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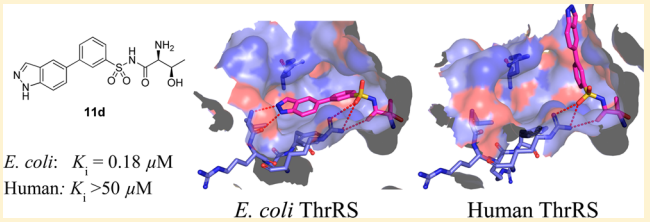
Identification of Bacteria-Selective Threonyl-tRNA Synthetase Substrate Inhibitors by Structure-Based Design

Min Teng,*[,†] Mark T. Hilgers,[‡] Mark L. Cunningham,[§] Allen Borchardt,[†] Jeffrey B. Locke,[§] Sunny Abraham,[†] Gregory Haley,[†] Bryan P. Kwan,[§] Courtney Hall,[‡] Grayson W. Hough,[∥] Karen J. Shaw,[§] and John Finn[†]

[†]Medicinal Chemistry, [‡]Structural Biology, [§]Biology, and [∥]Analytical Chemistry, Trius Therapeutics, Inc., 6310 Nancy Ridge Drive, San Diego, California 92121, United States

Supporting Information

ABSTRACT: A series of potent and bacteria-selective threonyl-tRNA synthetase (ThrRS) inhibitors have been identified using structure-based drug design. These compounds occupied the substrate binding site of ThrRS and showed excellent binding affinities for all of the bacterial orthologues tested. Some of the compounds displayed greatly improved bacterial selectivity. Key residues responsible for potency and bacteria/human ThrRS selectivity have been identified. Antimicrobial activity has been achieved against wild-type *Haemophilus influenzae* and efflux-deficient mutants of *Escherichia coli* and *Burkholderia thailandensis*.



INTRODUCTION

Infections caused by antibiotic-resistant pathogens have led to a crisis in health care, especially for those due to Gram-negative organisms. These disease-causing microbes have developed several different classes of resistance mechanisms, one of which is target-based mutations that reduce drug affinity, as in the case of fluoroquinolones.[1] Alternatively, as seen for the aminoglycosides, enzymatic modification of a drug may lead to high-level resistance.[2] There are resistance mechanisms to all major antibacterial drugs currently in use, and many strains are resistant to multiple drug classes. Thus, new classes of agents that bind to novel targets would be a significant addition to the antimicrobial armamentarium to combat resistant pathogens.

Aminoacyl-tRNA synthetases (aaRSs) are a class of enzymes that have been validated as antimicrobial targets.[3] Mupirocin, which inhibits bacterial isoleucyl-tRNA synthetase, is approved for the topical treatment of impetigo due to *Staphylococcus aureus* and *Streptococcus pyogenes*.[4] With the exception of glutaminyl-tRNA synthetase (GlnRS), absent in many Gram-positive bacteria, all of the other 19 synthetases are highly conserved and perform essential activities in clinically important microorganisms.[5] In addition to mupirocin, whole cell screening of natural products has identified multiple aaRS inhibitors with antibacterial activity.[6] These include borrelidin (ThrRS), granaticin (LeuRS), indolmycin (TrpRS), ochratoxin A (PheRS), and cispentacin (ProRS). In recent years, other aaRS inhibitors have emerged, including those that are mimics of the aminoacyl adenosine monophosphate intermediate (aa-AMP), such as **1** (Figure 1).[7] Although most of the aa-AMP mimics demonstrated low nanomolar binding affinities against their corresponding aaRSs, they generally displayed weak antimicrobial activity due to poor bacterial cell permeability.[3f,6,8]

In screening marine natural products for antibacterial activity, we identified borrelidin (**2**) as the active component of one of the extracts we prioritized for structural elucidation. Borrelidin inhibits ThrRS[9] and has an interesting antibacterial profile that includes activity on *Yersinia pestis*, *Haemophilus influenzae*, and *Francisella tularensis* (unpublished results). However, the antibacterial potency and spectrum of borrelidin need to be significantly improved to generate a useful antibacterial agent that targets ThrRS. In addition, borrelidin lacks selectivity for bacterial ThrRS versus human ThrRS, raising concerns of mammalian toxicity. Indeed, borrelidin has been shown to be a potent inhibitor of angiogenesis.[10] Although previous efforts sought to optimize the antibacterial activity of borrelidin, none of these efforts utilized X-ray crystallographic data to guide optimization. To address these issues, we cocrystallized borrelidin with *Escherichia coli* and human ThrRS (which will be reported on separately).[11] Comparison of the human and *E. coli* enzymes reveals that the borrelidin binding site is highly conserved between the two ThrRS enzymes and that the ATP/substrate binding site displays more structural variability than the borrelidin site. The *E. coli* and human ThrRS selectivity ratios for a number of semisynthetic analogues of borrelidin confirm that achieving a good bacterial selectivity with borrelidin analogues may be a challenging task. Thus, we explored totally synthetic inhibitors of bacterial ThrRS that specifically target the substrate binding sites of ThrRS. In this paper we summarizes our effort to design novel small-molecule

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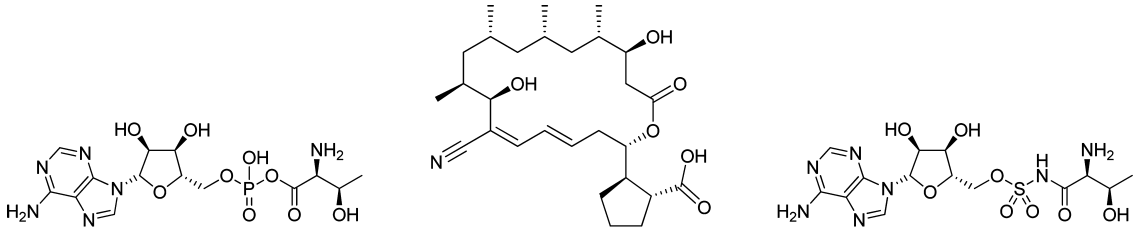

Figure 1. Structures of known ThrRS inhibitors: threonyl-AMP (**1**), borrelidin (**2**), threonyl-AMS (**3**).

antibacterial agents that selectively inhibit bacterial ThrRS enzymes.

The starting point for this program was 5′-*O*-[*N*-(threonyl)-sulfamoyl] adenosine (AMS, **3**), a known competitive inhibitor of ThrRS.[12] The crystal structure of **3** bound to *E. coli* ThrRS has been reported.[11] As an adenosine analogue, **3** lacks antibacterial activity due to poor (too polar) drug properties and lacks selectivity for the bacterial enzymes (Table 1). We aimed to address both of these issues by designing compounds with more typical heterocyclic fragments found in drugs and by tailoring the shape of the interaction sites of the inhibitors to achieve bacterial selectivity.

CHEMISTRY

Compound **4** was synthesized through a coupling of sulfonamide **5** and Boc-protected L-threonine **6** followed by deprotection of the Boc (Scheme 1). The synthesis of **10a−f** and **11a** was initiated with a coupling reaction between dioxaborolane **8a** with **6** to form intermediate **9a** (Scheme 2). Suzuki coupling of **9a** with a corresponding ArBr followed by deprotection of Boc yielded **10a−f** and **11a**. Similarly, Suzuki coupling of **9b** with a corresponding $ArB(OH)_2$ followed by deprotection of Boc yielded **11c,d**. Upon NCS treatment, **11d** was converted to **11b**. The synthesis of **10g,i** started with **12**, which was synthesized using a known procedure.[13] The synthesis of **10j** started with a reaction of 7-bromo-1,2,3,4-tetrahydroisoquinoline with sulfamoyl chloride to furnish **13**. A coupling reaction of **12** or **13** with **6** afforded **14a,b**. A conversion of Br in **14a,b** to borane pinacol ester yielded **15a,b**. A Suzuki coupling reaction of **15a,b** to a corresponding ArCl followed by Boc deprotection furnished **10g,i,j** (Scheme 3). A coupling reaction of **6** with (3-(aminomethyl)phenyl)boronic acid by HOAT and EDCI furnished **16**. After a Suzuki coupling reaction with 7-bromo-2-chloroquinazolin-4-amine followed by deprotection of the Boc, **16** was converted to **17** (Scheme 4). Compound **19** was synthesized through (3-bromophenyl)-hydrazine. Conversion of the hydrazine to threonyl hydrazide **18a** was effected by threonyl chloride generated in situ. Conversion of Br in **18a** to borane pinacol ester yielded **18b**. After a Suzuki coupling followed by deprotection of Boc, **18b** was converted to **19** (Scheme 5). The synthesis of **22** started with a conversion of **6** into its primary amide, which was subsequently treated with LAH to form **20**. This intermediate was treated with 3-bromobenzenesulfonyl chloride under basic conditions to form **21a.** A conversion of Br **21a** into borane pinacol ester yielded **21b** (Scheme 6). Suzuki coupling of **21b** with 7-bromo-2-chloroquinazolin-4-amine followed by deprotection of Boc yielded **22**. The synthesis of **24** started with methylation of the acylsulfonamide **9b** with iodomethane followed by a Suzuki coupling with 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)isoindolin-1-one and deprotection of the Boc (Scheme 7). The synthesis of **10h** and **26−29** started with a Heck reaction of 6-(3-bromophenyl)pyrimidin-4-amine with vinylsulfonamide to yield **25**. After coupling with a corresponding acid and deprotection of the Boc and TBS under acidic conditions, intermediate **25** furnished **10h** and **26−29** (Scheme 8).

RESULTS AND DISCUSSION

In the initial designs, we simplified **3** by replacing the polar adenosine moiety with a 4-phenoxyphenyl moiety, but retained the acylsulfonamide, as in **4**. These changes resulted in loss of potency due to the loss of the H-bonding network that is present with adenosine. As a result, compound **4** had only modest inhibition at 50 μM concentration. Enzymatic assays showed that **4** was competitive with respect to both ATP and the amino acid substrate (which will be reported on separately). Despite the low potency, we were able to obtain a cocrystal structure of **4** with *E. coli* ThrRS. This structure demonstrated that compound **4** binds to an "open form" of the ATP binding site that more closely resembles the apo conformation of ThrRS than the ATP-bound conformation (Figure 2). The threonylacylsulfonamide portion of the molecule binds to the Zn atom, as anticipated from the Thr-AMS structure. The 4-phenoxy group is largely disordered, suggesting that it has negligible contributions to binding. These data revealed opportunities for further optimization of the binding affinity.

