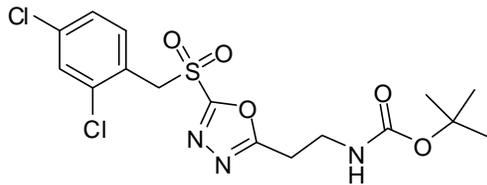


## Project: Specific Inhibitors of AmpC $\beta$ -Lactamase (Chemotype 2)

Probe:

**Covalent Modifier:**

tert-butyl 2-(5-(2,4-dichlorobenzylsulfonyl)-1,3,4-oxadiazol-2-yl)ethylcarbamate



[SID: 864201](#)

Covalent modifier

*Probe Synonyms:* NCGC00161790 (internal ID)

**PubChem Bioassay Identifiers (AID):** 584

SID	IC50 ( $\mu$ M)	Antitarget	Selectivity*
<b>Covalent modifier 864201</b>	<b>0.066 <math>\mu</math>M</b>	<b>Cruzain; Chym; MDH 410; &gt;1; .600</b>	<b>Cruzain = 6200 Chym = 15.2 MDH = 9.1</b>

\*Counterscreen in 0.01% Triton X-100; Selectivity = antitarget IC50/target IC50

**Assigned Assay Grant #:** MH079825-01 and MH079825-02

**Screening Center Name:** NIH Chemical Genomics Center

**Principal Investigator of Screening Center:** Christopher Austin

**Assay Submitter & Institution:** Brian Shoichet, University of California, San Francisco

**Assay or Pathway Target:** AmpC  $\beta$ -Lactamase

### ***Assay provider information***

*Specific Aim:* AmpC has been studied since the early 1970's and comprises a major class of plasmid-mediated  $\beta$ -lactamases with extended hydrolysis profiles for cephalosporins, penicillins and aztreonam. A major issue within the broad-spectrum treatment of bacterial infections involves the multidrug resistance associated with the AmpC  $\beta$ -lactamases.<sup>1,2</sup> As such, we explored a screen and optimization program for novel inhibitors (both covalent and non-covalent) of this important enzyme class. The primary screen was performed in the presence and absence of the detergent Triton X-100 in an effort to identify molecules that behave as promiscuous enzyme inhibitors through the phenomenon of colloidal aggregation. Additionally, this study offered a unique opportunity to provide a comprehensive analysis of the screening results to determine the mechanism of every active molecule from concomitant experimental and virtual screens of an unbiased library of 70,563 molecules against  $\beta$ -lactamase.

*Significance:* AmpC  $\beta$ -lactamases have emerged as an important target of study based upon their clinical relevance to antibiotic resistant bacteria. Most of these enzymes are cephalosporinases but are capable of hydrolysing all  $\beta$ -lactams to some extent. The hydrolytic properties of AmpC  $\beta$ -lactamases are similar. Unfortunately, these enzymes have demonstrated drug resistant properties for the third generation cephalosporins presenting immense future challenges to health providers.<sup>1,2</sup> A second goal of this study was to further characterized the molecular libraries collection for small molecules prone to aggregation and promiscuous, non-selective inhibitory mechanisms. A third goal of this study was to directly compare experimental screening with computational docking in an attempt to gage the level of overlap and accuracy that novel computational methods provide.

*Rationale:* The search for novel inhibitors of AmpC was accomplished using purified AmpC in a standard enzymatic assay. The aggregation profiling approach exploits the sensitivity of aggregate formation to detergent by analyzing the inhibition of  $\beta$ -lactamase by library members measured in the presence and absence of 0.01% Triton X-100. Compounds that inhibit only in the absence of detergent are considered likely promiscuous aggregators. Confirmation assays were accomplished via a cuvette-based screen done in the Assay providers laboratory.

## **Screening center information**

### **Assay Implementation and Screening**

#### **PubChem Bioassay Names:**

AID 584: Promiscuous and Specific Inhibitors of AmpC Beta-Lactamase (assay with detergent)

AID 585: Promiscuous and Specific Inhibitors of AmpC Beta-Lactamase (assay without detergent)

AID 1002: Confirmation Concentration-Response Assay for Inhibitors of AmpC Beta-Lactamase (assay with detergent).

AID 1003: Confirmation Cuvette-Based Assay for Inhibitors of AmpC Beta-Lactamase (assay with detergent).

