Journal of Medicinal Chemistry

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Article

In Vitro and In Vivo Inhibition of the *Mycobacterium tuberculosis* Phosphopantetheinyl Transferase PptT by Amidinoureas

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Cite This: https://doi.org/10.1021/acs.jmedchem.1c01565

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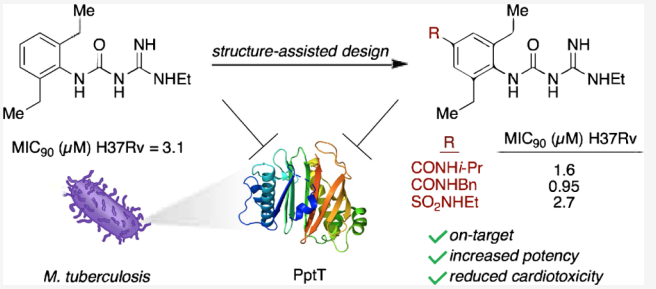
ABSTRACT: A newly validated target for tuberculosis treatment is phosphopantetheinyl transferase, an essential enzyme that plays a critical role in the biosynthesis of cellular lipids and virulence factors in *Mycobacterium tuberculosis*. The structure−activity relationships of a recently disclosed inhibitor, amidinourea (AU) 8918 (**1**), were explored, focusing on the biochemical potency, determination of whole-cell on-target activity for active compounds, and profiling of selective active congeners. These studies show that the AU moiety in AU 8918 is largely optimized and that potency enhancements are obtained in analogues containing a para-substituted aromatic ring. Preliminary data reveal that while some analogues, including **1**, have demonstrated cardiotoxicity (e.g., changes in cardiomyocyte beat rate, amplitude, and peak width) and inhibit $Ca_v1.2$ and $Na_v1.5$ ion channels (although not hERG channels), inhibition of the ion channels is largely diminished for some of the para-substituted analogues, such as **5k** (*p*-benzamide) and **5n** (*p*-phenylsulfonamide).



INTRODUCTION

Nearly 75 years after the introduction of streptomycin, tuberculosis (TB) remains a worldwide threat to human health that is estimated to have resulted in more than 1.5 million deaths in 2020.[1] Drug treatment of TB today is much the same as it was when rifampicin was introduced in 1968 and came to replace streptomycin as one of the first-line agents used against TB, along with isoniazid, ethambutol, and pyrazinamide. The most prevalent mechanisms of action of older TB drugs are the disruption of cell wall synthesis, inhibition of DNA synthesis and transcription, protein translation, and folate synthesis. Although much is known about the effects of pyrazinamide, one of the most important TB drugs, its specific mechanism of action is still being unraveled and is likely to depend on the location of its intracellular localization in macrophages.[2−4] A typical course of TB therapy is 6−9 months long, with treatment of drug-resistant forms often requiring over 2 years. The challenges posed by the need for lengthy treatments and the concomitant increase of drug-resistant forms of the disease have continued to spur efforts to create new therapies, especially those that function by mechanisms differing from standard TB drugs.[5,6]

Mycobacterium tuberculosis (Mtb), the causative agent of TB, has a complex cell wall that presents a challenge to the permeability of future antibiotic molecules.[7] However, the cellular functions that create the cell wall provide numerous drug targets.[8] Mycolic acids are lipids characteristically found in the cell walls of mycobacteria, and two TB drugs introduced since 2012, delamanid and pretomanid, inhibit mycolic acid biosynthesis, although their mechanisms of action are not fully known.[9,10] One critical enzyme in early stage lipid biosynthesis in Mtb is phosphopantetheinyl phosphoryl transferase (PptT), which catalyzes the transfer of a 4-phosphopanetheinyl moiety of coenzyme A (CoA) to an inactive *apo* acyl carrier protein (ACP) or a peptidyl carrier protein (Figure 1).[11,12] This provides a thiol handle onto which a nascent lipid or peptide chain can be attached. The thus equipped, currently active *holo* ACP is used in orchestrating the elaboration of a growing biosynthetic product. Molecules that depend on PptT for their biosynthesis include fatty acids, such as mycolic acid, as well as a variety of virulence factors, including phthiocerol dimycocerosates,[7,13,14] siderophores,[3,13,15] or sulfolipid-1.[16] In 2012, Leblanc et al. showed that PptT is an essential enzyme for the

Received: September 3, 2021

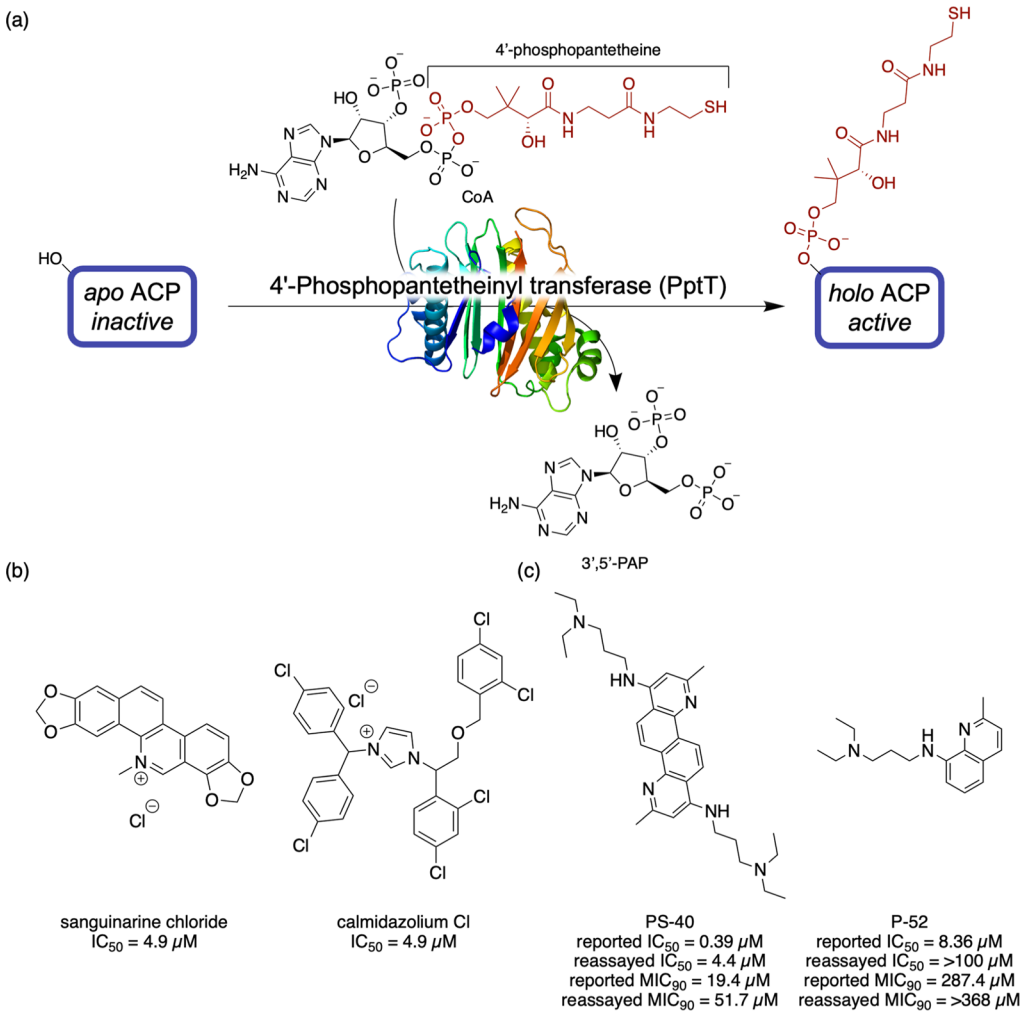


Figure 1. (a) Generalized mechanism for the function of PptT and selected inhibitors reported by (b) Vickery et al.[13] and (c) Rohilla et al.[18]

replication and survival of Mtb in mice, supporting the view that a PptT inhibitor would be a potential TB drug.[17]

The attractiveness of PptT as a target has led several investigators to develop high-throughput friendly assays[17,19,20] and seek small-molecule inhibitors as potential biological probes or drug candidates. The representative early hits reported by Vickery et al. are shown in Figure 1b.[13] More recently, Rohilla et al. used published crystal structures of Mtb's PptT in the native state[13] or as a fusion protein with maltose binding protein[21] to carry out a virtual screen that yielded a number of potential binders, 13 of which were reported to inhibit the enzyme with IC_{50} values <10 μM (two examples are shown in Figure 1c). We briefly considered carrying out optimization studies based on PS-40 and P-52. However, upon resynthesis and reassay, we found that both exhibited weaker inhibitory activity than that published and therefore abandoned plans to work in these series (synthesis procedure is given in the Supporting Information and reassay data is shown in Figure 1c).