One of the strategies to enhance the binding affinity is to replace the distal phenoxy in **4** with a suitable group which can induce the "closed form" of the ATP pocket. Inhibition of the closed form of the enzyme was preferred, as it targets key highly conserved residues that are required for enzymatic activity. Because of their biochemical importance, these residues are inherently difficult to mutate, thereby making target-based resistance to the new inhibitors less likely. Furthermore, it is well appreciated that targeting the enzymatic transition states is a useful strategy for generating highly potent inhibitors.[14]

Computational modeling using Discovery Studio with structural information from **3** suggested that compound **10a**, with an aminoquinazoline fragment, could fit the adenosine site of the closed form, as the pyrimidine ring of **10a** would bind to the ThrRS enzyme in a fashion similar to that of the pyrimidine ring of **3**. As predicted by the modeling, compound **10a** was a strong inhibitor of *E. coli* ThrRS (Table 1). The cocrystal structure of **10a** bound to *E. coli* ThrRS showed that this compound did indeed bind to the closed form of the ATP pocket with the quinazoline ring sandwiched between Arg520 and Phe379 (Figure 3). In addition, the quinazoline ring formed a number of important H-bonds with nearby residues. The amino group formed bidentate H-bonds with Glu365 and

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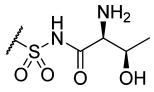
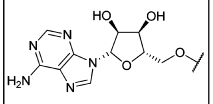
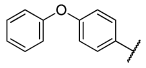
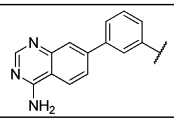
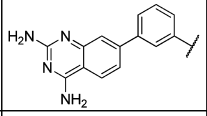
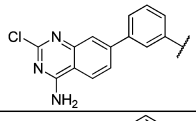
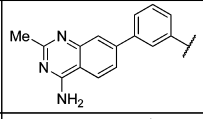
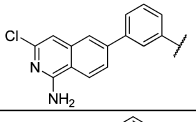
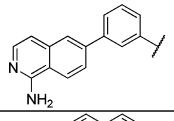
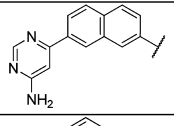
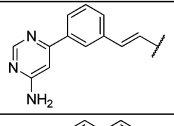
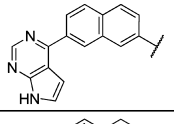
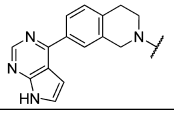
Table 1. Binding Affinities and Bacterial ThrRS Selectivity of 3, 4, and 10a−j



ID	R	ThrRS K_i (nM)				selectivity ratio
		E. coli	*B. thail*	*Y. pestis*	Human	Human/*E. coli*
3		13.1	2.8	9.8	13.4	1
4		36500	193000	33100	36800	1
10a		2.9	0.9	2.2	3.3	1.1
10b		10.7	2.9	8.2	10.1	0.9
10c		0.8	0.3	0.7	1.2	1.5
10d		17.2	12.2	9.3	72	4
10e		20	23	25	463	23
10f		90	89	115	2242	25
10g		2.7	0.7	2	3	1.1
10h		2.7	1.1	1.8	4.1	1.5
10i		2.4	0.6	1.8	1.8	0.8
10j		9.5	4	5.7	5.2	0.5

Val376, similar to the amino group in the adenine. In addition, atoms N1 and N3 formed H-bonds with Ser517 and Val376, respectively. The threonyl group bound to the Zn atom, as observed for Thr-AMS. The acylsulfonamide formed a H-bond network with its nearby residues, as predicted from the threonyl-AMS binding mode. The net result of all these interactions was that compound **10a** is a highly potent inhibitor of *E. coli* ThrRS (K_i of 2.9 nM).

Additional quinazoline analogues **10b−f**, together with a few rearranged skeletons, **10g−j**, were prepared to explore the structure−activity relationship (SAR) of the ATP binding pocket. Table 1 shows the ThrRS enzymatic binding activity and bacterial selectivity for these analogues. The addition of a C2 amino group, as in **10b**, resulted in weaker *E. coli* ThrRS binding. The cocrystal structure of **10b** with *E. coli* ThrRS showed that although this new amine group forms a hydrogen bond to the carbonyl of Leu373, this is a weak hydrogen bond due to poor geometry and length. Furthermore, compound **10b** may be paying a desolvation penalty. The incorporation of a chlorine atom at C2, as shown in **10c**, produced a significant improvement in potency. The distance from Cl to the carbonyl of Leu373 is 2.8 Å. This is shorter than the sum of the van der Waals radii of Cl and O (3.3 Å), indicating a halogen bond formed (Figure 4).[15] Compound **10d**, with a methyl group at C2, lost potency. The cocrystal structure of **10d** in *E. coli* ThrRS demonstrated that the quinazoline ring was rotated by 180°, avoiding a steric clash between the methyl group and Leu373. To accommodate the size of the quinazoline ring of **10d**, Glu365 was found in an alternative orientation (Figure 4). The binding affinity of **10d** could be partially attributed to the donor−acceptor H-bonds with Val376. More dramatic loss of potency was observed when a quinazoline was changed into an isoquinoline. Isoquinolines **10e** and **10f** lost 30−100-fold binding affinity, at least in part due to the loss of a H-bond with Ser517. Lastly, other nonquinazolines, **10g−j**, were examined, in which an aminopyrimidine or pyrrolopyrimidine replaced the aminoquinazoline ring, and a naphthyl-, a styryl-, or a tetrahydroisoquinolinyl moiety replaced the phenyl ring. These compounds retained good enzymatic potency, which indicates that different chemical scaffolds can be developed to fit tightly to the ATP binding pocket.

It is worth mentioning that **10e** and **10f**, though less potent, showed good selectivity for all bacterial ThrRS enzymes tested versus the human (cytoplasmic-1) ThrRS enzyme. These data suggest that the H-bond to Ser517 is more critical to binding to human ThrRS than to bacterial ThrRS and that a subtle change in the structure can achieve bacteria/human selectivity in the ATP binding site.

To explore the opportunity for selective bacterial ThrRS inhibitors and to fully understand the structural components necessary to achieve such a level of selectivity, we examined **11a−d**. These compounds, missing the critical H-bond to Ser517, were designed to have weaker potency against human ThrRS. Table 2 shows that compounds **11a−d** exploit human/bacterial differences in a unique and dramatic way. The cocrystal structure of **11d** bound to *E. coli* ThrRS shows that **11d** induced the expected conformational changes in the *E. coli* enzyme that are typically seen upon substrate binding, specifically the collapse of regions around both the threonine and ATP binding sites (Figure 5a). (The same collapse has been verified with the cocrystal structure of **11d** bound to the *B. thailandensis* enzyme.) The structure of **11d**−human ThrRS enzyme, however, showed that the human enzyme undergoes the comparable collapse around the threonine site, but *not* in the ATP site (Figure 5b).[16] These results demonstrate that the human and bacterial ATP sites are distinct when bound to this class of compounds. In particular, the conformation and lack of flexibility of Phe379, immobilized by Leu361, and therefore the inability of the polypeptide backbone (around Val376) to

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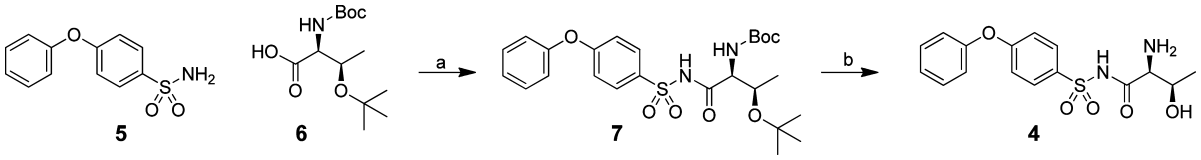
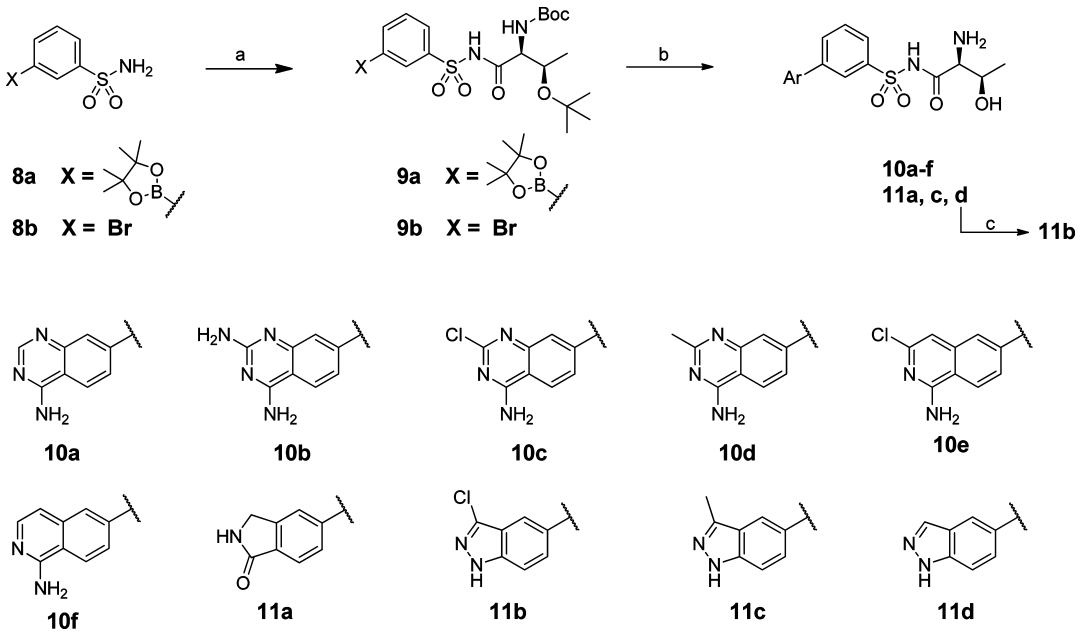
Scheme 1. Synthesis of 4[a]



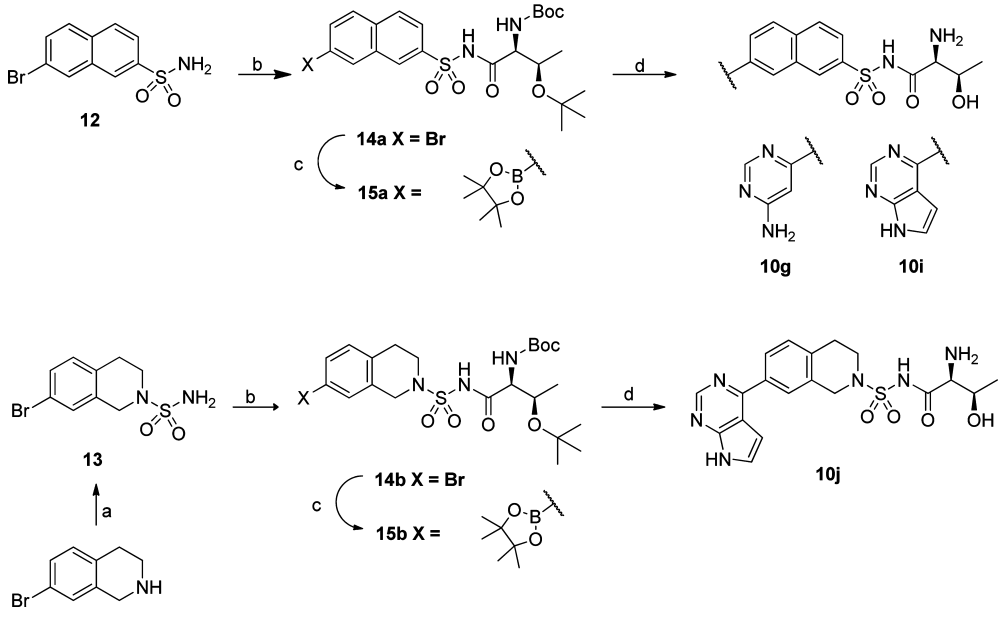
[a]Reagents and conditions: (a) EDCI, DBU, HOAT, DCM, rt, 2 h, 65%; (b) 4 N HCl in dioxane, rt, 30 min, 90%.

Scheme 2. Synthesis of Analogues 10a–f and 11a–d[a]



[a]Reagents and conditions: (a) **6**, EDCI, DBU, HOAT, DCM, rt, 2 h, 55–95%; (b) (i) $PdCl_2(TPP)_2$, ArBr (for **10a–f** and **11a**) or $ArB(OH)_2$ (for **11c,d**), Na_2CO_3, microwave 140 °C, 5 min; (ii) 4 N HCl in dioxane, 15–79%; (c) NCS, CH_3CN, 70 °C, 1 h, 68%.