#### **List of PubChem bioassay identifiers generated for this screening project (AIDs):**

AID	Target	Concentration	Bioassay type
584	AmpC Beta-Lactamase	30 $\mu$ M – 0.7 nM <sup>a</sup>	Primary qHTS
585	AmpC Beta-Lactamase	30 $\mu$ M – 0.7 nM <sup>a</sup>	Profile qHTS (no detergent)
1002	AmpC Beta-Lactamase	30 $\mu$ M – 0.7 nM <sup>a</sup>	Confirmation qHTS
1003	AmpC Beta-Lactamase	30 $\mu$ M – 0.7 nM <sup>a</sup>	Cuvette-Based Assay

<sup>a</sup>Seven point concentration-titration series was used covering the indicated range;1:5 dilutions.

#### **Primary Assay Description as defined in PubChem:**

##### **Overview:**

This aggregation profiling approach exploits the sensitivity of aggregate formation to detergent. Inhibition of  $\beta$ -lactamase is measured in the presence and absence of 0.01% Triton X-100. This particular assay had the presence of 0.01% Triton X-100. See PubChem assay "Promiscuous and Specific Inhibitors of AmpC Beta-Lactamase (assay without detergent)" for related screen. Compounds that inhibit only in the absence of detergent are considered likely promiscuous aggregators.

The assay is based on that used in a screen of 1,030 compounds performed in Brian Shoichet's lab in 96-well format<sup>3</sup>. AmpC activity was measured by following the absorbance at 480 nm. Nitrocefin is a chromogenic substrate: while nitrocefin is yellow (Imax 390 nm), the product of its hydrolysis is red (Imax 486 nm, 17,000 M<sup>-1</sup>). AmpC beta lactamase was provided by Brian Shoichet and Brian Feng, UCSF.

**Protocol:**

Buffer: 20 mM phosphate buffer, pH 7.0

Triton X-100: No Detergent screen: 0.0001%, Detergent screen: 0.01%.

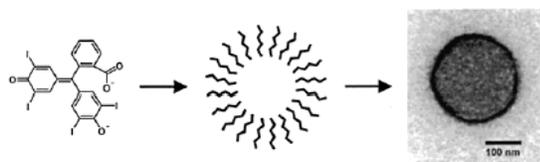
Reagents/Controls: Buffer in columns 3 and 4 as negative control (no enzyme).

5.3 nM AmpC in columns 1, 2, 5-48. Titration of UCSF compound 9 control (from 10 mM, then 1:2) pin-transferred to column 2. Column 1 is neutral (100% activity).

Assay Steps: Six uL of reagents were dispensed to 1536-well Greiner black clear bottom plates. Compounds and controls (23 nL) were transferred via Kalypsys PinTool. The plates were incubated for 15 min at room temperature, and then 2 uL of 800 uM solution of nitrocefin was added to start the reaction. The plates were immediately transferred to and read every 20 seconds for 4 min on ViewLux (Perkin-Elmer) High-throughput CCD imager using 480 nm absorbance protocol. During dispense, reagent bottles were kept submerged into 4 deg C water bath.

**Center Summary of the Primary Screen:**

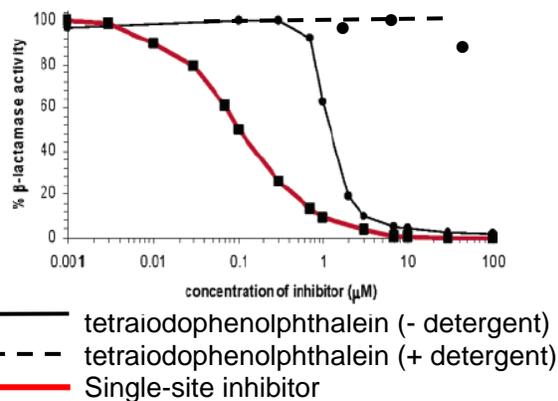
*Assay background:*



Shoichet, B.K. *J. Med. Chem.* 2006, **49**, 7274

Ryan, A.J. et al., *J. Med. Chem.* 2003, **46**, 3448

McGovern, S.L. et al. *J. Med. Chem.*, 2002, **45**, 1712



**Figure 1:** qHTS assay principle. Colloidal-size aggregates can block enzyme active sites non-specifically and can act as reproducible inhibitors. Inclusion of detergent (0.01 % Triton X-100) in the assay medium prevents aggregate formation and allows the detection of genuine actives (Nat. Chem. Biol., 2005, 1, 146).

