, H.; Inagaki, K. Recombinant

Expression, Biochemical Characterization and Stabilization through Proteolysis of an L-Glutamate Oxidase from *Streptomyces sp.* X-119-6. *J. Biochem.* **2003**, *134*, 805-812. (2) Conway, M. E.; Hutson, S. M. Mammalian Branched-Chain Aminotransferases. *Methods Enzymol.* **2000**, *324*, 356-356). The assay monitors the production of L-glutamate from branch chain amino-acids and α-ketoglutarate through the coupling of hBCATm activity to two additional enzymes, L-Glutamate Oxidase (L-GOx) and Horseradish Peroxidase (HRP). L-GOx catabolises L-glutamate to generate α-Ketoglutarate and hydrogen peroxide, the later being utilised by HRP and leading to the formation of fluorescent resorufin from the redox sensitive dye Amplex Red. The BCATm fluorescent assay was carried out in low volume 384-well plates (Greiner Bio-one, Stonehouse, UK) at a final volume of 10 μL per well. Test compounds were added to plates as 50 nL solution in DMSO using an Echo 555 acoustic dispenser (Labcyte, Sunnyvale, CA) prior to the addition of assay components. Additionally, 50 nL DMSO or positive control compound was included in two columns each to give 100% activity and 100% inhibition controls, respectively. Single-concentration testing was at 10 μM compound concentration. For pIC_{50} determination, compounds were tested using an 11-point, three-fold dilution series from either 625 μM or 6.25 μM prepared using a Biomek FX (Beckman Coulter, Wycombe, UK). To these compound plates, 4 μL of an enzyme-PLP solution containing 20 nM BCATm and 40 nM PLP in assay buffer was added. Following this, 4 μL of a coupling solution containing 3 mM L-Leucine, 0.5 mM α-ketogluterate, 10 units/mL HRP and 80 μM Amplex red in assay buffer was added to initiate the reaction. The coupling solution was incubated on a roller at room temperature in a 15 mL tube with 1 mL Agarose immobilised Catalase (Sigma-Aldrich Ltd.) per 10 mL of coupling solution to 'scrub' the coupling solution prior to addition of Amplex Red and remove background levels of hydrogen peroxide. After a 10 minute incubation,

2 μL of 100 mM 4-methyl-2-oxovalerate was added to stop the reaction. Final assay concentrations were 10 nM BCATm, 20 nM PLP, 5 units per mL HRP, 1.5 mM L-Leucine, 0.25 mM alpha-ketoglutarate and 40 μM Amplex Red. All additions were performed using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA). Plates were transferred to an EnVision reader (PerkinElmer) (excitation filter 525/20 nm; emission filter 598/25 nm).

BCATm/PLP protein production and crystallisation. Human branched-chain amino acid aminotransferase mitochondrial (BCATm) (residues 28-392) deleted of the N-terminal mitochondria signal peptide (residues 1-27) was cloned into Kanamycin resistant pET28a via NdeI/XhoI restriction endonuclease sites with an N-terminal 6His-Thrombin cleavage site. The protein was expressed in Escherichia coli in inclusion bodies. Purification required 4 M Urea lysis followed by refolding and elution by NiSephHP ultrafiltration (UF). HiPrep Desalting was then performed using Superdex200 prep grade column followed by Thrombin cleavage to remove the tag and final clarification by UF on a Butyl Sepharose HP - HiPrep desalting column. The purified protein was stored in buffer (25 mM HEPES pH 7.5, 25 mM NaCl, 20 mM DTT, 20 mM EDTA, 2.5 % glycerol) and concentrated to 7.6 mg/mL for crystallography. Cocrystals of BCATm with compound **15e** were grown by hanging drop vapour diffusion at 20 °C using protein supplemented with 25 mM compound **15e** (5% DMSO) and mixed in a 1:1 drop ratio with a microseed solution comprising BCATm seeds in MDL Morpheus™ screen condition B2 supplemented with 10 mM DTT. The Hampton Research seed-bead method was used to generate microseeds from a crystal grown in the absence of compound. The crystals had a yellow hue suggestive of bound PLP cofactor despite none being added during purification or crystallisation.

X-ray crystal structure of BCATm/PLP complexed with inhibitor 15e. A cocrystal was soaked for an additional 3 days in buffer comprising MDL Morpheus™ screen condition B2 supplemented with 20 mM DTT and 25mM compound **15e.** A soaked cocrystal was harvested in a cryo-loop and mounted within a 100K liquid nitrogen cryostream on the goniometer of an in-house RIGAKU FR-E$^+$ SUPERBRIGHT generator/Mardtb/MAR345 detector system. Diffraction data were collected and processed using MOSFLM (Leslie, A.G.W. & Powell, H.R. 2007. Processing diffraction data with MOSFLM. *in* Evolving Methods for Macromolecular Crystallography, Read R.J & Sussman, J.L. (eds), Springer Press, 41-51) and scaled using AIMLESS (-Evans, P.R. & Murshudov, G.N. Acta Cryst. (2013) **D69**, 1204-1214) within the CCP4 programming suite(Collaborative Computational Project, Number ***4.*** "*The CCP4 Suite: Programs for Protein Crystallography*." Acta Cryst. (1994) **D50,** 760-763). The structure was solved by Fourier synthesis using REFMAC(Murshudov, G.N., Vagin, A.A. and Dodson, E.J. Acta Cryst. (1997) **D53**, 240-255) starting from a previously determined in-house structure, model-building was performed using COOT (P. Emsley and K. Cowtan Acta Cryst. (2004) **D60**, 2126-2132) and refinement using REFMAC. The statistics for the data collection and refined co-ordinates are given in Table 1 of Supporting Information section. The final crystal structure is deposited in the Protein Data Bank under the accession code 5CR5.

Table 1. X-ray data summary table for 15e

Parameter[a]

Data collection	
Space Group	$P2_12_12_1$
Cell Dimensions	
a,b,c (Å)	69.55,105.59,107.05

a, b, γ (°)	90.00,90.00, 90.00
Resolution (Å)	1.61 (1.63)
Rmerge[b]	0.058 (0.791)
Average I/σI	17.4 (2.2)
Completeness (%)	97.4 (76.0)
Redundancy	6.9 (5.8)
No. Reflections	695861
No. Unique Reflections	101131 (3832)
Refinement	
Resolution (Å)	20.00-1.61
Rwork/Rfree	0.168/0.199
No. Reflections	95729
No. atoms	
Protein (chain A/B)	2939/2914
Cmpd (L1/L2)	35/35
B- factors [Å2]	
Protein (chain A/B)	22.7/24.0
Cmpd (L1/L2)	18.9/19.1
R.m.s deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.360
Crystal soaking conditions	*Cocrystal* (grown with 25mM compound, 10% DMSO, 20mM DTT) + *soak* (at 25mM compound, 10% DMSO, 20mM DTT, 3 days)

[a] Data for the highest resolution shell are given in parentheses.

[b] $R_{merge} = \Sigma | I_j - \langle I_j \rangle | / \Sigma \langle I_j \rangle$.