Scheme 3. Synthesis of 10g,i,j[a]



[a]Reagents and conditions: (a) sulfamoyl chloride, DMA, 24 h, 43%; (b) **6**, EDCI, DBU, HOAT, DCM, rt, 2 h, 69.7%; (c) bis(pinacolato)diboron, $PdCl_2(dppf)CH_2Cl_2$, KOAc, DMF, 80 °C, 1 h, 60–80%; (d) (i) ArCl, $Pd(TPP)_4$, Na_2CO_3, dioxane, microwave 10 min, 130 °C; (ii) 4 N HCl in dioxane, 13–46% (two steps).

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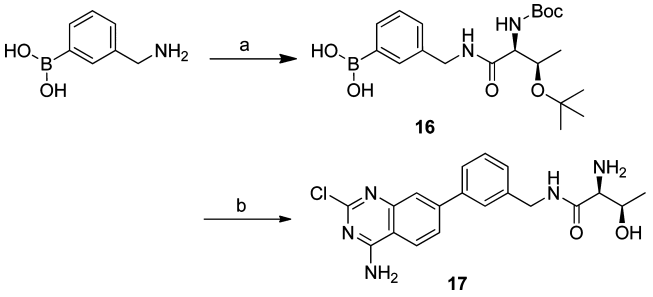
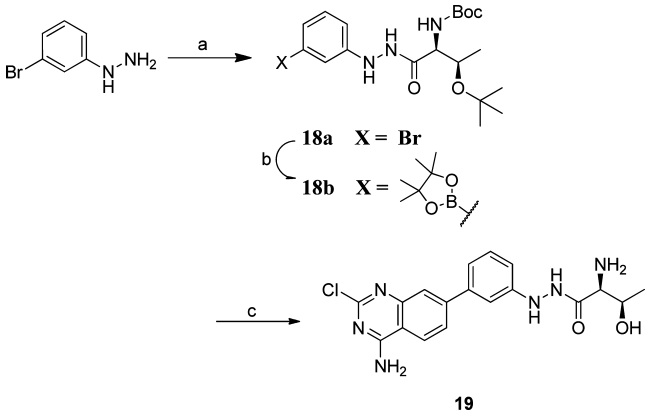
Scheme 4. Synthesis of 17[a]



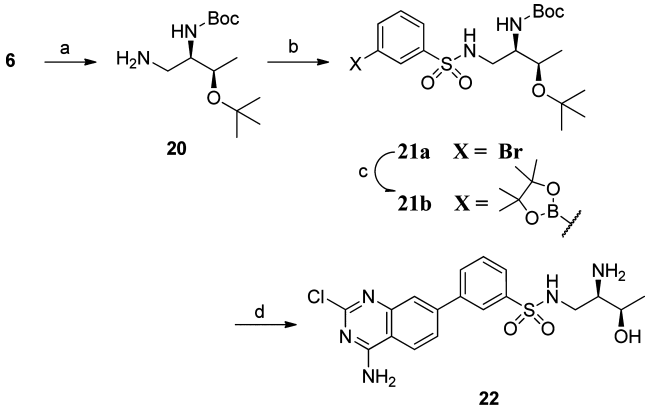
[a]Reagents and conditions: (a) **6**, EDCI, DBU, HOAT, DCM, rt, 12 h, 99%; (b) (i) $PdCl_2(TPP)_2$, 7-bromo-2-chloroquinazolin-4-amine, Na_2CO_3, microwave 140 °C, 6 min; (ii) 4 N HCl in dioxane, 12% (two steps).

Scheme 5. Synthesis of 19[a]



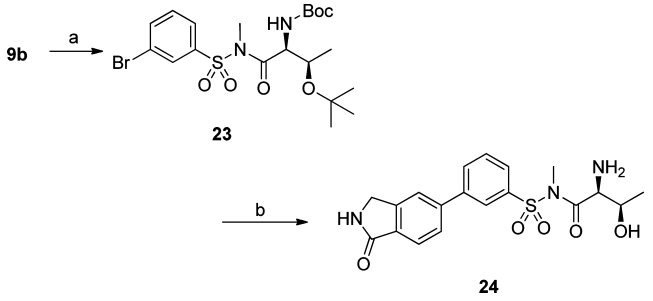
[a](a) **6**, ethyl chloroformate, *N*-methylmorpholine, THF, 15 min, 66%; (b) bis(pinacolato)diboron, $PdCl_2(dppf)CH_2Cl_2$, KOAc, DMF, 80 °C, 1 h, 44%; (c) (i) $PdCl_2(TPP)_2$, 7-bromo-2-chloroquinazolin-4-amine, Na_2CO_3, microwave 140 °C, 6 min; (ii) 4 N HCl in dioxane, 31% (two steps).

Scheme 6. Synthesis of 22[a]



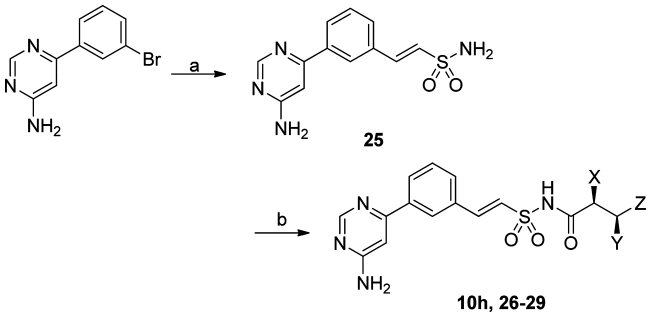
[a]Reagents and conditions: (a) (i) ethyl chloroformate, *N*-methylmorpholine, ammonium hydroxide, THF, rt, overnight, 96%; (ii) LAH, THF, 50 °C, 4 h, 96%; (b) 3-bromobenzenesulfonyl chloride, TEA, DCM, 2 h, 25%; (c) bis(pinacolato)diboron, $PdCl_2(dppf)$-CH_2Cl_2, KOAc, DMF, 80 °C, 1 h, crude; (d) (i) $PdCl_2(TPP)_2$, 7-bromo-2-chloroquinazolin-4-amine, Na_2CO_3, microwave 140 °C, 6 min, 22%; (ii) 4 N HCl in dioxane, 39%.

Scheme 7. Synthesis of 24[a]



[a]Reagents and conditions: (a) MeI, Cs_2CO_3, 65 °C, 6 h, DMF, 54%; (b) (i) $PdCl_2(TPP)_2$, $ArB(OH)_2$, Na_2CO_3, microwave 140 °C, 5 min, 55%; (ii) 4 N HCl in dioxane, 47%.

Scheme 8. Synthesis of 10h and 26−29[a]



[a]Reagents and conditions: (a) vinylsulfonamide, $Pd(OAc)_2$, TPP, DMF, microwave, 120 °C, 0.5 h, 69%; (b) (i) a corresponding acid, EDCI, DBU, HOAT, DCM, rt, 2 h; (ii) 4 N HCl in dioxane, rt, 30 min, 10−68% (two steps).

hydrogen bond with the indazole portion of the molecule in this context forced **11d** to adopt a much less energetically favorable binding mode in the human enzyme (Figure 6). As a consequence, **11d** is selective for bacterial ThrRS with a K_i >50 μM for the human ThrRS enzyme.

The same explanations account for the observed selectivity for **10e,f**. When the H-bond between N1 and Ser517 is replaced by the repulsive interactions between CH and Ser517 as in **10e,f**, the isoquinoline ring will rotate to minimize the undesired interactions. As anticipated, bacterial ThrRS enzymes can accommodate such a rotation better than the human ThrRS enzyme. These results represented an important proof-of-concept that bacterial ThrRS can be targeted with good selectivity, and that the ATP site is likely the best region to achieve this.

The SAR around the threonylsulfonamide moiety has been studied. Cocrystal structures of a number of our inhibitors indicated that the acylsulfonamide, engaged in several H-bonds with nearby residues, appeared indispensable to the binding of the inhibitors (Figure 7). The relative contribution of each of the acylsulfonamide components to binding was examined with the synthesis of **17**, **19**, **22**, and **24** (Table 3). Using **10c** and **11a** as reference compounds in this study, we replaced the SO_2 group with a CH_2 group as in **17** and with an NH as in **19**. Both changes caused >100-fold reduction in enzymatic potency compared to that of **10c**. Removal of the threonine carbonyl group as in **22** abolished all activities. The NH of the acylsulfonamide was "masked" with a methyl group as in **24**. This change also abolished all activity seen in **11a**. These data

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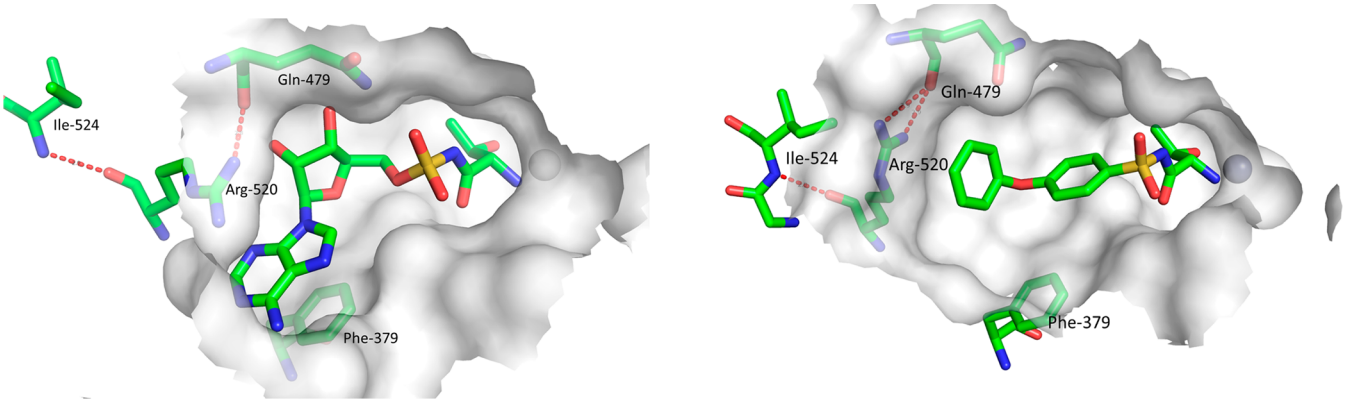


Figure 2. Cocrystal structures of **3** (left) and **4** (right) in *E. coli*. ThrRS. The cocrystal structure of **3** in *E. coli* ThrRS reveals that the side chain containing Arg520 folds down to be closer to the adenosine moiety to form sandwiched interactions together with Phe379 (the "closed form"). In contrast, the apo form of the enzyme adapts a more open form in which Arg520 is engaged in H-bonds with Ile524 and Gln479. The 4-phenoxy moiety in **4**, due to a lack of H-bond interactions as in adenine, is largely disordered upon binding to the enzyme, which allows the ATP binding site to remain "open".

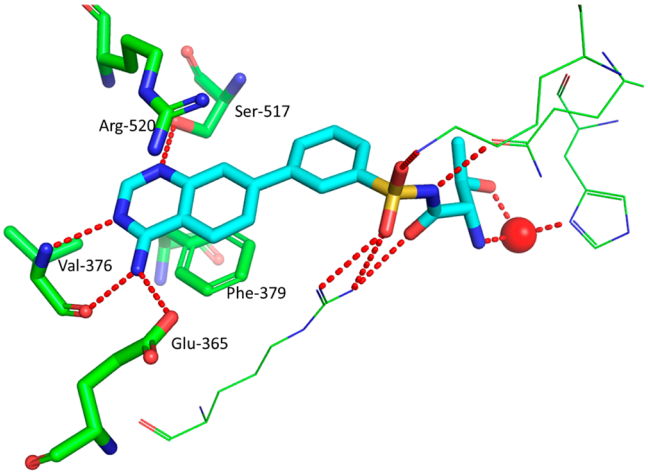


Figure 3. Part of the H-bond networks of compound **10a** in *E. coli* ThrRS. Residues that interact with the quinazoline ring are shown in bold.