Assay protocol:

<b>AmpC Beta-Lactamase qHTS Protocol</b>			
<b>Step</b>	<b>Parameter</b>	<b>Value</b>	<b>Description</b>
1	Reagent	6 uL	AmpC (4 nM in -D, 3 nM in +D)
2	Control	23 nL	Control plate
3	Compound	23 nL	Compound library
4	Time	10 min	Room temp
5	Reagent	2 uL	Nitrocefin Substrate (200 uM final)
6	Detector	Absorbance	Viewlux
<b>Step</b>	<b>Notes</b>		
1	Dispenser 1 (tip 1-4) Head B substrate		
2	Control plate		
3	Compound library 33 $\mu$ M-0.3 nM; 1:5 dilutions		
4	Room temperature incubation		
5	Dispenser 2 (tips 1-4) = 1X; col 3, 4: no enzyme		
6	Viewlux in kinetic mode, 485 nm Absorbance		

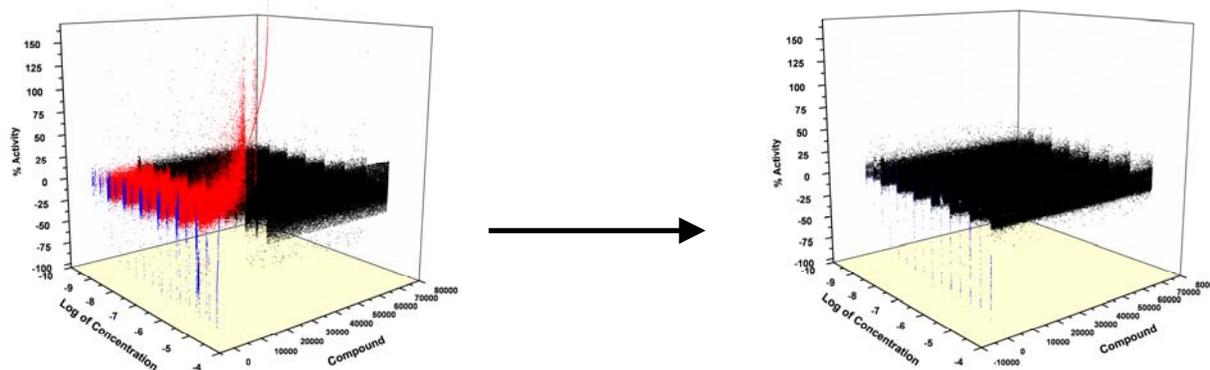
**Table 1:** qHTS Protocol for AmpC with and without detergent. AmpC activity was measured by following the absorbance at 485 nm. Nitrocefin is a chromogenic substrate: while nitrocefin is yellow ( $\lambda_{max}$  390 nm), the product of its hydrolysis is red ( $\lambda_{max}$  486 nm, 17,000 M<sup>-1</sup>).

A control titration was pin-transferred to column 2 in addition to neutral and no enzyme controls used in assay. Control titration compound was "UCSF compound 9" (transferred from 10 mM, then 1:2, in duplicates)

*qHTS Summary of Assay results:*

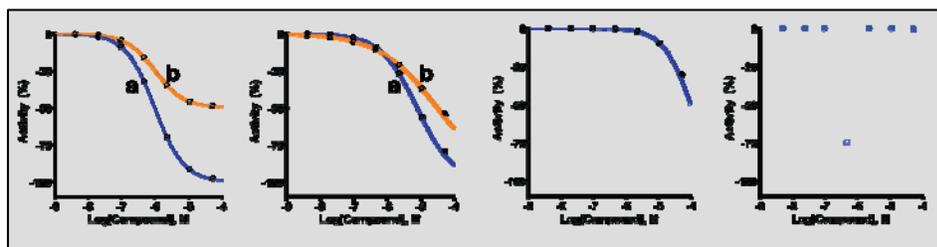
<b>qHTS Assays Performance Summary</b>		
<b>Parameter</b>	<b>Without Detergent</b>	<b>With Detergent</b>
Plates Screened	437	437
Plates Failed QC	0	0
Compound (total # tested)	70,563	70,563
Samples (total wells)	671,232	671,232
Number of Data Points	8,054,784	6,041,088
% 7 pt-titrations	85%	85%
% 15 pt-titrations	15%	15%
Data Layers	Read 1 Read 2 through 12 Delta (Activity Layer)	Read 1 Read 2 through 9 Delta (Activity Layer)
Average Z' [Delta Activity]	0.79	0.83