In 2019, we reported the results of a phenotypic screen seeking molecules able to inhibit the growth of Mtb strain H37Rv, known to be virulent in mice and humans.[22] The screen yielded a hit molecule, amidinourea (AU) 8918 (Figure 2a), with an IC_{50} value of 2.3 μM. Subsequent studies led to the identification of 10 strains resistant to AU 8918, 3 of which had mutations in the gene coding essential PptT, while the rest were mutants in a previously unidentified gene, *rv2795c*. We ultimately determined that *rv2795c*, which is present on the same operon as PptT, encodes phosphopantetheinyl hydrolase (PptH). PptH removes phosphopantetheine from holo-CPs, regenerating apo-CPs, but, as it has been shown to be non-essential to Mtb growth,[23] it was unlikely to be the target of AU 8918. AU 8918 kills Mtb in mice and is active in vitro against *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG but is not effective against other bacteria, yeast, or animal cells. In addition, the sensitivity of Mtb to AU 8918 increases in PptT knockdown (depletion) strains proportionally to the degree of knockdown. Taken together, the above experiments provide strong evidence for PptT inhibition being the mechanism of AU 8918's action against Mtb.

AU 8918 (**1**) has an interesting chemical structure that is reminiscent of lidamidine (**2**), an antidiarrheal agent, and the clinically used antimalarial drug proguanil (**3**), which itself is a prodrug of cycloguanil, a dihydrofolate reductase inhibitor (Figure 2a). Proguanil was shown to have no activity against Mtb, and lidamidine was poorly active (see below), suggesting a steep structure−activity relationship (SAR) for the AU moiety and its attached substituents. Insights into the structural basis for PptT inhibition by AU 8918 were provided by the X-ray crystal structure of the inhibitor/PptT cocrystal (Figure 2b). Early crystallographic work on PptT[13,21] revealed a narrow hydrophobic channel that accommodates the

https://doi.org/10.1021/acs.jmedchem.1c01565
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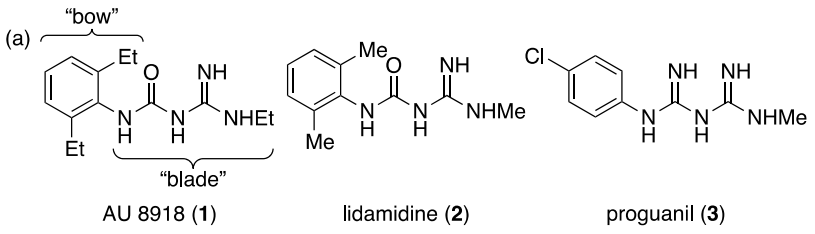
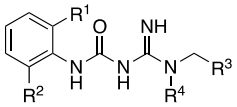


Figure 2. (a) Structure of AU 8918 and related compounds. (b) Portion of X-ray structure of AU 8918 bound to PptT (PDB 6CT5), showing key interactions between the ligand and protein.[22] The figure was generated using Chimera;[24] the surface is colored according to the electrostatic potential (±5 kT/e).

Table 1. Effect of Adding Methyl Groups on Inhibition of PptT and *Mtb* Growth



						IC_{50} (μM)		
entry	comp	R^1	R^2	R^3	R^4	BpsA[a]	FP[b]	MIC_{90} (μM)[c]
1	**2** (lidamidine)	Me	Me	H	H	35		>100[d]
2	**4a**	Et	Me	H	H	6.2		64
3	**4b**	Et	Et	H	H		0.99	24
4	**1** (AU 8918)	Et	Et	Me	H	2.3	0.27	3.1
5	**4c**	Et	Et	Et	H	10.4		10.6
6	**4d**	Et	Et	Me	Me	>100	>100	>100
7	**4e**	Et	H	Me	H	51.4		89.3
8	**4f**	*i*-Pr	*i*-Pr	Me	H	1.2		2.2

[a]BpsA assay of inhibition of PptT (average of duplicate experiments).[22] [b]FP assay of inhibition of PptT (average of duplicate experiments). [c]MIC_{90} of *Mtb* growth in vitro. [d]MIC_{50} for lidamidine was determined to be ca. 125.

pantetheinyl arm of a bound CoA substrate. We note the resemblance of AU 8918 to a physical key, with the aromatic moiety corresponding to the bow and the AU part the blade. In addition, the phosphoadenosine phosphate (PAP) portion of CoA present was explicitly visualized. In our structure, the AU shaft occupies this channel, with the aromatic moiety extending into a relatively expansive antechamber. This structure also suggested a charge−charge interaction between the protonated AU moiety and a nearby glutamate moiety (E157) in binding the AU group.

The discovery of AU 8918 as an inhibitor of PptT active against Mtb both in vitro and in vivo suggests it as a lead for a possible TB therapeutic. In this paper, we describe SAR studies of AU 8918. The new compounds synthesized were assayed for biochemical potency against PptT in vitro as well as against wild-type, a PptT-knockdown strain and a PptH knockout strains of Mtb in culture, the latter of which determines whether the activity of a specific analogue is due to on-target PptT inhibition. The docking studies and preliminary pharmaceutical studies of selected inhibitors are also described.

RESULTS AND DISCUSSION

SAR Studies. Most of the new compounds were examined for biochemical potency using the previously reported kinetic assay, that is, by following the transfer of 4′-phosphopantetheine from CoA to the non-ribosomal peptide synthase apo-

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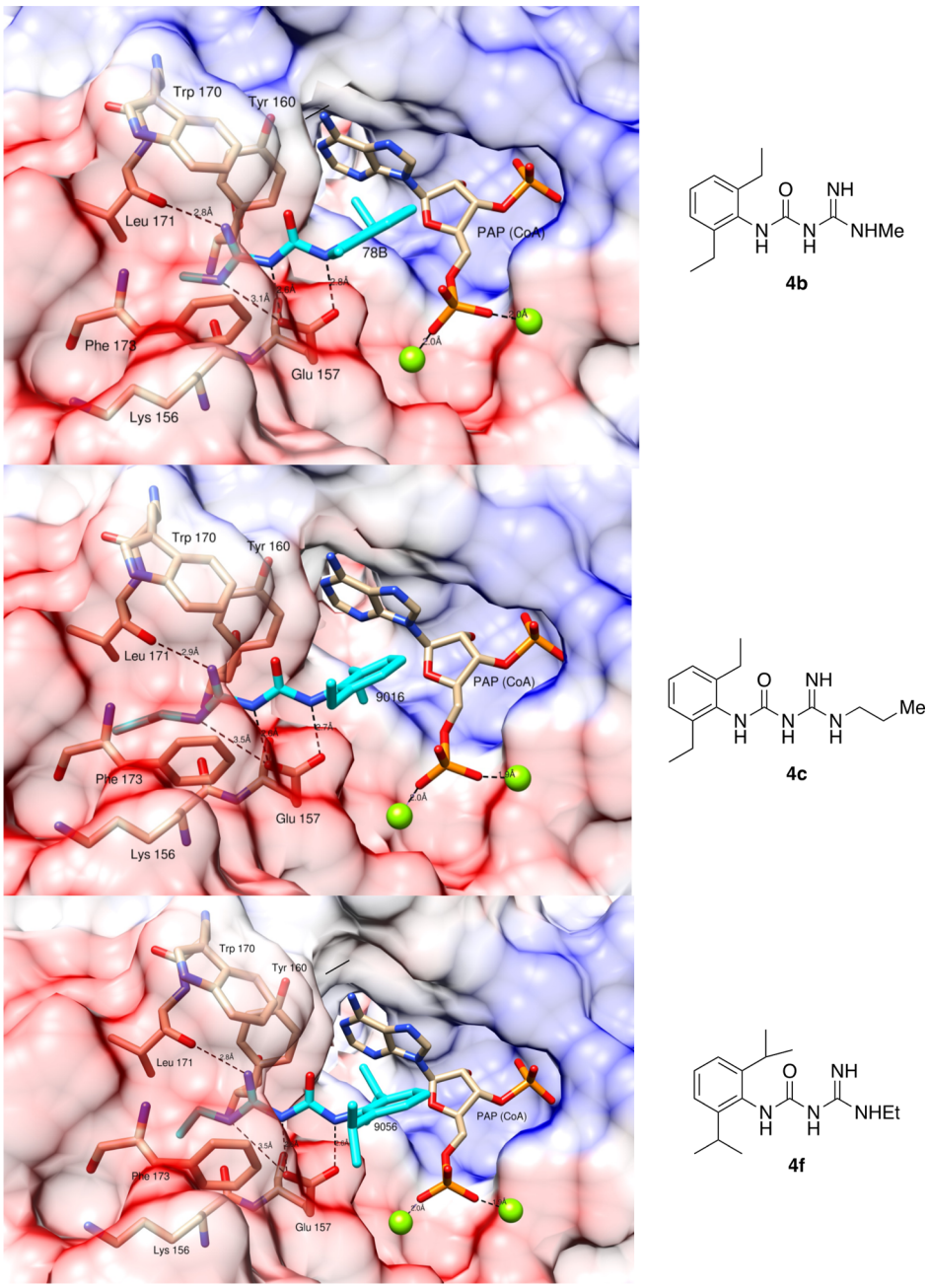

Figure 3. Portions of crystal structures of **4b** (refined to 1.57 Å; PDB 7N8M), **4c** (refined to 1.74 Å; PDB 7N8E), and **4f** (refined to 2.26 Å; PDB 7N8L) bound to PptT. The images were generated using Chimera;[24] the surfaces are colored according to the electrostatic potential (±5 kT/e). See the Supporting Information (Table S1) for data collection and refinement statistics.