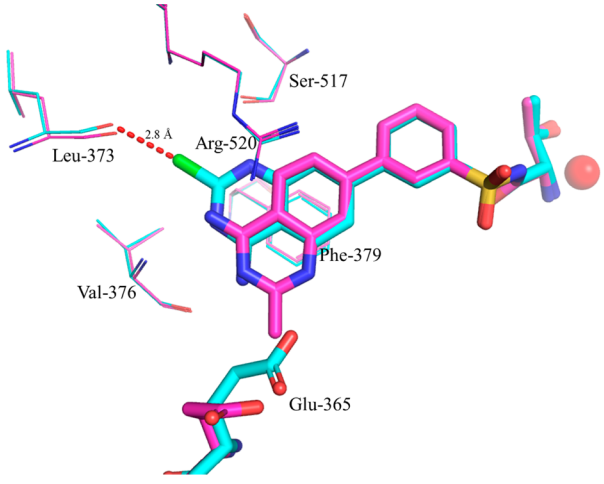
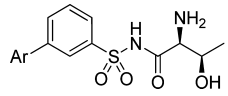


Figure 4. Overlay of cocrystal structures **10c** (aqua) and **10d** (cyan). The Glu365 (shown bold) residue moves in the presence of **10d**. The halogen bond is indicated with a dashed line.

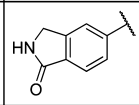
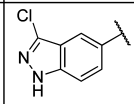
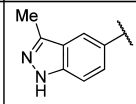
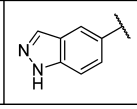
reiterated the importance of acylsulfonamide to the binding affinities of our ThrRS inhibitors. The carbonyl group appeared particularly critical, possibly due to its roles in mediating the pK_a of both threonine and acylsulfonamide.

Finally, analogues that outline the importance of the Zn chelating portion of our ThrRS inhibitors were prepared. Although it is generally believed that ThrRS enzymes recognize L-threonine as a substrate, in our study we have expanded the SAR surrounding the methyl group of L-threonine. The corresponding diamine and diol had also been evaluated. Table 4 summarizes the results for these compounds. These data showed that there is a limitation in the Zn binding pocket to accommodate size, with threonine being the optimal followed by ethylthreonine **26** and isopropylthreonine **27**. The human (cytoplasmic-1) and *B. thailandensis* ThrRS enzymes are particularly sensitive to the size of the head piece, which offers an opportunity for selectivity optimization. The recognition of threonine analogues by ThrRS appeared to be specific in this study, as compounds **28** and **29** showed no appreciable enzymatic activity.

Table 2. Binding Affinity and Improved Bacterial Selectivity of 11a–d



11a-d

ID	Ar	ThrRS K_i (nM)				selectivity ratio
		E. coli	*B. thail.*	*Y. pestis*	Human	Human/*E. coli*
11a		7.7	7.4	3.9	91	12
11b		52	77	39	1518	29
11c		127	143	83	5820	46
11d		182	164	132	>50,000	>270

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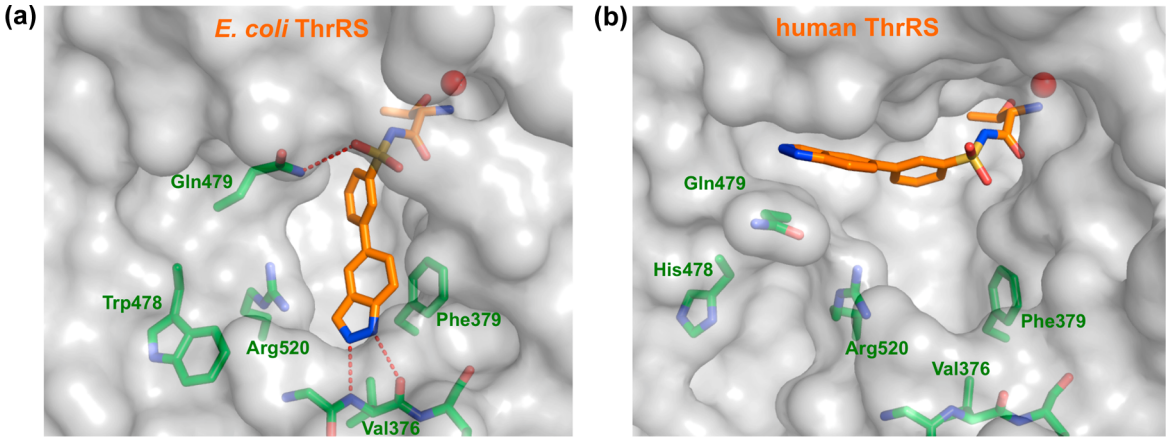


Figure 5. Phe379 is more flexible in the bacterial ThrRS enzymes such that it can shift slightly away from the active site and accommodate **11d** (a). In the human ThrRS orthologue (b), Phe379 is unable to shift, and the inhibitor is forced to adopt a very different and less energetically favorable conformation.

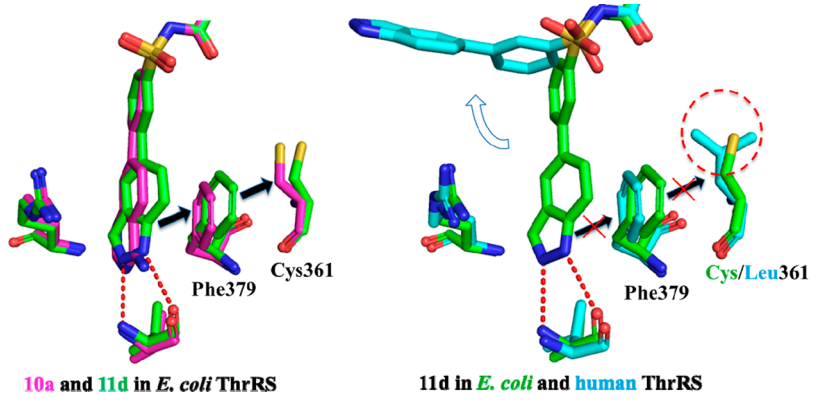


Figure 6. Bacterial selectivity is due to differences in underlying ThrRS residues that prevent Phe379 movement to accommodate **11d**. The tilted conformation of selective inhibitors, such as **11d**, presses tightly against the sides of the ATP pocket (compared with the conformation of the nonselective **10a**). In the bacterial enzymes, Phe379 can accommodate this tilt due to the presence of a less rigid underlying residue, Cys361. In the human orthologue, however, the underlying residue Leu361 prevents movement of Phe379 and the inhibitor must find an alternate (less energetically favorable) conformation.

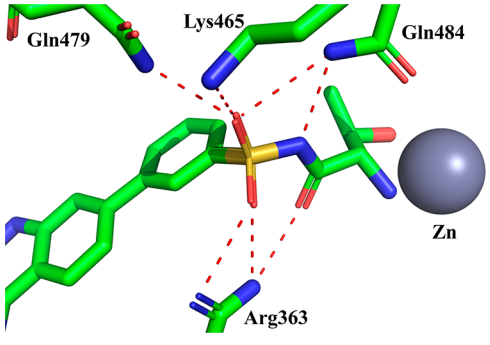
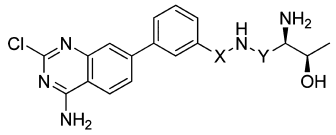


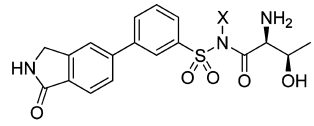
Figure 7. Acylsulfonamide H-bond network.

Through these SAR studies, we were able to evaluate antimicrobial activity for a few potent ThrRS inhibitors. This was carried out through measurement of antimicrobial activity against a panel of Gram-negative wild-type strains. In addition, antibacterial activity was measured versus *E. coli imp*, *E. coli tolC*, and a *B. thailandensis* pump knockout strain to measure the impact of permeability and efflux pumps on antimicrobial activity. Table 5 shows the minimal inhibitory concentration (MIC) values against clinically important pathogens and BSL2 surrogate strains of important biodefense pathogens. Compared

Table 3. Binding Affinities for 10c, 11a, 17, 19, 22, and 24



10c, X = SO_2, Y = C=O
17, X = CH_2, Y = C=O
19, X = NH, Y = C=O
22, X = SO_2, Y = CH_2



11a, X = H
24, X = CH_3

ID	ThrRS K_i (nM)			
	E. coli	*B. thailandensis*	*Y. pestis*	human
10c	0.8	0.3	0.7	1.2
17	329	140	436	935
19	136	34	70	399
22	>50 μM	>50 μM	>50 μM	>50 μM
11a	7.7	7.4	3.9	91.0
24	>50 μM	>50 μM	>50 μM	>50 μM

to threonyl-AMS (**3**), the newly identified ThrRS inhibitors showed improved antibacterial activity against wild-type *H. influenzae*, *E. coli tolC*, and *B. thailandensis* pump mutant strains, although all compounds had poor antimicrobial activity against wild-type strains of *E. coli* and *B. thailandensis*. As these

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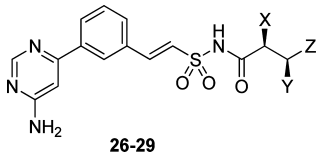
Table 4. ThrRS Inhibitors Bearing Different Head Pieces



26-29

				ThrRS K_i (nM)			
ID	X	Y	Z	*E. coli*	*B. thailandensis*	*Y. pestis*	human
10h	NH_2	OH	CH_3	2.7	1.1	1.8	4.1
26	NH_2	OH	C_2H_5	4.3	14.1	2.7	32
27	NH_2	OH	*i* -Pr	32.4	588	22.5	601
28	OH	OH	CH_3	>50 μM	>50 μM	>50 μM	>50 μM
29	NH_2	NH_2	CH_3	>50 μM	2262	>50 μM	12576

Table 5. MIC Values of Representative Compounds[a]

	MIC (μg/mL)					
ID	*E. coli*	*E. coli imp*	*E. coli tolC*	*H. influenzae*	*B. thail.*	*B. thail. (KO)*
3	>128	>128	>128	>128	>128	64
10a	>128	>128	64	16	>128	8
10b	>128	>128	64	32	>128	4
10c	>128	128	16	8	>128	32
10g	>128	>128	64	4	>128	4
10h	>128	>128	64	8	>128	32
10i	>128	>128	16	16	>128	32

[a]Values that are ≤32 μg/mL are shaded light blue.

compounds have significantly increased activity on the pump-deficient strains, they are likely efficient substrates for efflux pumps and are more limited in this respect than in their ability to permeate cellular membranes. Further structural modifications will be needed to enhance the antimicrobial activity of the inhibitors.

In summary, we have identified a series of ThrRS synthetase inhibitors using SBDD. These compounds bind to the substrate binding site of ThrRS synthetase and show excellent binding affinities for the bacterial enzymes. Several compounds possess greatly improved bacterial selectivity. Key residues responsible for potency and bacteria/human ThrRS selectivity have been identified. Improvements in antimicrobial activity, as compared to AMS, have been achieved against *H. influenzae*. Further work is directed toward the improvement of potency and the expansion of the antibacterial spectrum. These results will be reported in due course.