**Table 2:** Performance of the two AmpC screens of 70, 563 compounds each. Data were collected in multiple time points and all time courses were processed and stored for detailed examination of compound behavior. The delta of the last and first time points was used for activity calculations.<sup>4</sup>



**Figure 2.** A 3D scatter plot of qHTS data. Concentration-response relationships for all 70,563 molecules are shown, colored as: no relationship (black), inhibition (blue), or activation (red). Comparison between no detergent (AID: 585) and detergent (AID: 584)<sup>4</sup>

*Identification of Active Clusters:* Following the qHTS, the concentration response curves (CRCs) data are subjected to a classification scheme to rank the quality of the CRCs as described in Inglese and co-workers<sup>5</sup> (see also scheme 1) Briefly, CRCs are placed into four classes. Class 1 contains complete concentration-response curves showing both upper and lower asymptotes and  $r^2$  values  $> 0.9$ . Class 2 contains incomplete CRCs lacking the lower asymptote and shows  $r^2$  values greater than 0.9. Class 3 curves are of the lowest confidence because they are defined by a single concentration point where the minimal acceptable activity is set at 3 SD of the mean activity calculated as described above. Finally, class 4 contains compounds that do not show any CRCs and are therefore classified as inactive. Once an active set of compounds was identified, hierarchical agglomerative clustering with a 0.7 Tanimoto cutoff was performed by using Leadscape (Leadscape Inc., Columbus, OH) fingerprints. For each cluster, maximal common substructures (MCS) were extracted, a manual step of MCS trimming was performed to create a list of scaffolds, and any overlapping scaffolds were abridged to a canonical set. Each scaffold was then represented as a precise definition to indicate descriptors such as the number of attachment points or the ring size variability. All filters were then relaxed to include the entire negative (class 4) assay data.



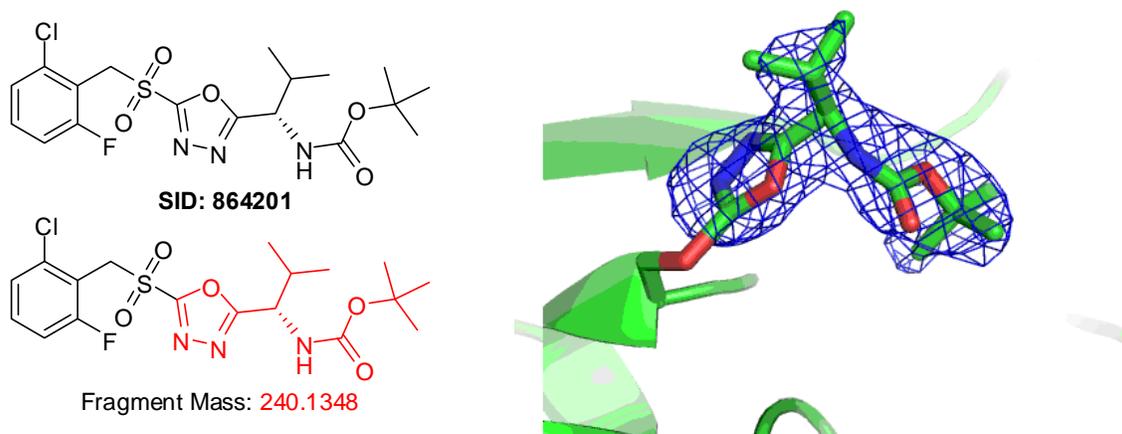
**Scheme 1:** Example classification scheme for assignment of resulting curve-fit data into classes. Curves fitting the following classification criteria are then used to establish SAR: Class 1 curves display two asymptotes,

an inflection point, and  $r^2 \geq 0.9$ ; subclasses 1a vs. 1b are differentiated by full ( $>80\%$ ) vs. partial ( $\leq 80\%$ ) response. Class 2 curves display a single left-hand asymptote and inflection point; subclasses 2a and 2b are differentiated by a max response and  $r^2$ ,  $>80\%$  and  $>0.9$  or  $<80\%$  and  $<0.9$ , respectively. Class 3 curves have a single left-hand asymptote, no inflection point, and a response  $>3SD$  the mean activity of the sample field. Class 4 defines those samples showing no activity across the concentration range.