BpsA from *Streptomyces lavendulae* and measuring holo-BpsA's catalysis of L-glutamine to indigoidine at 590 nm.[22] A few analogues were examined using a fluorescence polarization (FP) assay.[20] In general, the FP IC_{50} values are ca. 10-fold more potent than those determined by the BpsA assay, which is likely due to the bodipy-CoA used in the FP assay not fitting completely into the binding pocket of PptT, making it easier to displace with an inhibitor. The activity against replicating, virulent *M. tuberculosis* H37Rv was determined as previously published.[22]

The structural similarity between lidamidine and AU 8918 led us to systematically examine the effect of adding methyl groups to the structures (Table 1). As previously reported,[22] lidamidine has only marginal inhibitory activity (entry 1, cf. MIC_{50}). Substantial increases in activity were observed when successively adding methyl groups to the two *ortho* groups on the phenyl substituent and the N-terminal methyl group, culminating in AU 8918 **1** (entry 4). An improvement in activity was realized by replacing both *ortho* Et groups in **1** with *i*-Pr moieties (entry 8), but an analogue bearing only a single *ortho* Et group was greatly diminished in potency and efficacy (entry 7). The two ortho substituents on the aryl group likely stabilize a conformation which places the aryl group and the amidinyl groups in a perpendicular orientation to one another, as observed in the X-ray structures of **1** (Figure 2b) and **4f** (Figure 3b). Finally, methylation at the terminal nitrogen of **1** led to loss of activity in **4d**, emphasizing the need for a hydrogen bonding group in this position.

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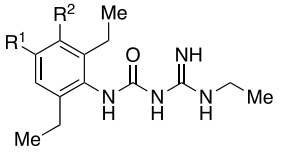
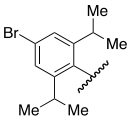
Table 2. Effect of Varying Aromatic Ring Substituents on the Inhibition of PptT and *Mtb* Growth



entry	comp	R^1	R^2	IC_{50}[a] (μM)	MIC_{90}[b] (μM)
1	**1** (AU 8918)	H	H	2.3	3.1
2	**5a**	Cl	H	6.3	3.0
3	**5b**	Br	H	2.4	1.8
4	**5c**	CN	H	2.2	5.0
5	**5d**	COOH	H	ND	9.86
6	**5e**	CO_2Me	H	7.1	1.0
7	**5f**	CO_2Et	H	2.6	0.48
8	**5g**	CO_2Bn	H	0.21	3.0
9	**5h**	CONHMe	H	3.6	4.7
10	**5i**	CONHEt	H	4.9	2.5
11	**5j**	CONH*i*-Pr	H	2.8	1.6
12	**5k**	CONHBn	H	2.5	0.95
13	**5l**	CONH (4-OMe) C_6H_4	H	3.6	2.1
14	**5m**	NSO_2Me	H	5.2	4.7
15	**5n**	$NHSO_2Ph$	H	3.8	4.5
16	**5o**	$NHSO_2Tol$	H	0.7	10.2
17	**5p**	SO_2NHEt	H	3.5	2.7
18	**5q**	H	SO_2NHEt	6.0	>100
19	**5r**	SO_2NHPh	H	3.6	10.8
20	**5s**	SO_2NHBn	H	36.2	5.9
21	**5t**			3.5	2.0
22	**5u**			>100	21.5

[a]BpsA assay of inhibition of PptT (average of duplicate experiments).[22] [b]MIC_{90} for the inhibition of *Mtb* growth in vitro.

Since the co-crystal structure of AU 8918 with PptT showed the AU moiety tucked into the channel that also binds to the pantetheinyl arm of CoA, we sought to determine whether larger groups could be accommodated at this position. Comparison of the N-terminal Me, Et, *i*-Pr series (entries 3−5) suggests that the Et group is optimal at this position. X-ray structures of these three analogues (**1**, **4b**, and **4c**; Figures 2b and 3b) bound to PptT show similar binding overall modes, with **4b** having comparable potency to **1**. Although a ca. 5-fold loss of biochemical potency was observed for compound **4c**, there were no obvious steric clashes noted that would explain this. Compound **4f** was slightly more active than **1** in both biochemical and cellular assays, possibly because the two *ortho* isopropyl groups pick up additional van der Waals interactions in the active site.

In addition, a series of compounds bearing extended alkyl groups (C4−C8), branched alkyl groups or those containing aryl groups or heteroatoms, were made, and all were found substantially less active than AU 8918 (Table S1; Supporting Information). We prepared the iminyl *N*-Boc derivative of **1** as a synthetic intermediate and were somewhat surprised that it had an MIC_{90} value of 6.6 μM against Mtb, although it was not active against the purified enzyme (Table S2). This behavior, which was also observed in several aromatic analogues, led us to examine a concise set of other groups containing modified AUs and several analogues in which the group was replaced by a squaramide moiety (Table S3). None of these analogues were associated with on-target biological activity (as described below) and were not pursued further. It is possible that the residual activity of Boc-**1** is due to the removal of the Boc group, affording **1**, in Mtb, but this was not experimentally checked.

Along with the results in Table 1, these observations and synthetic expedience led us to prepare additional analogues where the scaffold of AU 8918 was held intact. Specifically, we hypothesized that the phenyl group would be relatively tolerant of substitution since AU 8918, as presented in the co-crystal structure, projects into the relatively expansive "antechamber" in the bound conformation. The data show that, indeed, numerous modifications of the aromatic moiety render active analogues (Table 2).

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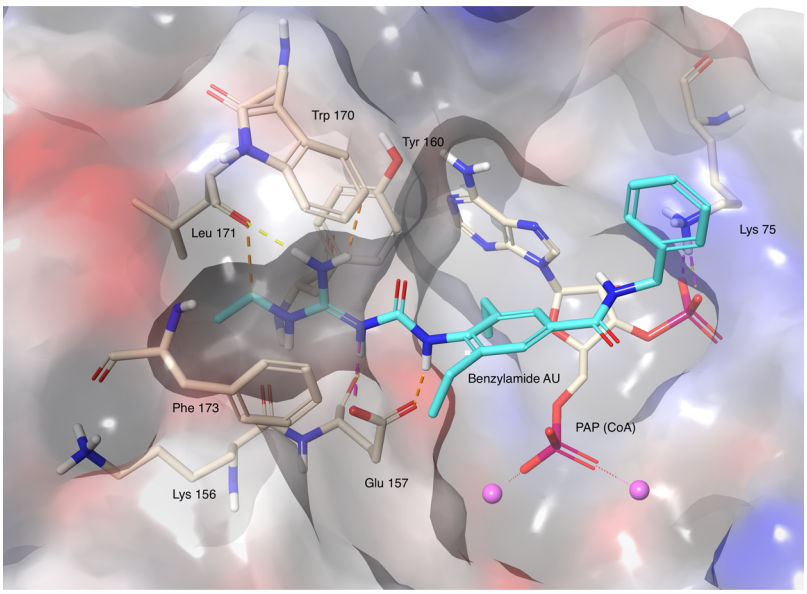

Figure 4. Model of compound **5k** bound (cyan) to PptT, illustrating potential stabilizing interactions with K75. The surface is colored by electrostatic potential, and magnesium ions are illustrated in pink. Compound **5k** was docked with Schrödinger's Glide 2020 and the figure generated in the Maestro Suite 2021-3: Glide, Maestro, Schrödinger, LLC, New York, NY 2021.