EXPERIMENTAL SECTION

General Procedures. Flash chromatography was performed on silica gel (280 mesh). ^{1}H NMR spectra were obtained with a Bruker 300 MHz spectrometer equipped with a 5 mm QNP probe or a Bruker 600 MHz spectrometer equipped with a Bruker BioSpin TCI 1.7 mm MicroCryoProbe. Chemical shifts are reported in parts per million relative to tetramethylsilane, and the following multiplicity abbreviations were used: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. HRES-LC/MS was conducted on a Micromass Q-TOF Premier (quadrupole time-of-flight) mass spectrometer (Beverly, MA) coupled with an Agilent 1100 (Santa Clara, CA) HPLC system equipped with a UV diode array detector monitoring 254 nm. HPLC was equipped with a Phenomenex kinetex-C18 column (100 × 3.0, 2.6 μm). Unless indicated otherwise, reagents and solvents were obtained from commercial sources and used as received.

General Method A for the Synthesis of Acylsulfonamide. A solution of **6** (1.65 g, 6.0 mmol), HOAT (0.817 g, 6.0 mmol), and EDC (1.15 g, 6.0 mmol) in DCM (20 mL) was stirred at room temperature for 30 min, followed by the addition of sulfonamide (4.0 mmol) and DBU (1.81 mL, 12 mmol). Reaction was monitored by LC/MS and was completed in 2 h. The reaction mixture was diluted with EtOAc, washed with 1.0 N HCl and water, dried over Na_2SO_4, concentrated, and either used as is or purified by flash chromatography (0−100% ethyl acetate/hexane). Yield: 55−95%.

General Method B for Boc Deprotection. A solution of Boc-protected threonylacylsulfonamide (0.063 mmol) was added to 4.0 N HCl in dioxane (0.5 mL) at room temperature. The reaction was stirred for 2 h, then stripped of volatiles, and purified by RPLC (10−50% ACN in water) to get the desired compound as a TFA salt. Yield: 30−97%.

General Method C for the Suzuki Coupling Reaction. A flask was charged with **9a** (108 mg, 0.20 mmol), ArCl (0.20 mmol), bis(triphenylphosphine)palladium(II) chloride (7.02 mg, 0.010 mmol), and aq sodium carbonate (0.2 mL, 1.0 M aq), vacuumed, flushed with N_2 five times, and then heated in the microwave at 140 °C for 6 min. The crude mixture was neutralized with acetic acid (1.0 N), concentrated, and purified by RPLC (0−90% ACN in water). Yield: 15−87%.

(2*S*,3*R*)-2-Amino-3-hydroxy-*N*-((4-phenoxyphenyl)sulfonyl)-butanamide (4). Using methods A and B, the title compound was synthesized in 58% yield in two steps from **6**. LC: 98%. ^{1}H NMR (600 MHz, DMSO): δ 8.01 (br, 3H), 7.85 (t, J = 16.6 Hz, 2H), 7.45 (t, J = 7.7 Hz, 2H), 7.23 (dd, J = 18.1, 10.8 Hz, 1H), 7.09 (t, J = 9.8 Hz, 2H), 7.03 (t, J = 13.0 Hz, 2H), 3.95 (dd, J = 22.0, 16.0 Hz, 1H), 3.47 (dd, J = 21.2, 16.9 Hz, 1H), 1.07 (d, J = 6.3 Hz, 3H). HRMS (ESI): m/z calcd for $C_{16}H_{18}N_2O5S$ $(M + H)^+$ 351.1014, found 351.1013.

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***tert*-Butyl ((2*S*,3*R*)-3-(*tert*-Butoxy)-1-oxo-1-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzenesulfonamido)butan-2-yl)carbamate (9a).** Following general method A, **9a** (1.05 g) was produced from **8a** (0.99 g, 55%). ^{1}H NMR (300 MHz, DMSO): δ 12.05 (br, 1H), 8.23 (s, 1H), 8.16 (s, 1H), 8.06 (s, 2H), 7.64 (br, 2H), 7.37 (s, 1H), 6.06 (s, 1H), 3.93 (m, 1H), 3.34 (m, 1H), 1.35 (m, 21H), 1.10 (s, 3H), 0.91 (s, 9H).

***tert*-Butyl ((2*S*,3*R*)-1-(3-Bromobenzenesulfonamido)-3-(*tert*-butoxy)-1-oxobutan-2-yl)carbamate (9b).** Following general method A, **9b** (1.87 g) was produced from **8b** (0.94 g, 95%). ^{1}H NMR (300 MHz, DMSO): δ 12.21 (br, 1H), 8.04 (s, 1H), 7.94 (d, J = 8.0 Hz, 2H), 7.87−7.47 (t, J = 7.0 Hz, 1H), 6.13 (d, J = 9.2 Hz, 1H), 4.35−3.61 (m, 1H), 3.21 (m, 1H), 1.37 (s, 9H), 0.97 (m, 12H). HRMS (ESI): m/z calcd for $C_{19}H_{29}BrN_2O_6S$ (M + H)$^+$ 493.1008, found 493.1029.

((2*S*,3*R*)-2-Amine-*N*-((3-(4-aminoquinazolin-7-yl)phenyl)-sulfonyl)-3-hydroxybutanamide (10a). Using methods C and B, **10a** was produced from **9a** (79%). LC: 99%. ^{1}H NMR (300 MHz, DMSO): δ 9.81 (br, 2H), 8.89 (s, 1H), 8.55 (d, J = 8.7 Hz, 1H), 8.27 (s, 1H), 8.13 (d, J = 8.7 Hz, 1H), 8.07−8.01 (m, 2H), 7.99 (d, J = 8.0 Hz, 1H), 7.84 (br, 2H), 7.72 (t, J = 7.8 Hz, 1H), 4.01−3.87 (m, 1H), 3.25 (m, 1H), 1.10 (d, J = 6.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{19}N_5O_4S$ (M + H)$^+$ 402.1235, found 402.1245.

(2*S*,3*R*)-2-Amino-*N*-((3-(2,4-diaminoquinazolin-7-yl)phenyl)-sulfonyl)-3-hydroxybutanamide (10b). Using methods C and B, **10b** was produced from **9a** (41%). LC: 96%. ^{1}H NMR (600 MHz, DMSO): δ 9.10 (s, 1H), 8.87 (s, 1H), 8.49−8.28 (m, 1H), 8.17 (s, 1H), 7.86 (m, 2H), 7.82−7.67 (m, 3H), 7.63 (s, 1H), 7.48 (s, 1H), 7.35−7.06 (m, 1H), 3.94 (m, 1H), 1.08 (d, J = 6.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{20}N_6O_4S$ (M + H)$^+$ 417.1345, found 417.1350.

(2*S*,3*R*)-2-Amino-*N*-((3-(4-amino-2-chloroquinazolin-7-yl)-phenyl)sulfonyl)-3-hydroxybutanamide (10c). Using methods C and B, **10c** was produced from **9a** (15%). LC: 93%. ^{1}H NMR (300 MHz, DMSO): δ 8.45 (br, 1H), 8.38 (d, J = 9.0 Hz, 1H), 8.27 (s, 1H), 8.05 (d, J = 8.2 Hz, 1H), 7.84 (m, 3H), 7.69 (t, J = 7.7 Hz, 1H), 4.05−3.77 (m, 1H), 3.5 (m, 1H), 1.10 (d, J = 6.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{18}ClN_5O_4S$ (M + H)$^+$ 436.0846, found 436.0839.

((2*S*,3*R*)-2-Amino-*N*-((3-(4-amino-2-methylquinazolin-7-yl)-phenyl)sulfonyl)-3-hydroxybutanamide (10d). Using methods C and B, **10d** was produced from **9a** (55%). LC: 99%. ^{1}H NMR (CD_3OD): δ 8.34 (m, 1H), 8.3 (d, J = 7.2 Hz, 1H), 8.01 (d, J = 6 Hz, 1H), 7.89 (m, 2H), 7.81 (dd, J_1 = 9 Hz, J_2 = 3 Hz, 1H), 7.63 (t, J = 9 Hz, 1H), 4.13 (m, 1H), 3.56 (m, 1H), 2.50 (s, 3H), 1.25 (d, J = 6 Hz, 3H). HRMS (ESI): m/z calcd for $C_{19}H_{21}N_5O_4S$ (M + H)$^+$ 416.1392, found 416.1390.

(2*S*,3*R*)-2-Amino-*N*-((3-(1-amino-3-chloroisoquinolin-6-yl)-phenyl)sulfonyl)-3-hydroxybutanamide (10e). Using methods C and B, **10e** was produced from **9a** (15%). LC: 85%. ^{1}H NMR (300 MHz, DMSO): δ 8.36 (d, J = 9.0 Hz, 1H), 8.30 (s, 1H), 8.18 (m, 3H), 8.02 (m, 2H), 7.81 (d, J = 6.0 Hz, 2H), 7.10 (s, 1H), 4.05 (m, 1H), 3.76 (m, 1H), 1.11 (d, J = 6.3 Hz, 3H). HRMS (ESI): m/z calcd for $C_{19}H_{19}ClN_4O_4S$ (M + H)$^+$ 435.0894, found 435.0905.

(2*S*,3*R*)-2-Amino-*N*-((3-(1-aminoisoquinolin-6-yl)phenyl)-sulfonyl)-3-hydroxybutanamide (10f). Using methods C and B, **10f** was produced from **9a** (36%). LC: 98%. ^{1}H NMR (300 MHz, DMSO): δ 13.54 (s, 1H), 9.33 (s, 2H), 8.79 (d, J = 8.7 Hz, 1H), 8.37 (s, 2H), 8.19 (m, 4H), 8.05 (d, J = 8.0 Hz, 1H), 7.87−7.66 (m, 2H), 7.33 (t, J = 11.5 Hz, 1H), 4.17−3.92 (m, 1H), 3.76 (br, 1H), 1.14 (d, J = 6.6 Hz, 3H). HRMS (ESI): m/z calcd for $C_{19}H_{20}N_4O_4S$ (M + H)$^+$ 401.1283, found 401.1292.

***tert*-Butyl ((2*S*,3*R*)-1-(7-Bromonaphthalene-2-sulfonamido)-3-(*tert*-butoxy)-1-oxobutan-2-yl)carbamate (14a).** Following general method A, **14a** (0.192 g) was produced from **12** (0.145 g, 69.7%). LC: 90%. ^{1}H NMR (300 MHz, DMSO): δ 12.19 (s, 1H), 8.64 (s, 1H), 8.55 (s, 1H), 8.17 (d, J = 8.8 Hz, 1H), 8.03 (d, J = 8.8 Hz, 1H), 7.95 (d, J = 8.7 Hz, 1H), 7.85 (dd, J = 8.8, 1.9 Hz, 1H), 6.08 (d, J = 9.3 Hz, 1H), 4.04−3.91 (m, 1H), 3.91−3.71 (m, 1H), 1.33 (s, 9H), 0.95 (d, J = 5.7 Hz, 3H), 0.89 (s, 9H). HRMS (ESI): m/z calcd for $C_{23}H_{31}BrN_2O_6S$ (M + H)$^+$ 543.1164, found 543.1174.