## Probe Characterization

*Prior Art for AmpC:* The general class of  $\beta$ -lactams are the current best in class inhibitors for AmpC, however, resistance for the third generation cephalosporins.<sup>6</sup>

Lead identification and synthetic preparation of selected analogues: Among the most intriguing chemotypes revealed via qHTS was a collection of sulfonyl-oxadiazoles that inhibited the enzyme potently, with  $IC_{50}$  values ranging from 0.015 to 8  $\mu$ M within the primary qHTS. One important analogue was **SID: 864201**. This compound was found to inhibit AmpC in a detergent insensitive manner at high potencies. In collaboration with Professor Brian Shoichet, this agent was among the 1274 actives that were characterized by secondary assays, mass analysis, and co-crystallization studies. While the majority of these compounds were found to be detergent sensitive colloidal aggregates, **SID: 864201** was noted to be a covalent modifier of AmpC based upon mass analysis (AmpC was noted to increase in mass by 240.1 AMU). Further, the exact mechanism of covalent modification was determined through co-crystallization studies with the enzyme (Figure 3). This compound represents an important new chemotype for AmpC inhibition.<sup>7</sup>



**Figure 3.** Structure of **SID: 864201** and crystal structure showing the portion of the molecule that covalently modifies AmpC.

Comparison of screening with detergent and without detergent: The detailed analysis of the hits from qHTS allowed us to categorize all the active molecules as either promiscuous aggregators (95%), known inhibitors (2%), irreproducible (assay format dependent) (2%), and promiscuous covalent (1%). Ultimately, no specific, reversible inhibitors were discovered. However, the results also highlight the robustness and accuracy of the screen. The qHTS not only worked well in identifying known inhibitors of  $\beta$ -lactamases but also further advanced new SAR surrounding the  $\beta$ -lactam class of small molecules. Further, the primary screen revealed a series of oxadiazoles that represent a novel non- $\beta$ -lactam class of covalent modifier of AmpC  $\beta$ -lactamase. The compounds that were identified as actives in the primary screen but were ultimately shown to be non-reproducible upon follow up analysis represented a minor percentage of related compounds found in the library (2 compounds of 87). Additionally, those actives that did not reproduce were revealed in the primary screen as curve class 2.2 or higher (partial curves and single point actives) and are not typically chosen for follow-up in the absence of supporting Class 1-2.1 curves (see scheme 1). Thus, despite the lack of a new, novel reversible inhibitor of AmpC the qHTS screen was demonstrably accurate, robust and successful. While high false-positive rates

remain a major issue in HTS, from these results its apparent the qHTS paradigm aids in the ability to avoid the early bad decisions that historically have plagued follow-up efforts.

### Secondary Assay Descriptions as defined in PubChem:

AID 1003: Confirmation Cuvette-Based Assay for Inhibitors of AmpC Beta-Lactamase (assay with detergent).

#### Overview:

This aggregation profiling approach exploits the sensitivity of aggregate formation to detergent. Inhibition of b-lactamase is measured in the presence and absence of 0.01% Triton X-100 (Feng 2007). This particular assay is a confirmation of previous qHTS (Inglese, 2006), Pubchem AID 584, assay with presence of 0.01% Triton X-100. For a related assay without detergent, see AID 585. Compounds that inhibit only in the absence of detergent are considered likely promiscuous aggregators. This confirmation assay is a cuvette-based screen of an oxadiazole carbamate series which is an expansion around docking hit Pubchem SID 4244870.

#### Protocol:

Reactions were performed in methylacrylate cuvettes in 1 mL of reaction volume. The beta-lactamase assay contained 1 nM AmpC beta-lactamase, 200 uM Nitrocefin and varying amounts of Triton X-100 (either 0.00002% or 0.1%). Compound and enzyme were incubated for 5 minutes before the reaction was initiated by addition of substrate. Nitrocefin hydrolysis was monitored as an increase in absorption at 482 nm on an Agilent 8453 UV-visible spectrophotometer at room temperature.