Aside from the above-noted preference for two ortho substituents, substitution on other positions of the aromatic ring, particularly the para site, were generally tolerated and, in several notable cases, led to potency increases. Of practical consequence is the observation that para carboxylic acids, esters, and amides either retain or, in the case of benzyl ester, increase the potency against recombinant PptT and activity against Mtb relative to AU 8918 (cf. entry 1 with entries 8, 12, and 13). We modeled these compounds in the active site using the crystallographic binding pose of AU 8918 as a starting point. The para substituents were comfortably accommodated in these models. In some cases, a potentially stabilizing cation−π interaction between a suitably placed aromatic ester and K75 was noted (shown for benzamide **5k** in Figure 4), although we note the lack of potency increase for the relatively electron-rich **5l**. The greatest increase in potency against the recombinant enzyme was observed for the benzyl ester **5g**, but the likely instability of the ester in dosing humans led us to concentrate instead on compounds **5j**, **5k**, **5n**, and **5p** for further characterization (below). Additional inactive analogues bearing substituted phenyl groups or in which the phenyl group is replaced by bicyclic aromatic and a few hetero-aromatic rings are presented in the Supporting Information (Tables S4 and S5, respectively). The aliphatic analogue **5u** bearing an adamantyl group had an MIC_{90} value of 21.5 μM when measured against replicating Mtb but did not inhibit PptT in vitro, suggesting that the antitubercular activity of this analogue operates through another mechanism.

Chemistry. The modular assembly of AU derivatives was accomplished using straightforward condensation reactions as shown in the two general routes summarized in Scheme 1. Thus, displacement of a thiomethyl group from methyl carbamimidothiolate sulfate by reaction with ethylamine or *n*-propylamine afforded intermediates **6a** and **6b**, respectively.[25] A subsequent reaction with substituted isocyanate afforded compounds **1**, **4c−f**, and **5u**.[26] However, most analogues were prepared by the double-displacement route outlined in Scheme 1B. This entailed amide displacement of the thiomethyl from the bis-Boc derivative of methyl carbamimidothiolate to afford amidines **7a−c**, which were reacted with a second amine to displace one of the NHBoc substituents in **7**; deprotection of the remaining Boc group with TFA afforded the final products.[27,28]

Most of the amine components were commercially available, but some of the p-substituted anilines were made using coupling methods (Scheme 2), including a palladium-catalyzed carbonylation reaction reported by Baburajan et al.[29] (Scheme 2A) and the palladium-catalyzed coupling reaction between **10** and substituted sulfonamides (Scheme 2B).[30] Similarly, intermediates **16a−16c** were made using a sequence of palladium-catalyzed bromine−sulfur coupling, oxidative chlorination to afford sulfonyl chloride **14**,[30] alkyl amine coupling, and final deprotection.

Additional routes afforded building blocks for single analogues (Scheme 3). Synthesis of intermediate **17** en route to AU derivative **5a** entailed a Suzuki cross-coupling between 4-chloro-2,6-dibromo aniline and ethylboronic acid (Scheme 3A),[31] whereas reductive debenzylation of **5g** afforded carboxylic acid **5d** (Scheme 3B). Synthesis of the intermediate **20** needed for AU derivative **5q** used the meta-selective Friedel−Crafts sulfonylation of isobutyl-carbamate protected 2,6-diethyl aniline, which was subsequently reacted with ethanamine and deprotected with TBAF (Scheme 3C).[32] Finally, a copper-catalyzed coupling reaction[33] converted 4-bromo-2,6-diethylaniline to cyano-substituted aniline **21**, which was used to make AU **5c**.

The syntheses of additional and generally inactive analogues are described in the Supporting Information. Synthesis schemes, experimental details, and full characterization for these additional analogues can be found there.

Additional Characterization of Selected Compounds for On-Target Activity and Preliminary PK. The selected compounds having potencies close to or exceeding that of AU 8918 were further characterized biochemically and for potential pre-clinical advancement. The fact that the ability of many of these compounds to kill *Mtb* tracks with their

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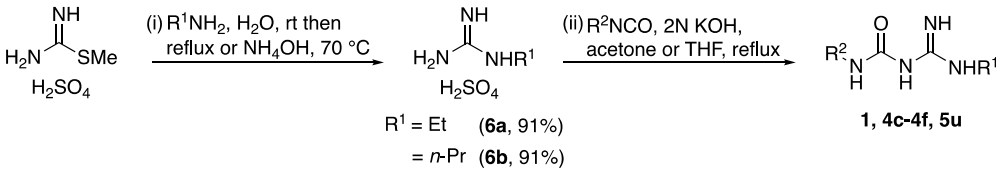
Scheme 1. Assembly of AU Analogues

A. From methyl carbamimidothioate sulfate

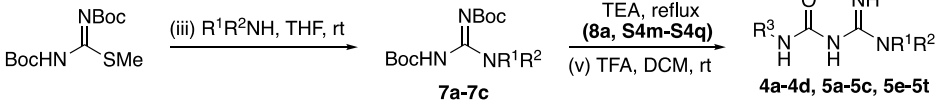


R^1 = Et (**6a**, 91%)
= *n*-Pr (**6b**, 91%)

1, 4c-4f, 5u

comp	R^1	R^2	yield (step ii, %)
1 (AU 8918)	Et	2,6-diethylphenyl	54
4c	*n*-Pr	2,6-diethylphenyl	72
4e	Et	2-ethylphenyl	62
4f	Et	2,6-diisopropyl	94
5u	Et	adamantyl	85

B. From N,N'-diBoc methyl carbamimidothioate



7a-7c

4a-4d, 5a-5c, 5e-5t

R^1,R^2 = Et, H (**7a**, 84%)
= Me, H (**7b**, 97%)
= Me, Me (**7c**, 83%)

comp	R^1	R^2	R^3	yield (steps iv+v, %)
4a	Me	H	2-ethyl-6-methylphenyl	66
4b	Me	H	2,6-diethylphenyl	67
4d	Me	Me	2,6-diethylphenyl	11
5a	Et	H	4-chloro-2,6-diethylphenyl	17
5b	Et	H	4-bromo-2,6-diethylphenyl	32
5c	Et	H	4-CN-2,6-diethylphenyl	24
5e	Et	H	4-COOMe-2,6-diethylphenyl	22
5f	Et	H	4-COOEt-2,6-diethylphenyl	32
5g	Et	H	4-COOBn-2,6-diethylphenyl	18
5h	Et	H	4-CONHMe-2,6-diethylphenyl	36
5i	Et	H	4-CONHEt-2,6-diethylphenyl	16
5j	Et	H	4-CONH*i*-Pr-2,6-diethylphenyl	18
5k	Et	H	4-CONHBn-2,6-diethylphenyl	18
5l	Et	H	4-CONHBn(4-OMe)-2,6-diethylphenyl	45
5m	Et	H	4-$NHSO_2$Me-2,6-diethylphenyl	43
5n	Et	H	4-$NHSO_2$Ph-2,6-diethylphenyl	26
5o	Et	H	4-NHTs-2,6-diethylphenyl	57
5p	Et	H	4-SO_2NHEt-2,6-diethylphenyl	27
5q	Et	H	3-SO_2NHEt-2,6-diethylphenyl	60
5r	Et	H	4-SO_2NHPh-2,6-diethylphenyl	48
5s	Et	H	4-SO_2NHBn-2,6-diethylphenyl	71
5t	Et	H	4-bromo-2,6-diisopropylphenyl	48

biochemical potency suggests that the in vitro activity is mostly due to PptT inhibition. More rigorously, the on-target activity was investigated by determining the whole-cell activity against the following strains: Mtb WT H37Rv, an Mtb PptT knockdown (using SspB-controlled proteolysis of a DAS-tagged PptT allele), and an Mtb Δ*pptH* knockout (Figure 5).[34−36] An on-target inhibitor of PptT should display equipotent activity against WT Mtb and Mtb DAS-PptT grown with ATC (no knockdown), approximately >30-fold lower MIC_{90} values (more potent) against Mtb DAS-PptT in the absence of ATC, and >30-fold higher MIC_{90} against the Mtb Δ*pptH* knockout. By these criteria, AU 8918 and the related AUs of comparable or greater biochemical potency are active against Mtb via PptT inhibition (Table 3; the additional data for other, less potent, compounds are provided in Table

https://doi.org/10.1021/acs.jmedchem.1c01565
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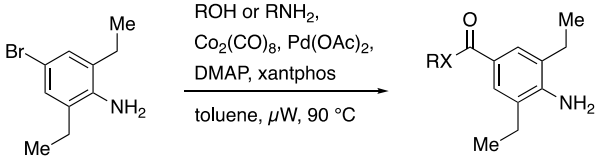
Scheme 2. Synthesis of Building Blocks for AU Synthesis (Multiple Analogues)