***tert*-Butyl ((2*S*,3*R*)-3-(*tert*-Butoxy)-1-oxo-1-(7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)naphthalene-2-sulfonamido)-butan-2-yl)carbamate (15a).** A solution of bis(pinacolato)diboron (0.373 g, 1.5 mmol), $PdCl_2(dppf)CH_2Cl_2$(cat.), KOAc (0.360 g, 3.67 mmol), and **14a** (0.665 g, 1.224 mmol) in DMF (7.0 mL) was flushed with argon and heated at 80 °C for 2.5 h in a sealed tube. The reaction mixture was diluted with ethyl acetate and washed with water. The organic layer was separated and concentrated to a solid (0.58 g, 80%) which was used directly in the next reaction.

(2*S*,3*R*)-2-Amino-*N*-((7-(6-aminopyrimidin-4-yl)naphthalen-2-yl)sulfonyl)-3-hydroxybutanamide (10g). Using methods C and B, **10g** was produced from **15a** (45.8%). LC: 94%. ^{1}H NMR (300 MHz, DMSO): δ 8.89 (s, 1H), 8.75 (m, 2H), 8.64 (br, 1H), 8.27 (t, J = 7.9 Hz, 2H), 8.18 (m, 3H), 8.05 (dd, J = 8.7, 1.7 Hz, 1H), 7.21 (s, 1H), 4.10−3.94 (m, 1H), 3.72 (m, 1H), 1.10 (d, J = 6.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{19}N_5O_4S$ (M + H)$^+$ 402.1236, found 402.1230.

***(E)*-2-(3-(6-Aminopyrimidin-4-yl)phenyl)ethenesulfonamide (25).** Triphenylphosphine (0.03 equiv), 6-(3-bromophenyl)pyrimidin-4-amine (0.1 g, 1.0 equiv), diacetoxypalladium (0.03 equiv), ethenesulfonamide (1.2 equiv), and TEA (1.2 equiv) were mixed in DMF (1.5 mL) and degassed. The reaction was microwaved at 120 °C for 1 h. The reaction mixture was concentrated and purified by column chromatography, eluting with ethyl acetate, to give the title compound as a white solid (69%). ^{1}H NMR (DMSO-d_6): δ 8.60 (s, 1H), 8.13 (s, 1H), 7.93 (d, J = 9 Hz, 1H), 7.83 (d, J = 7.5 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.63 (br, 2H), 7.50 (d, J = 15.6 Hz, 1H), 7.25 (d, J = 15.0 Hz, 1H), 7.10 (s, 1H). 6.03 (br, 2H).

(2*S*,3*R*)-2-Amino-*N*-(((*E*)-3-(6-aminopyrimidin-4-yl)styryl)-sulfonyl)-3-hydroxybutanamide (10h). Following general methods A and B, the title compound was produced as a white solid from **25** (68%). HPLC: 99%. ^{1}H NMR (CD_3OD): δ 8.66 (s, 1H), 8.15 (s, 1H), 7.94 (t, J = 9 Hz, 2H), 7.8 (d, J = 15 Hz, 1H), 7.70 (t, J = 6.0 Hz, 2H), 7.50 (d, J = 15.6 Hz, 1H), 7.07 (s, 1H), 4.18 (m, 1H), 3.72 (d, J = 4.5, 1H), 1.34 (d, J = 6.0 Hz, 3H). HRMS (ESI): m/z calcd for $C_{16}H_{19}N_5O_4S$ (M + H)$^+$ 378.1235, found 378.1232.

(2*S*,3*R*)-*N*-((7-(7*H*-Pyrrolo[2,3-*d*]pyrimidin-4-yl)naphthalen-2-yl)sulfonyl)-2-amino-3-hydroxybutanamide (10i). Using the same procedure as for the synthesis of **10g**, **10i** was produced from **15a** (13%). HPLC: 99%. ^{1}H NMR (600 MHz, DMSO): δ 9.05 (s, 1H), 8.93 (d, J = 5.0 Hz, 2H), 8.54 (d, J = 8.4 Hz, 1H), 8.24 (t, J = 9.5 Hz, 2H), 8.15 (br, 3H), 8.00 (d, J = 8.4 Hz, 1H), 7.76 (s, 1H), 7.21 (s, 1H), 5.5 (br, 1H), 4.07−3.97 (m, 1H), 3.73 (m, 1H), 1.09 (d, J = 6.3 Hz, 3H). HRMS (ESI): m/z calcd for $C_{20}H_{19}N_5O_4S$ (M + H)$^+$ 426.1236, found 426.1236.

7-Bromo-3,4-dihydroisoquinoline-2(1*H*)-sulfonamide (13). A solution of 7-bromo-1,2,3,4-tetrahydroisoquinoline (0.424 g, 2.0 mmol) in DMA (3 mL) was cooled to 0 °C. To this was added sulfamoyl chloride (0.254 g, 2.2 mmol) dissolved in DMA (2.0 mL). The reaction mixture was stirred at room temperature overnight and was purified by RPLC as a white solid (43%). HPLC: 89%. ^{1}H NMR (300 MHz, DMSO): δ 7.43 (s, 1H), 7.41−7.30 (d, J = 9.0 Hz, 1H), 7.14 (d, J = 9.0 Hz, 1H), 6.94 (s, 2H), 4.21 (s, 2H), 3.26 (t, J = 5.9 Hz, 2H), 2.94−2.79 (t, J = 6.0 Hz, 2H). HRMS (ESI): m/z calcd for $C_9H_{11}BrN_2O_2S$ (M + H)$^+$ 290.9803, found 290.9805.

***tert*-Butyl ((2*S*,3*R*)-1-(7-Bromo-1,2,3,4-tetrahydroisoquinoline-2-sulfonamido)-3-(*tert*-butoxy)-1-oxobutan-2-yl)-carbamate (14b).** Using procedure A, **14b** was synthesized from **13** (69.4%). This compound was used without further handling.

***tert*-Butyl ((2*S*,3*R*)-3-(*tert*-butoxy)-1-oxo-1-(7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1,2,3,4-tetrahydroisoquinoline-2-sulfonamido)butan-2-yl)carbamate (15b).** Using the same procedure as for the synthesis of **15a**, **15b** was synthesized (59.7%) and was used without further handling.

(2*S*,3*R*)-*N*-((7-(7*H*-Pyrrolo[2,3-*d*]pyrimidin-4-yl)-3,4-dihydroisoquinolin-2(1*H*)-yl)sulfonyl)-2-amino-3-hydroxybutanamide (10j). Using methods C and B, **10j** was produced from **15b** as a HCl salt (17%). HPLC: 77%. ^{1}H NMR (300 MHz, DMSO): δ 13.08 (s, 1H), 12.46 (s, 1H), 9.01 (s, 1H), 8.39 (br, 3H), 8.00 (d, J = 7.6 Hz, 2H), 7.92 (s, 1H), 7.49 (d, J = 8.1 Hz, 1H), 7.13 (s, 1H), 4.71 (q, J = 15.7 Hz, 2H), 4.17−3.96 (m, 1H), 3.84 (br, 1H), 3.75−3.51 (m, 2H),

1.20 (t, J = 6.0 Hz, 3H). HRMS (ESI): m/z calcd for $C_{19}H_{22}N_6O_4S$ $(M + H)^+$ 431.1501, found 431.1498.

(2*S*,3*R*)-2-Amino-3-hydroxy-*N*-((3-(1-oxoisoindolin-5-yl)-phenyl)sulfonyl)butanamide (11a). Using methods C and B, **11a** was produced from **9a** as a HCl salt (36%). HPLC: 99%. ^{1}H NMR (300 MHz, DMSO): δ 8.67 (s, 1H), 8.25 (m, 4H), 8.09 (d, J = 7.8 Hz, 1H), 7.99 (d, J = 7.9 Hz, 1H), 7.92 (s, 1H), 7.87−7.65 (m, 3H), 4.47 (s, 2H), 4.16−3.94 (m, 1H), 3.84 (s, 1H), 1.11 (d, J = 6.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{19}N_3O_5S$ $(M + H)^+$ 390.1123, found 390.1127.

(2*S*,3*R*)-2-Amino-3-hydroxy-*N*-((3-(3-methyl-1*H*-indazol-5-yl)phenyl)sulfonyl)butanamide (11c). Using methods C and B, **11c** was produced from **9b** as a TFA salt (18%). LC: 99%. ^{1}H NMR (300 MHz, DMSO): δ 8.21 (br, 1H), 8.05 (d, J = 5.2 Hz, 2H), 8.03 (s, 1H), 7.88 (d, J = 8.3 Hz, 1H), 7.72 (s, 1H), 7.70 (s, 1H), 7.67 (dd, J = 4.3, 2.2 Hz, 1H), 7.65 (m, 1H), 7.61 (s, 1H), 7.58 (s, 1H), 4.08−3.91 (m, 1H), 3.61 (m, 1H), 2.57 (s, 3H), 1.10 (d, J = 6.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{20}N_4O_4S$ $(M + H)^+$ 389.1283, found 389.1281.

***tert*-Butyl ((2*S*,3*R*)-1-(3-(1*H*-Indazol-5-yl)-benzenesulfonamido)-3-(*tert*-butoxy)-1-oxobutan-2-yl)-carbamate (11d).** Using methods C and B, **11d** was produced from **9b** as a TFA salt (29%). HPLC: 98%. ^{1}H NMR (600 MHz, DMSO): δ 8.13 (s, 2H), 8.04 (s, 1H), 7.86 (br, 3H), 7.91−7.70 (m, 2H), 7.65 (s, 2H), 7.58 (t, J = 7.6 Hz, 1H), 4.01−3.84 (m, 1H), 3.48 (d, J = 11.0 Hz, 1H), 1.14−0.95 (d, J = 6.0 Hz, 3H). HRMS (ESI): m/z calcd for $C_{17}H_{18}N_4O_4S$ $(M + H)^+$ 375.1127, found 375.1138.

(2*S*,3*R*)-2-Amino-*N*-((3-(3-chloro-1*H*-indazol-5-yl)phenyl)-sulfonyl)-3-hydroxybutanamide (11b). A solution of **11d** (0.083 mmol) and NCS (0.014 g, 0.11 mmol) in ACN (3 mL) was heated to 70 °C for 1 h. The reaction was cooled to room temperature and was concentrated to an oil. The product was purified by RPLC to give the desired product as a TFA salt (68%). HPLC: 99%. ^{1}H NMR (300 MHz, DMSO): δ 13.43 (s, 1H), 8.16 (s, 1H), 7.89 (d, J = 10.6 Hz, 2H), 7.83 (m, 4H), 7.72 (d, J = 9.0 Hz, 1H), 7.58 (t, J = 7.7 Hz, 1H), 4.01−3.82 (m, 1H), 3.5 (m, 1H), 1.10 (d, J = 6.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{17}H_{17}ClN_4O_4S$ $(M + H)^+$ 409.0737, found 409.0733.

(3-(((2*S*,3*R*)-3-(*tert*-Butoxy)-2-((*tert*-butoxycarbonyl)amino)-butanamido)methyl)phenyl)boronic acid (16). To a round-bottom flask charged with **6** (716 mg, 2.6 mmol), (3-(aminomethyl)-phenyl)boronic acid hydrochloride (375 mg, 2.00 mmol), HOAT (272 mg, 2.00 mmol), and diisopropylethylamine (1.4 mL, 8.0 mmol) in DCM (6.7 mL) was added EDCI (767 mg, 4.00 mmol) in one portion at room temperature. LC/MS after 12 h showed complete conversion. Workup: the reaction was concentrated and purified by reversed-phase liquid chromatography (5−100% acetonitrile/water). The yield of the product as a colorless foam was 99% (810 mg). ^{1}H NMR (300 MHz, DMSO): δ 8.21 (t, J = 5.6 Hz, 1H), 7.98 (s, 2H), 7.74−7.62 (m, 2H), 7.38−7.18 (m, 2H), 6.05 (d, J = 8.9 Hz, 1H), 4.33 (dd, J = 14.8, 5.9 Hz, 1H), 4.21 (dd, J = 14.8, 5.2 Hz, 1H), 3.92 (d, J = 9.4 Hz, 2H), 1.40 (s, 9H), 1.07 (s, 9H), 1.02 (d, J = 5.8 Hz, 3H). HRMS (ESI): m/z calcd for $C_{20}H_{33}BN_2O_6$ $(M + H)^+$ 409.2510, found 409.2523.