### Known probe properties:

Properties of probe compounds as reported in PubChem:

#### **SID: 864201:**

Molecular Weight	436.31016 [g/mol]
Molecular Formula	C <sub>16</sub> H <sub>19</sub> Cl <sub>2</sub> N <sub>3</sub> O <sub>5</sub> S
XLogP	4.2
H-Bond Donor	1
H-Bond Acceptor	7
Rotatable Bond Count	8
Tautomer Count	2
Exact Mass	435.042247
MonoIsotopic Mass	435.042247
Topological Polar Surface Area	111
Heavy Atom Count	27

#### Canonical SMILES:

C1=CC=C2C(=C1)C=CC=C2C[C@H](C(=O)O)N3C(=O)C4=C(C3=O)C=C(C=C4)C(=O)O

#### InChi:

InChI=1/C22H15NO6/c24-19-16-9-8-14(21(26)27)10-17(16)20(25)23(19)18(22(28)29)11-13-6-3-5-12-4-1-2-7-15(12)13/h1-10,18H,11H2,(H,26,27)(H,28,29)/t18-/m1/s1/f/h26,28H

*Has this compound been provided to the MLSMR:*

Yes, this compound is part of the MLSMR (MLS000041932).

*Probe availability:*

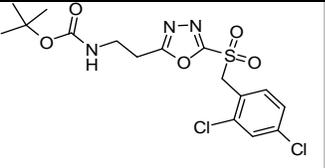
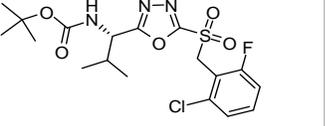
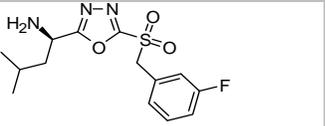
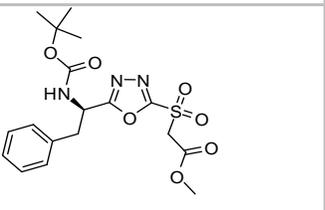
The probe is available from InterBioScreen (ID: STOCK5S-11605).

*Compound preparation:*

Compounds are prepared in DMSO at 10 mM stock concentration.

Appendices:

**Table S1:** Sulfonyl-Oxadiazole Series - Covalent inhibitors

Pubchem CID NCGC ID	Structure	IC50 [uM]	LogP	Compound Source
CID: 665449 NCGC00067552 PROBE		0.066	3.2	InterBioScreen STOCK5S-11605
CID: 663743 NCGC00067197		1.8	3.18	InterBioScreen STOCK4S-42975
CID: 6603229 NCGC00073961		8.6	1.15	InterBioScreen STOCK4S-49370
CID: 665535 NCGC00067511		9.4	1.79	InterBioScreen STOCK5S-10760

***Bibliography:***

- (1) Walsh, C. (2000) Molecular mechanisms that confer antibacterial drug resistance. *Nature* 406 (6797), 775-781
- (2) Baudry, P.J. et al. (2008) Comparison of antimicrobial resistance profiles among extended-spectrum-beta-lactamase-producing and acquired AmpC beta-lactamase-producing *Escherichia coli* isolates from Canadian intensive care units. *Antimicrob Agents Chemother* 52 (5), 1846-1849
- (3) Feng, B.Y. et al. (2005) High-throughput assays for promiscuous inhibitors. *Nat Chem Biol* 1 (3), 146-148
- (4) Feng et al. (2007) A High-Throughput Screen for Aggregation-Based Inhibition in a Large Compound Library. *J. Med. Chem.* 50, 2385-2390
- (5) Inglese, J. et al. (2006) Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci U S A* 103 (31), 11473-11478
- (6) Yamawaki, K. et al. (2008) A novel series of parenteral cephalosporins exhibiting potent activities against both *Pseudomonas aeruginosa* and other gram-negative pathogens. Part 2: Synthesis and structure-activity relationships. *Bioorg Med Chem* 16 (4), 1632-1647
- (7) Babaoglu, K et al (2008). Comprehensive Mechanistic Analysis of Hits from High-Throughput and Docking Screens against Beta-Lactamase. *J. Med. Chem.* 51, 2502-2511.