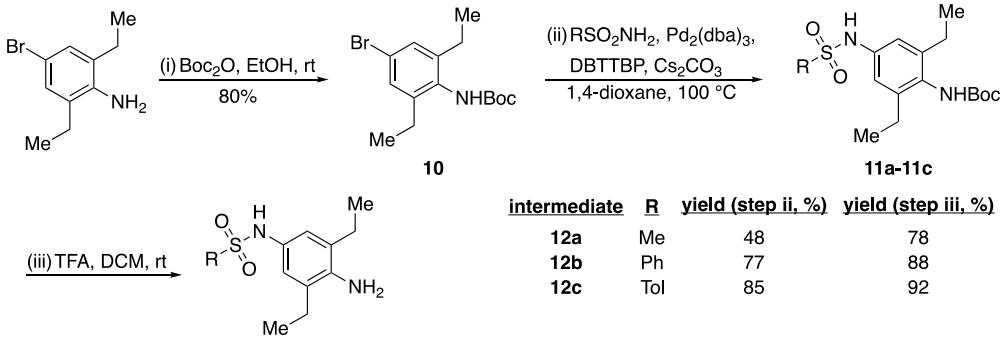
A. *p*-Substituted Esters and Amides for Compounds 5e-5l



9a-9h

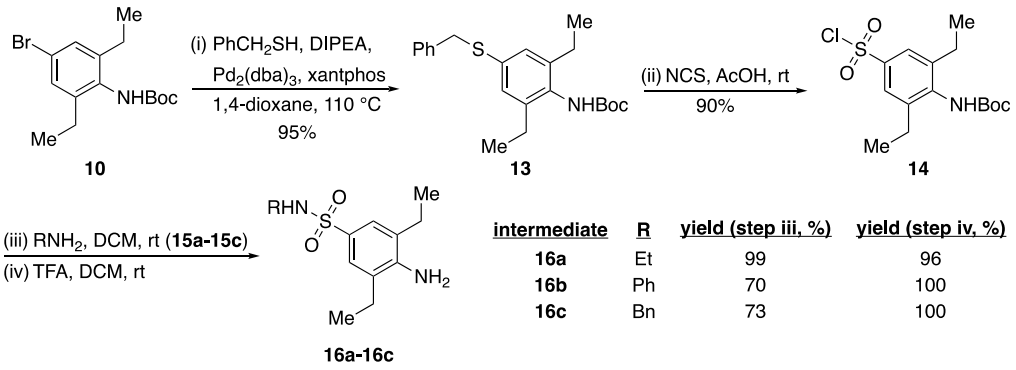
intermediate	X	R	yield (%)
9a	O	Me	70
9b	O	Et	64
9c	O	Bn	67
9d	NH	Me	87
9e	NH	Et	89
9f	NH	*i*-Pr	86
9g	NH	Bn	23
9h	NH	4-OMeBn	50

B. *p*-Substituted Sulfonamides for Compounds 5m-5o



intermediate	R	yield (step ii, %)	yield (step iii, %)
12a	Me	48	78
12b	Ph	77	88
12c	Tol	85	92

C. *p*-Substituted Sulfonamides for Compounds 5p-5s



intermediate	R	yield (step iii, %)	yield (step iv, %)
16a	Et	99	96
16b	Ph	70	100
16c	Bn	73	100

S2 in the Supporting Information). Additional studies to confirm this are ongoing.

In the course of profiling, it was determined that AU 8918 was not toxic to HEPG2 or THP1 (macrophage) cells but that many close analogues caused changes in cardiomyocyte beat rate, Na^+ slope, Na^+ amplitude, and peak width, portending serious cardiotoxicity issues that would preclude development as drugs (Table 4). While most analogues were found not to cause hERG inhibition, they displayed some degree of inhibition of $Ca_v1.2$ and $Na_v1.5$ channels in vitro, which are often associated with cardiotoxicity issues.[37] For comparison, we note that the clinically used lidamidine, while clean with respect to the channels examined, has similar properties in cardiomyocytes as do some of the new compounds reported here (entry 2). We have found that aromatic AU analogues bearing para substitution, in addition to leading to increases in potency, have diminished interactions with $Ca_V1.2$ and $Na_V1.5$ channels (entries 4−6). Although many PptT-active AUs were cardiotoxic, this work identified a series of p-substituted AUs with both potent on-target activity and reduced cardiotoxicity, suggesting that the cardiotoxicity is not an intrinsic feature of PptT inhibition.

https://doi.org/10.1021/acs.jmedchem.1c01565
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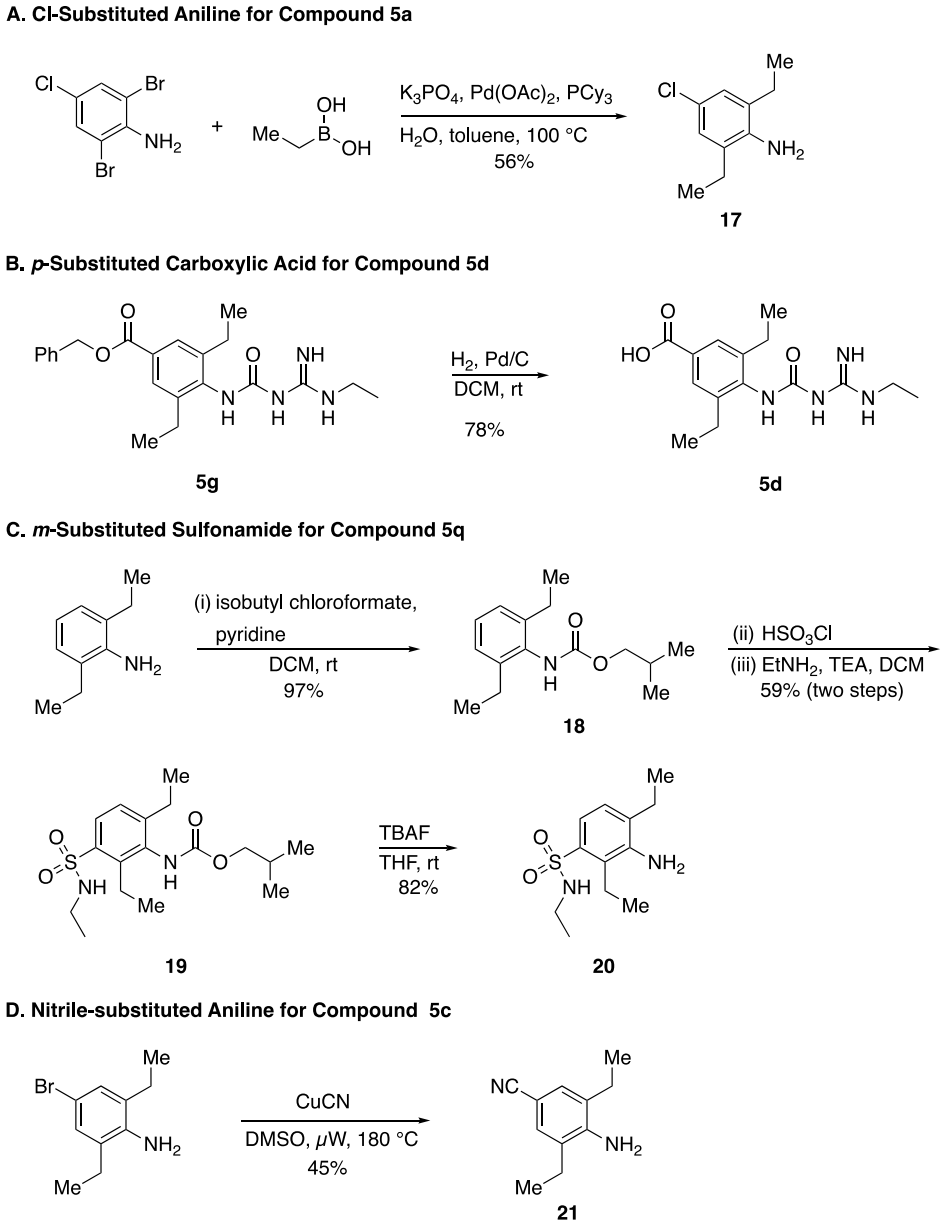
Scheme 3. Synthesis of Building Blocks for AU Synthesis (Single Analogues)



Most of the data obtained are within or close to the desirable values associated with drug-like properties, including low toxicity, good solubility, and lack of inhibition of CYP3A4 (Table 5). These compounds are reasonably stable in mouse and human plasma, but half-lives are under 1 h when exposed to human liver microsomes (they are more stable to the effects of mouse microsomes). Despite the relatively unusual AU chemotype, these preliminary data suggest that further multiparameter optimization of suitable analogues such as **5j**, **5n**, and **5p** is warranted.