(2*S*,3*R*)-2-Amino-*N*-(3-(4-amino-2-chloroquinazolin-7-yl)-benzyl)-3-hydroxybutanamide (17). Using general methods B and C, the title compound was synthesized from **16** as a HCl salt in 12% combined yield for two steps. HPLC: 94%. ^{1}H NMR (300 MHz, DMSO): δ 9.06 (s, 1H), 8.71 (s, 1H), 8.37 (m, 1H), 8.16 (s, 2H), 7.88 (m, 1H), 7.84 (m, 2H), 7.49 (m, 3H), 5.61 (br, 1H), 4.48 (m, 1H), 3.96 (m, 1H), 3.51 (m, 2H), 1.19 (d, J = 6.0 Hz, 3H). HRMS (ESI): m/z calcd for $C_{19}H_{21}ClN_5O_2$ $(M + H)^+$ 386.1384, found 386.1390.

***tert*-Butyl ((2*S*,3*R*)-1-(2-(3-Bromophenyl)hydrazinyl)-3-(*tert*-butoxy)-1-oxobutan-2-yl)carbamate (18a).** A round-bottom flask charged with **6** (275 mg, 1.00 mmol) and ethyl chloroformate (115 μL, 1.2 mmol) was treated with 4-methylmorpholine (132 μL, 1.2 mmol) dropwise over 5 min. After the solution was stirred for 5 min at room temperature, (3-bromophenyl)hydrazine hydrochloride (223 mg, 1.00 mmol) was added followed by 4-methylmorpholine (264 μL, 2.4 mmol). After 15 min, LC/MS showed consumption of the starting material. Workup: the reaction was concentrated and purified by reversed-phase liquid chromatography (5−100% acetonitrile/water). The yield of the light yellow foam was 66% (292 mg). HPLC: 95%. ^{1}H NMR (300 MHz, DMSO): δ 9.64 (s, 1H), 8.05 (s, 1H), 7.06 (t, J = 6.0 Hz, 1H), 7.00 (s, 1H), 6.83 (d, J = 9 Hz, 1H), 6.78 (d, J = 6.0 Hz, 1H), 6.30 (d, J = 8.4 Hz, 1H), 3.99 (dd, J = 8.5, 4.1 Hz, 1H), 3.92−3.80 (m, 1H), 1.42 (s, 9H), 1.18 (s, 9H), 1.06 (d, J = 6.2 Hz, 3H). HRMS (ESI): m/z calcd for $C_{19}H_{30}BrN_3O_4$ $(M + H)^+$ 444.1498, found 444.1506.

***tert*-Butyl ((2*S*,3*R*)-3-(*tert*-Butoxy)-1-oxo-1-(2-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)hydrazinyl)butan-2-yl)carbamate (18b).** A septum-capped vial charged with **18a** (178 mg, 0.4 mmol), 4,4,4′,4′,5,5,5′,5′-octamethyl-2,2′-bis(1,3,2-dioxaborolane) (112 mg, 0.440 mmol), and KOAc (118 mg, 1.20 mmol) in dry DMSO (1.2 mL) was vacuum flushed with nitrogen five times, then treated with bis(diphenylphosphino)ferrocene]dichloropalladium(II) complex with dichloromethane (9.8 mg, 0.012 mmol), and placed in an 80 °C heating block. LC/MS after 60 min showed ∼50% conversion. After 2 h, the reaction was diluted with ether, filtered through Celite, concentrated, and purified by reversed-phase liquid chromatography (5−100% acetonitrile/water), giving 86 mg (44% yield) of the title compound. ^{1}H NMR (300 MHz, DMSO): δ 10.06 (s, 1H), 8.19 (s, 1H), 7.91 (s, 1H), 7.16−7.07 (m, 2H), 7.04 (t, J = 7.0 Hz, 1H), 6.93 (d, J = 7.9 Hz, 1H), 6.21 (d, J = 9.1 Hz, 1H), 4.00 (m, 1H), 3.86 (m, 1H), 1.42 (s, 9H), 1.29 (s, 12H), 1.16 (s, 9H), 1.07 (d, J = 5.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{25}H_{42}BN_3O_6$ $(M + H)^+$ 492.3245, found 492.3275.

(2*S*,3*R*)-2-Amino-*N*′-(3-(4-amino-2-chloroquinazolin-7-yl)-phenyl)-3-hydroxybutanehydrazide (19). Using general methods C and B, the title compound was synthesized from **18b** in 31% combined yield in two steps. HPLC: 98%. ^{1}H NMR (300 MHz, DMSO): δ 10.30 (s, 1H), 8.30 (m, 3H), 8.12 (s, 1H), 7.91−7.81 (m, 1H), 7.79 (s, 1H), 7.76−7.72 (m, 1H), 7.54 (t, J = 7.4 Hz, 1H), 7.33 (t, J = 8.0 Hz, 1H), 7.25−7.13 (m, 2H), 6.90 (d, J = 8.7 Hz, 1H), 5.70 (s, 1H), 4.00 (br, 1H), 3.66 (br, 1H), 1.25 (d, J = 6.2 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{19}ClN_6O_2$ $(M + H)^+$ 387.1336, found 387.133.

***tert*-Butyl ((2*R*,3*R*)-1-Amino-3-(*tert*-butoxy)butan-2-yl)-carbamate (20).** A solution of **6** (826 mg, 3.0 mmol) and ethyl chloroformate (1.2 equiv) in THF (15 mL) was added to TEA (1.2 equiv) slowly. After the solution was stirred at room temperature for 5 min, aq ammonium hydroxide (5 equiv) was added. The reaction was stirred overnight. LC/MS revealed the product as M + Na (297). Workup: the reaction was concentrated and purified by RPLC (0−100% ACN in water, over 15 min). The yield of the colorless foam was 787 mg (96%). To a round-bottom flask charged with the above-synthesized foam (490 mg, 1.79 mmol) dissolved in dry THF (4.5 mL) at 0 °C was added LAH (5.36 mL, 5.36 mmol, 1.0 M solution in THF). The reaction was allowed to warm to room temperature and stirred for a total of 4 h. Reaction progress was monitored by TLC (10% MeOH/DCM with ammonia, visualized with ninhydrin stain). Workup: the reaction was cooled in an ice bath and then slowly quenched with NaOH (aq, 1.0 N) until a thick white precipitate/emulsion was formed. The emulsion was gently extracted with ether (5 × 10 mL) and concentrated to a colorless oil (445 mg, 96%) that was used without further purification. ^{1}H NMR (300 MHz, DMSO): δ 7.15 (s, 2H), 5.96 (d, J = 8.9 Hz, 1H), 3.95−3.74 (m, 2H), 3.33 (m, 2H), 1.40 (s, 9H), 1.13 (s, 9H), 1.02 (d, J = 6.2 Hz, 3H). HRMS (ESI): m/z calcd for $C_{12}H_{28}N_2O_3$ $(M + H)^+$ 261.2178, found 261.2179.

***tert*-Butyl ((2*R*,3*R*)-1-(3-Bromobenzenesulfonamido)-3-(*tert*-butoxy)butan-2-yl)carbamate (21a).** To a round-bottom flask charged with crude **20** (445 mg, 1.71 mmol) and 3-bromobenzene-1-sulfonyl chloride (480 mg, 1.88 mmol) in DCM (5.7 mL) was added TEA (310 μL, 2.22 mmol) dropwise over 5 min. LC/MS, after 1 h, showed complete conversion to the product. Workup: the reaction was concentrated, filtered, and purified by reversed-phase liquid chromatography (5−100% acetonitrile/water). The desired product was obtained as a colorless oil (194 mg, 25%) and was used without further handling.

***tert*-Butyl ((2*S*,3*R*)-3-(*tert*-Butoxy)-1-oxo-1-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzenesulfonamido)butan-

2-yl)carbamate (21b). A Teflon-capped vial charged with **21a** (112 mg, 0.234 mmol), 4,4,4′,4′,5,5,5′,5′-octamethyl-2,2′-bis(1,3,2-dioxaborolane) (65 mg, 0.257 mmol), and KOAc (69 mg, 0.70 mmol) in dry DMF (0.584 mL) was vacuum flushed with nitrogen five times, treated with bis(diphenylphosphino)ferrocene]dichloropalladium(II) complex with dichloromethane (5.7 mg, 7.0 mmol), and placed in an 80 °C heating block. After 1 h, LC/MS showed complete consumption of the starting material. Workup: the reaction mixture was concentrated, diluted with ethyl acetate, filtered through Celite, concentrated to dryness, and used in the next step without further purification.

(2*S*,3*R*)-2-Amino-*N*-((3-(4-amino-2-chloroquinazolin-7-yl)-phenyl)sulfonyl)-3-hydroxybutanamide (22). Using general methods B and C, the title compound was synthesized from **21b** in 9% combined yield for the final three steps starting from **21a**. HPLC: 97%. ^{1}H NMR (300 MHz, DMSO): δ 8.41 (br, 1H), 8.39 (d, J = 8.6 Hz, 1H), 8.25 (s, 1H), 8.18 (d, J = 7.9 Hz, 1H), 7.98 (d, J = 1.5 Hz, 1H), 7.95 (d, J = 1.7 Hz, 1H), 7.90 (d, J = 8.7 Hz, 2H), 7.80 (m, 2H), 5.41 (d, J = 6.0 Hz, 1H), 3.81 (m, 1H), 2.97 (m, 3H), 1.13 (d, J = 6.0 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{20}ClN_5O_3S$ $(M + H)^+$ 422.1053, found 422.1051.

***tert*-Butyl ((2*S*,3*R*)-1-(3-Bromo-*N*-methylbenzenesulfonamido)-3-(*tert*-butoxy)-1-oxobutan-2-yl)carbamate (23).** To a solution of **9b** (0.17 g, 0.35 mmol) and Cs_2CO_3 (0.135 g, 0.413 mmol) in DMF (3 mL) was added iodomethane (0.15 g, 3.0 equiv). The reaction mixture was heated to 65 °C for 12 h under argon. The reaction mixture was added to water and sonicated. The solids were filtered and collected as the desired product (0.095 g, 54.3%). ^{1}H NMR (300 MHz, DMSO): δ 8.09 (s, 1H), 7.96 (d, J = 7.8 Hz, 2H), 7.60 (t, J = 8.0 Hz, 1H), 6.42 (d, J = 8.3 Hz, 1H), 4.67 (m, 1H), 3.92 (m, 1H), 3.36 (s, 3H), 1.35 (s, 9H), 1.08 (s, 9H), 0.98 (d, J = 6.0 Hz, 3H).