CONCLUSIONS

We have reported a series of amidino-derived compounds for the inhibition of PptT, a new and attractive target for the treatment of TB. One feature of our approach was the simultaneous determination of on-target activity for each compound as the SAR was being explored. Moreover, the substantial reduction in the activity of compounds in Mtb strains lacking PptH, the enzyme that undoes the action of PptT, reflects an interesting example of biochemical potentiation of inhibitor action in the natural state, where PptH is present. The SAR studies above indicate that, while the AU portion of the inhibitors is very nearly optimized for its interactions with the target enzyme, numerous changes in the aromatic moiety are tolerated and, in particular, para substitution leads to greater potency and a reduction in what at this time are the most concerning off-target effects at Ca^{2+} and Na^{+} channels. Particularly interesting analogues identified to date include **5k** and **5n**, which have potencies comparable to **1** but less activity against channels associated with cardiotoxicity. Work to effect further optimization and to find additional chemotypes is ongoing.

EXPERIMENTAL SECTION

Chemistry. *General Information.* All chemicals were used as received from a commercial source without further purification, unless otherwise noted. Thin-layer chromatography (TLC) was performed

https://doi.org/10.1021/acs.jmedchem.1c01565
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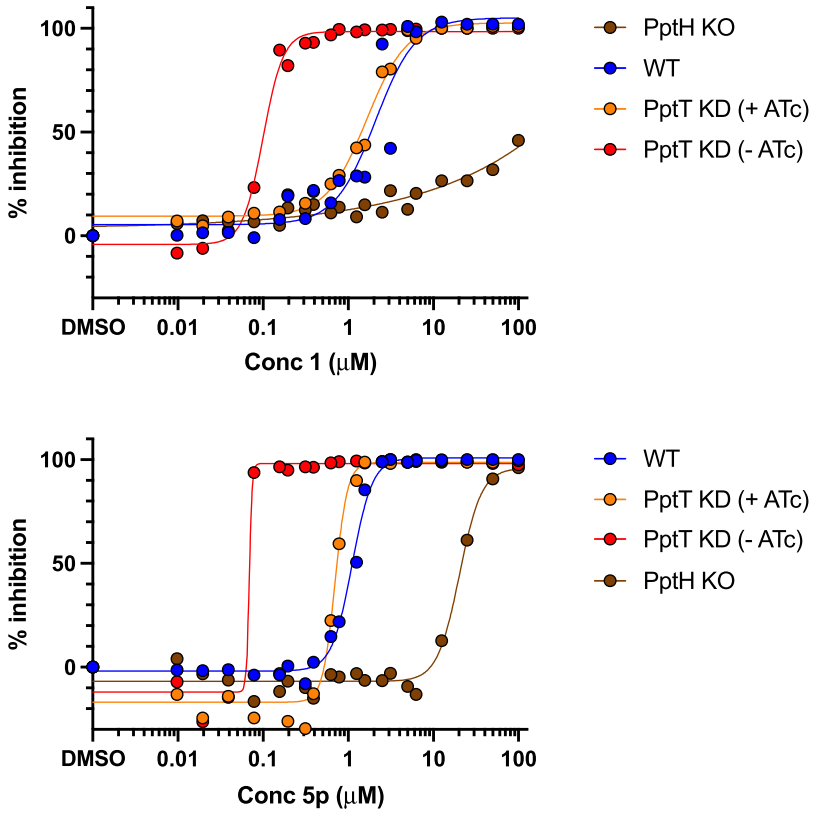
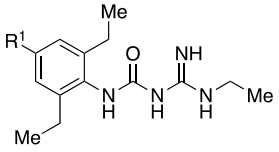


Figure 5. Representative activity of on-target PptT inhibitors (a) **1** and (b) **5p** against Mtb and modified organisms.

Table 3. Determination of On-Target Activity of Selected Analogues



				MIC$_{90}$ (μM)[b]			
entry	comp	R^1	IC$_{50}$ PptT (μM)[a]	H37Rv	DAS PptT −ATC	DAS PptT +ATC	PptH KO
1	**1** (AU 8918)	H	2.3	3.1	0.16	3.4	>100
2	**5b**	Br	2.4	1.8	<0.06	1.7	24.8
3	**5c**	CN	2.2	5.0	0.27	7.9	>100
4	**5j**	CONH*i*-Pr	2.8	1.6	0.098	1.8	32.7
5	**5k**	CONHBn	2.5	0.95	0.090	1.6	42.9
6	**5n**	NHSO$_2$Ph	3.8	4.5	0.17	3.6	86.9
7	**5p**	SO$_2$NHEt	3.5	2.7	0.073	1.8	60.2

[a]BpsA assay of in vitro inhibition of PptT.[22] [b]MIC$_{90}$ for inhibition of *Mtb* growth in vitro for H37Rv (WT *Mtb*), an Mtb PptT knockdown (using SspB-controlled proteolysis of a DAS-tagged PptT allele) in the presence or absence of anhydrotetracycline (ATC), and a PptH KO strain of *Mtb*.

Table 4. Channel Inhibition and Initial Cardiac Profiling of Selected Analogues

		IC$_{50}$ (μM)			MEC (μM)[a]				
entry	comp	hERG	hCa$_v$1.2	hNa$_v$1.5	G_{mean} cardiomyocytes[b]	avg beat period	avg Na$^+$ slope	avg Na$^+$ amplitude	avg field potential duration
1	**1** (AU 8918)	>30	8.35	3.75	0.7	0.5	0.5	0.5	0.6
2	**2** (lidamidine)	>30	>30	>30	8.9	16	5	5	16
3	**5j**	>30	>30	22.4					
4	**5k**	>30	22.5	24.0	6.7	5	5	5	16
5	**5n**	>30	>30	>30	8.9	16	5	5	16
6	**5p**	>30	>30	>30					

[a]Minimum effective concentration (μM). [b]Geometric mean of the MEC values for average beat period, Na$^+$ slope, Na$^+$ amplitude, and field duration (used for overall ranking of compounds, with higher values better).

https://doi.org/10.1021/acs.jmedchem.1c01565
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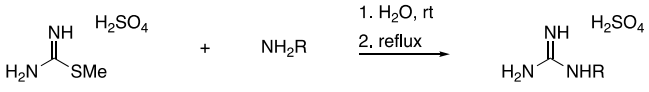
Table 5. Physicochemistry and ADMET Profiling of Selected Compounds

entry	comp	TC_{50} (μM) HEPG2/THP1[a]	log $D_{7.4}$[b]	sol $pH_{7.4}$ (μg/mL)[c]	P_{app} (nm/s) (recovery %)[d]	P_{app} (nm/s) + Elacridar (recovery %)[d]	microsome stab. CLint ($t_{1/2}$) h/m[e]	plasma stab. ($t_{1/2}$) h/m[f]	IC_{50} CYP3A4 (μM)[g]
1	**1** (AU 8918)	>30/>30	1.09	>1000	40 (88)	87 (91)	41/192 (7′)	>1000/>1000	>30
2	**5j**		1.36	>1000	1.1 (89)	13 (88)	6/3	>1000/>1000	
3	**5n**				1.4 (73)	31 (61)	31/97 (14′)	>1000/>1000	
4	**5p**				1.3 (85)	19 (81)	61 (23′)/190 (7′)	199/221	

[a]Cytotoxicity in the HepG2 cell line after 40 h of incubation (desirable value TC50 > 30 μM). [b]Chromatographic log *D* at pH 7.4 (desirable value: 1 < log *D* < 3 for oral route). [c]Thermodynamic solubility at pH 7.4 (μg/mL) (desirable value: solubility >100 μg/mL). [d]Caco-2 (A to B) apparent permeability −/+ the P-glycoprotein inhibitor elacridar; results above 20 nm/s suggest good oral absorption in humans. Elacridar is used as the P-glycoprotein transporter inhibitor. [e]Percentage metabolized by human/mouse liver microsomes following 20 min of incubation at 5 μM in the presence of 1 mM NADPH. CLint >100 μL/min/mg protein; no alert. [f]Measured in mouse and human plasma after 2 h of incubation of 1 μM test compounds at 37 °C; the test compounds were considered stable at 2 h if the percentage of stability was within the 80−120% range. [g]Inhibition in human liver microsomes of the CYP3A4-mediated metabolism of either midazolam (M) or testosterone (T) (desirable value > 30 μM.

using commercial silica gel 60 F_{254} coated aluminum-backed sheets. Visualization was accomplished with UV light (254 nm) and an iodine vapor chamber or *p*-anisaldehyde stain with heating. Purification was carried out on an automated flash chromatography/medium-pressure liquid chromatography (MPLC) system using normal-phase silica flash columns (4, 12, 24, or 40 g) or reversed-phase (RP) C-18 columns (15, 50, and 150 g). IR spectra were acquired as films or solids as indicated. All NMR (^{1}H and ^{13}C) spectra were recorded at either 400 MHz with a dual carbon/proton probe or 600 MHz with a dual carbon/proton cryoprobe instrument. NMR samples were recorded in D_2O, $CDCl_3$, CD_3OD, or DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and are referenced to either TMS (δ 0.0 ppm) or to the center line of the solvent (for D_2O, δ 4.79 ppm for ^{1}H; for $CDCl_3$, δ 7.26 ppm for ^{1}H NMR and 77.2 ppm for ^{13}C NMR; for CD_3OD, δ 4.87 and 3.31 for ^{1}H NMR and 49.0 ppm for ^{13}C NMR; for DMSO-d_6, δ 2.50 ppm for ^{1}H NMR and 39.5 ppm for ^{13}C NMR). Coupling constants are given in hertz (Hz). HRMS data were collected with a time-of-flight (TOF) mass spectrometer or with a hybrid linear trap quadrupole Fourier transform (LTQ FT) and an electrospray ion source (ESI). Melting points were determined in open capillary tubes using an automated melting point apparatus and are uncorrected. Purity was measured using a UPLC system with a photodiode array detector coupled to a mass spectrometer with an ESI source. Compound purity was determined on the basis of peak integration (area under curve) for the selected wavelengths of 214, 254, or 280 nm. All tested compounds were ≥95% pure except for **5a**, **5d**, **5o**, **S4o**, **S1h**, and **S3c**, all of which were 94% pure.

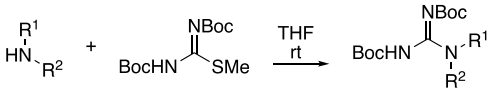
Due to strong similarities in the infrared (IR) spectra of the AU chemical classes, only representative values are given in Table S2 (Supporting Information).



6a-6b

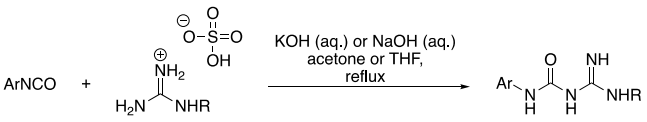
*General Procedure A for the Preparation of Guanidine Sulfates **6a−6b**.* Using a modified published procedure for 1-isopropylguanidine sulfate,[25] 2-methyl-2-thiopseudourea sulfate (5.3 mmol, 1.0 equiv) was dissolved in water (10 mL) at 0 °C, and a solution of amine in THF (2.0 M, 10.6 mmol, 2.0 equiv) was added dropwise via a syringe. The mixture was allowed to warm to rt and stirred for 16 h, at which time it was heated to reflux for 4 h. The reaction was cooled to room temperature, and the solvents were removed to afford an oil. EtOH (55 mL) was added, and the solution was warmed (ca. 60 °C) for 5 min. The mixture was allowed to cool to rt slowly, affording after 17 h a crystalline solid that was collected by vacuum filtration and used without further purification unless otherwise noted.

*General Procedure B for the Preparation of Boc-Protected Guanidines **7a−7c**.* Using a modified literature procedure,[27] to a mixture of 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (3.44 mmol, 1.0 equiv) in anhydrous THF (28 mL) was added ethylamine (2.0 M in THF, 3.44 mmol, 1.0 equiv). The solution was



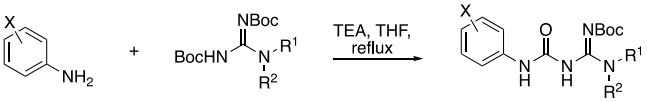
7a-7c

stirred overnight at rt, and then the solvents were removed to yield an off-white solid, which was purified by MPLC (100% hexanes to 20% EtOAc/hexanes).



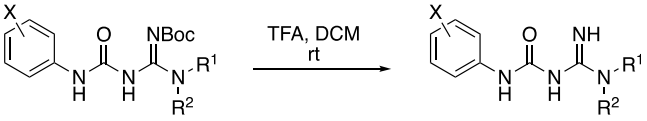
6a-6b **1, 4c-4f, 5u**

*General Procedure C for the Preparation of AUs **1, 4c−4f, and 5u**.* Using a modified literature procedure,[26] to a solution of alkyl guanidine sulfate **6a−6b** (3.91 mmol, 1.0 equiv) dissolved in 10% NaOH (aq) or 10% KOH (aq) (2.0 mL) and acetone or THF (31.8 mL) was added isocyanate (4.31 mmol, 1.1 equiv) dropwise. The solution was heated to reflux and monitored by UPLC for product formation. Following completion, the reaction was cooled, and the solvents were removed. The crude product was dissolved in water (4 mL) and acidified with 1 N HCl. The solution was then neutralized with sat. $NaHCO_3$ (aq), and the aqueous mixture was extracted with EtOAc (3 × 60 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. The crude oil was recrystallized using EtOH or purified using MPLC (DCM/MeOH or EtOAc/hexanes) to afford the product.



7a-7c **8a, S4m-S4q**

*General Procedure D for the Preparation of Boc-Protected AUs **8a and S4m−S4q**.* Using a modified literature procedure,[28] A Boc-protected guanidine **7a−7c** (0.35 mmol, 1.0 equiv), aniline (0.42 mmol, 1.2 equiv), and TEA or DIPEA (1.39 mmol, 4.0 equiv) in anhydrous THF (2.8 mL) were heated to reflux overnight or to 120 °C in a microwave reactor for 10 min and monitored by UPLC for product formation. After heating for the designated amount of time, the mixture was cooled to rt, and the solvents were removed *in vacuo*. The products were isolated following MPLC (hexanes/EtOAc or DCM/MeOH).



8a, S4m-S4q **5a, 5f, 5h, 5k, 5l, 5n**

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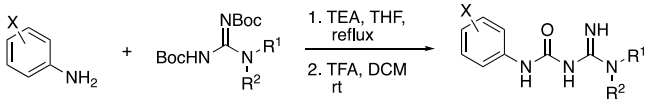
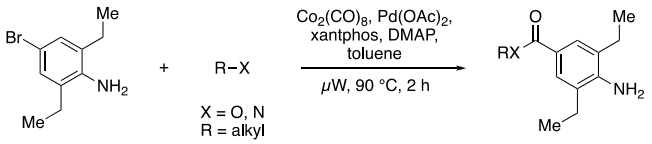
*General Procedure E for the Preparation of AUs **5a, 5f, 5h, 5k, 5l, and 5n**.* Using a modified literature procedure,[38] to a Boc-protected AU (0.19 mmol, 1.0 equiv) was added a mixture of TFA and DCM as a solvent (1:1 unless otherwise noted). The reaction mixture was stirred at rt and monitored by UPLC for the disappearance of the starting material. After 30 min, the solvents were removed, and the crude sample was dissolved in minimal EtOAc and neutralized with sat. aq $NaHCO_3$ or 10% aq NaOH (5 mL), and the aqueous mixture (pH = 7) was extracted with EtOAc (3 × 10 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. The products were isolated following MPLC.



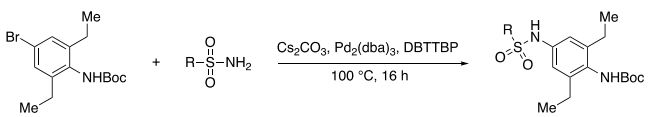
7a-7c → **4a, 4b, 4d, 5b, 5c, 5e, 5g, 5i, 5j, 5m, 5o-5t**

*General Procedure F for the Preparation of AUs **4a, 4b, 4d, 5b, 5c, 5e, 5g, 5i, 5j, 5m, and 5o−5t**.* Using a modified literature procedure,[28] A Boc-protected guanidine **7a−7c** (0.35 mmol, 1.0 equiv), substituted aniline (0.42 mmol, 1.2 equiv), and TEA or DIPEA (1.39 mmol, 4.0 equiv) in anhydrous THF (2.8 mL) were heated to reflux overnight or to 120 °C in a microwave reactor for 10 min and monitored by UPLC for product formation. After heating for the designated time, the mixture was cooled to rt, and the solvents were removed *in vacuo*. Following MPLC (hexanes/EtOAc or DCM/MeOH) or RP MPLC (water/MeCN or water/MeOH), a mixture of the Boc-protected AU product and starting aniline was obtained and treated with a mixture of TFA and DCM as a solvent (1:1 unless otherwise noted). The reaction mixture was stirred at rt and monitored by UPLC for the disappearance of the starting material. After ca. 30 min, the solvents were removed, and the crude sample was dissolved in minimal EtOAc and neutralized with sat. aq $NaHCO_3$ or 10% aq NaOH (5 mL). The aqueous mixture (pH = 7) was extracted with EtOAc (3 × 10 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. The products were isolated following MPLC.