(2*S*,3*R*)-2-Amino-3-hydroxy-*N*-methyl-*N*-((3-(1-oxoisoindolin-5-yl)phenyl)sulfonyl)butanamide (24). Using the same procedure as for the synthesis of **11a**, intermediate **23** produced *tert*-butyl ((2*S*,3*R*)-3-(*tert*-butoxy)-1-(*N*-methyl-3-(1-oxoisoindolin-5-yl)benzenesulfonamido)-1-oxobutan-2-yl)carbamate in 55.4% yield. ^{1}H NMR (300 MHz, DMSO): δ 8.60 (s, 1H), 8.19 (s, 1H), 8.11 (d, J = 7.8 Hz, 1H), 8.01 (d, J = 7.9 Hz, 1H), 7.94 (s, 1H), 7.85 (d, J = 8.3 Hz, 1H), 7.79 (m, 2H), 6.29 (d, J = 8.7 Hz, 1H), 4.82 (m, 1H), 4.46 (s, 2H), 3.98 (m, 1H), 3.33 (s, 3H), 1.30 (s, 9H), 1.06 (s, 9H), 1.02 (d, J = 6.0 Hz, 3H). This intermediate was treated with 4.0 N HCl in dioxane at room temperature to afford product **24** in 46.8% yield. ^{1}H NMR (600 MHz, DMSO): δ 8.67 (s, 1H), 8.23 (s, 1H), 8.13 (d, J = 7.4 Hz, 1H), 8.10 (d, J = 7.1 Hz, 1H), 7.95 (s, 1H), 7.82 (m, 3H), 5.76 (br, 1H), 4.61 (s, 1H), 4.45 (s, 2H), 4.13 (m, 1H), 3.33 (s, 3H), 1.14 (d, J = 6.5 Hz, 3H). HRMS (ESI): m/z calcd for $C_{19}H_{21}N_3O_5S$ $(M + H)^+$ 404.1280, found 404.1286.

(2*S*,3*R*)-2-Amino-*N*-(((*E*)-3-(6-aminopyrimidin-4-yl)styryl)-sulfonyl)-3-hydroxypentanamide (26). Using the same procedure as for the synthesis of **10h**, the title compound was produced in 15% yield in two steps using **25** and (2*S*,3*R*)-2-((*tert*-butoxycarbonyl)-amino)-3-((*tert*-butyldimethylsilyl)oxy)pentanoic acid (Annova Chem). ^{1}H NMR (300 MHz, DMSO): δ 8.40 (s, 1H), 8.16 (s, 1H), 7.98 (d, J = 6 Hz, 1H), 7.56 (d, J = 6.0 Hz, 1H), 7.48 (m, 1H), 7.36 (d, J = 15 Hz, 1H), 7.18 (d, J = 15.6 Hz, 1H), 7.09 (s, 1H), 4.17 (m, 1H), 3.75 (d, J = 4.5 Hz, 1H), 1.14 (m, 2H), 0.72 (t, J = 7.4 Hz, 3H). HRMS (ESI): m/z calcd for $C_{17}H_{21}N_5O_4S$ $(M + H)^+$ 392.1392, found 392.1402.

(2*S*,3*R*)-2-Amino-*N*-(((*E*)-3-(6-aminopyrimidin-4-yl)styryl)-sulfonyl)-3-hydroxy-4-methylpentanamide (27). Using the same procedure as for the synthesis of **10h**, the title compound was produced in 10% yield in two steps using **25** and (2*S*,3*R*)-2-((*tert*-butoxycarbonyl)amino)-3-((*tert*-butyldimethylsilyl)oxy)-4-methylpentanoic acid.[17] ^{1}H NMR (300 MHz, DMSO): δ 8.53 (s, 1H), 8.23 (s, 1H), 7.99 (d, J = 7.7 Hz, 1H), 7.80 (d, J = 7.1 Hz, 1H), 7.58 (t, J = 7.8 Hz, 1H), 7.45 (d, J = 14.0 Hz, 1H), 7.37 (m, 2H), 7.00 (s, 1H), 5.78 (d, J = 5.3 Hz, 1H), 3.94 (br, 1H), 3.58 (m, 1H), 1.72 (m, 1H), 0.93 (d, J = 5.8 Hz, 3H), 0.87 (d, J = 6.6 Hz, 3H). HRMS (ESI): m/z calcd for $C_{18}H_{23}N_5O_4S$ $(M + H)^+$ 406.1549, found 406.1546.

(2*S*,3*R*)-*N*-(((*E*)-3-(6-Aminopyrimidin-4-yl)styryl)sulfonyl)-2,3-dihydroxybutanamide (28). Using the same procedure as for the synthesis of **10h**, the title compound was produced in 19% yield in two steps using **25** and (2*S*,3*R*)-2,3-*O*-isopropylidene-2,3-dihydroxybutyric acid.[18] ^{1}H NMR (300 MHz, DMSO): δ 8.63 (s, 1H), 8.29 (s, 1H), 8.00 (d, J = 7.7 Hz, 1H), 7.94 (d, J = 7.9 Hz, 1H), 7.70 (d, J = 15 Hz, 1H), 7.65 (d, J = 6.0 Hz, 1H), 7.53 (d, J = 15 Hz, 1H), 7.00 (s, 1H), 3.84 (m, 2H), 1.06 (d, J = 6.0 Hz, 3H). HRMS (ESI): m/z calcd for $C_{16}H_{18}N_4O_5S$ $(M + H)^+$ 379.1076, found 379.1088.

(2*S*,3*R*)-2,3-Diamino-*N*-(((*E*)-3-(6-aminopyrimidin-4-yl)-styryl)sulfonyl)butanamide (29). Using the same procedure as for the synthesis of **10h**, the title compound was produced in 15% yield in two steps using **25** and (2*S*,3*R*)-2,3-bis((*tert*-butoxycarbonyl)amino)-butanoic acid.[19] ^{1}H NMR (600 MHz, DMSO): δ 8.70 (s, 1H), 8.43 (s, 1H), 8.34 (s, 1H), 8.14 (s, 1H), 7.88 (d, J = 7.4 Hz, 1H), 7.81 (d, J = 7.1 Hz, 1H), 7.62 (t, J = 7.8 Hz, 1H), 7.46 (d, J = 18 Hz, 1H), 7.35 (d, J = 18 Hz, 1H), 7.35 (s, 1H), 7.26 (s, 1H), 7.18 (s, 1H), 7.09 (s, 1H), 7.05 (s, 1H), 3.81 (m, 1H), 3.59 (m, 1H), 1.18 (d, J = 6.5 Hz, 1H). HRMS (ESI): m/z calcd for $C_{16}H_{20}N_6O_3S$ $(M + H)^+$ 377.1396, found 377.1414.

Enzyme Assays. The tRNA synthetases catalyze a two-step reaction. The first step is the ATP-dependent amino acid activation, resulting in the formation of an amino acid adenylate. In the second step, this activated amino acid is transferred to the relevant tRNA. The enzymatic activity of ThrRS was determined using a coupled spectrophotometric assay in which the stoichiometric release of pyrophosphate during the ATP-dependent amino acid adenylation step was followed. Serine was used as a surrogate amino acid substrate due to a significantly higher rate of turnover due to the increased rate of seryl adenylate hydrolysis.[20]

In ThrRS, the amino acid adenylation reaction proceeds via a random sequential mechanism in which either ATP or amino acid binds first to the enzyme, forming a binary complex, followed by binding of the second substrate to form a ternary enzyme−ATP−amino acid complex.[21] The reader is referred to the numerous texts which describe techniques used to obtain the key kinetic parameters required for the determination of inhibition binding constants.[22]

All reagents were obtained from Sigma-Aldrich unless indicated. Assays, typically 100 μL, consisted of 60 mM Tris, pH 7.6, 10 mM $MgCl_2$, 20 mM KCl, 200 μM MESG (Berry and Associates), 4 mM ATP, 200 mM L-serine, 0.5 mM DTT, 0.1 U of inorganic pyrophosphatase, and 0.001 U of purine nucleoside phosphorylase. DMSO concentrations were maintained at 2% (v/v) in all reactions. Due to weak potency, the conditions used for compound **4** were modified and contained 25 mM L-serine, 0.5 mM ATP, and 20 nM enzyme. An enzyme concentration of 5 nM was used for all orthologues. Data were collected using a Tecan Safire II microplate reader. Inhibition data were normalized to controls lacking inhibitor (maximum activity) and no enzyme (blank). Typically, IC_{50} values were obtained using a 16-point, 2-fold dilution series, from 50 μM to 1.5 nM, analyzed using a 4-parameter sigmoidal log/response plot with variable slope and maximum/minimum constraint. K_i values were calculated for a bisubstrate enzyme as described by Cheng and Prusoff.

Bacterial Strains and Culture Conditions. *E. coli imp* (BAS849; permeability mutant in the MCR106 background with Imp deletion Thr332−Tyr354),[23] *E. coli tolC* (efflux mutant in the BW25113 background with a TolC deletion from Leu13−Ala480) and wild type (ATCC 25922), and *B. thailandensis* strain E264[24] and the isogenic Δ*amrAB-oprA* Δ*bpeAB-oprB* double knockout efflux mutant strain (KO) (H. P. Schweizer, Colorado State University) were cultured on Mueller−Hinton II cation adjusted agar (MHA) or in MH broth (MHB; BD catalog no. 212322) at 37 °C. *H. influenzae* wild type (ATCC 31517) was cultured on chocolate agar plates or in *Haemophilus* test medium broth (Remel catalog no. R112380) at 37 °C in the presence of 5% CO_2.

Minimum Inhibitory Concentration (MIC) Assays. Broth microdilution MIC assays were performed according to CLSI guidelines with the exception that compound stocks were made up at 50× and the end point detection was determined colorimetrically via detection with alamarBlue (Invitrogen, catalog no. DAL1100).[25]

Stock solutions of all test compounds were prepared in 100% dimethyl sulfoxide (DMSO) at 6.4 mg/mL and then serially diluted in two steps. Two microliters from each dilution series was added to 98 μL of inoculated media, resulting in final test concentrations of 0.063−128 μg/mL. Serial dilutions of the starting inocula were plated to ensure that the assay contained roughly the desired 5×10^5 CFU/mL. MIC plates were incubated overnight at 37 °C in the appropriate conditions. The following day, 10 μL of alamarBlue was added to each well. The plates were shaken and incubated for another 1 h, and then the MICs were determined as the lowest concentration of compound where no color change was visible.

Protein Purification and Cocrystallization Conditions. The proteins were purified by metal chelate affinity chromatography, exploiting a vector-derived C-terminal 6 × His tag. Compounds were cocrystallized by hanging drop vapor diffusion using a mother liquor containing 12% (w/v) PEG 4000, 21% (v/v) MPD, and 0.1 M sodium citrate, pH 5.9. The resulting crystals were of space group $P2_12_12_1$ and typically diffracted to 1.8 Å or better on a rotating anode X-ray source. Crystals of the human enzyme (cytoplasmic isoform 1) were generated with an analogous construct and purification scheme.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full description of the crystallographic methods, protein purification methods, enzymatic assay, and MIC conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates for the cocrystal structures of compounds **10a,c,d** and **11d** bound to *E. coli* ThrRS and compound **11d** bound to the human ThrRS have been deposited in the Protein Data Bank (http://www.pdb.org) under accession codes 4HWO, 4HWS, 4HWP, 4HWR, and 4HWT, respectively.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mteng@triusrx.com. Phone: (858) 452-0370.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

B. thailandensis (KO), *B. thailandensis* pump knockout; ThrRS, threonyl-tRNA synthetase; aa-AMP, aminoacyl adenosine monophosphate; AMS, 5′-*O*-[*N*-(threonyl)sulfamoyl]-adenosine; TEA, triethylamine; SBDD, structure-based drug design; ThrRS, threonyl-tRNA synthetase

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