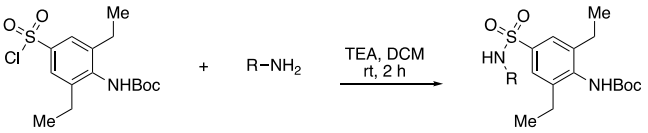
9a-9h

*General Procedure G for the Preparation of Ester and Amide para-Substituted Anilines **9a−9h**.* Using a modified literature procedure,[29] 4-bromo-2,6-diethylaniline (1.0 equiv), amine or alcohol (3.0−5.0 equiv or 1:3 with solvent), diacetoxypalladium (0.05 equiv), xantphos (0.1 equiv), and DMAP (2.0 equiv) were combined in a dry microwave vial, which was evacuated and purged with Ar. To the mixture was added toluene to a reaction molarity of 0.1−0.2 M, followed by dicobalt octacarbonyl (0.25 equiv). The vial was subsequently capped and irradiated in the microwave at 90 °C for 2 h. The reaction progress was monitored via UPLC for product formation. Upon reaction completion, a blue precipitate was filtered off, and the solvent was removed. The crude residue was purified via MPLC (hexanes/EtOAc).



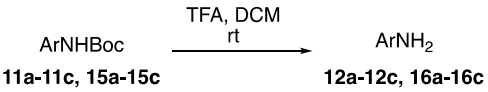
10 → **11a-11c**

*General Procedure H for the Preparation of Boc-Protected N-Connected Sulfonamides **11a−11c**.* Following a modified literature procedure,[30] a solution of *tert*-butyl(4-bromo-2,6-diethylphenyl)-carbamate **10** (0.31 mmol, 1.0 equiv) in 1,4-dioxane (4 mL) was treated with sulfonamide (0.47 mmol, 1.5 equiv), cesium carbonate (0.46 mmol, 1.5 equiv), and di-*tert*-butyl(2′,4′,6′-triisopropyl-3,4,5,6-tetramethyl-[1,1′-biphenyl]-2-yl)phosphane (DBTTBP, 0.05 mmol, 0.16 equiv). The solution was degassed, and tris-(dibenzylideneacetone)dipalladium(0) (0.33 mmol, 0.11 equiv) was added. The reaction was stirred at 100 °C for 16 h and monitored by UPLC. Upon reaction completion, the crude mixture was diluted with ca. 4 mL of ethyl acetate, filtered through Celite, and purified via normal-phase MPLC (hexanes/EtOAc).



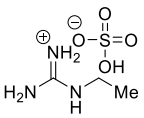
14 → **12a-12c**

*General Procedure I for the Preparation of S-Connected Sulfonamides **12a−12c**.* To a solution of *tert*-butyl-(4-(chlorosulfonyl)-2,6-diethylphenyl)carbamate **14** (0.42 mmol, 1.0 equiv) in dichloromethane (3 mL) was added an alkyl amine (0.46 mmol, 1.1 equiv) and triethylamine (0.84 mmol, 2.0 equiv). The reaction mixture was stirred at room temperature for 2 h, and the solvents were then removed to provide the crude product as a solid. The crude material was subject to normal-phase MPLC (hexanes/EtOAc) for purification.

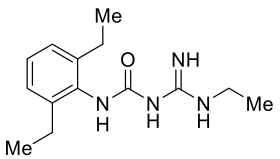


11a-11c, 15a-15c → **12a-12c, 16a-16c**

*General Procedure J for the Preparation of Intermediates **12a−12c and 16a−16c**.* Using a modified literature procedure,[38] to a Boc-protected compound (0.19 mmol, 1.0 equiv) was added a mixture of TFA and DCM as a solvent (1:1 unless otherwise noted). The reaction mixture was stirred at rt and monitored by UPLC for the disappearance of the starting material. After 30 min, the solvents were removed, and the crude sample was dissolved in minimal EtOAc and neutralized with sat. aq $NaHCO_3$ or 10% aq NaOH (5 mL), and the aqueous mixture (pH = 7) was extracted with EtOAc (3 × 10 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. The products were isolated following MPLC.



*1-Ethylguanidine Sulfate (**6a**).* Using general procedure A: 91% yield (4.52 g, 24.4 mmol). Procedure and characterization previously reported.[22]



*N-(2,6-Diethylphenyl)-N′-[(ethylamino)iminomethyl]-urea (**1**).* Using general procedure C with 1,3-diethyl-2-isocyanatobenzene (0.744 mL, 4.31 mmol, 1.1 equiv), 1-ethylguanidine sulfate **6a** (725 mg, 3.91 mmol, 1.0 equiv), 10% KOH (aq) (2.0 mL), and THF (31 mL). Following MPLC (100% DCM to 10:90 MeOH/DCM) and recrystallization from EtOH, the product was isolated in 54% yield (557 mg, 2.12 mmol). Purity (LC): >99%. Procedure and characterization previously reported.[22]

*N,N′-Bis(tert-butoxycarbonyl)-N″-ethylguanidine (**7a**).* Using general procedure B, following MPLC (100% hexanes to 20%

https://doi.org/10.1021/acs.jmedchem.1c01565
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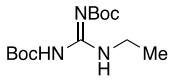
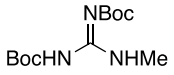
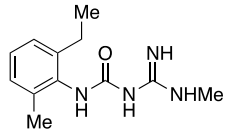


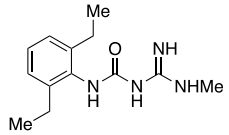
EtOAc/hexanes): 84% yield (836 mg, 2.91 mmol). White crystalline solid. mp 125.3−127.0 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 11.51 (s, 1H), 8.25 (s, 1H), 3.46 (qd, J = 7.3, 5.2 Hz, 2H), 1.50 (d, J = 6.3 Hz, 18H), 1.20 (t, J = 7.3 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 163.8, 156.1, 153.5, 83.2, 79.4, 35.9, 28.5, 28.2, 14.5. HRMS (LTQ FT): calcd for $C_{13}H_{26}N_3O_4$ $[M + H]^+$, 288.1918; found, 288.1931.



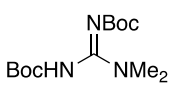
*N,N′-Bis(tert-butoxycarbonyl)-N″-methylguanidine (**7b**).* Using general procedure B, 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (250 mg, 0.86 mmol, 1.0 equiv) and methanamine (40% in water, 100 μL, 1.29 mmol, 1.50 equiv) in THF (7.0 mL). Following MPLC (100% hexanes to 100% EtOAc/hexanes): 97% yield (228 mg, 0.84 mmol). White solid. mp 90.2−94.3 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.45 (s, 1H), 8.27 (d, J = 5.0 Hz, 1H), 2.77 (d, J = 4.7 Hz, 3H), 1.47 (s, 9H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 163.01, 155.66, 151.88, 82.69, 78.00, 27.99, 27.73, 27.60. HRMS (LTQ FT): calcd for $C_{12}H_{24}N_3O_4$ $[M + H]^+$, 274.1761; found, 274.1753.



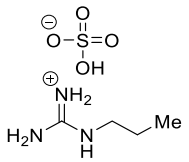
*N-(2-Ethyl-6-methylphenyl)-N′-[(methylamino)iminomethyl]-urea (**4a**).* Using general procedure F, with *N,N′*-bis(*tert*-butoxycarbonyl)-*N″*-methylguanidine **7b** (93.3 mg, 0.34 mmol, 1.0 equiv), 2-ethyl-6-methylaniline (57.2 μL, 0.41 mmol, 1.2 equiv) and TEA (178 μL, 1.28 mmol, 4.0 equiv) in anhydrous THF (2.59 mL) were heated to reflux overnight, followed by MPLC purification (100% hexanes to 40% EtOAc/hexanes). The resultant oil (39.8 mg, 0.12 mmol) was added a 1:1 mixture of TFA and DCM (1.0 mL). Following workup: 66% yield (27.9 mg, 0.04 mmol). Colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.17−7.03 (m, 3H), 2.72 (s, 3H), 2.61 (q, J = 7.4 Hz, 2H), 2.24 (s, 3H), 1.16 (t, J = 7.6 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 160.59, 158.11, 142.04, 136.79, 134.18, 128.15, 127.19, 126.29, 27.60, 25.01, 18.58, 14.72. HRMS (LTQ FT): calcd for $C_{12}H_{19}N_4O$ $[M + H]^+$, 235.1553; found, 235.1546. Purity (LC): >99%.



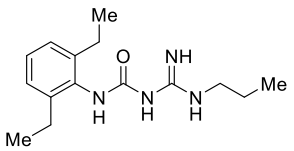
*N-(2,6-Diethylphenyl)-N′-[(methylamino)iminomethyl]-urea (**4b**).* Using general procedure F, with *N,N′*-bis(*tert*-butoxycarbonyl)-*N″*-methylguanidine **7b** (100 mg, 0.37 mmol, 1.0 equiv), 2,6-diethylaniline (72.3 μL, 0.44 mmol, 1.2 equiv) and TEA (204 μL, 1.46 mmol, 4.0 equiv) in anhydrous THF (2.97 mL) were heated to reflux overnight, followed by MPLC purification (100% hexanes to 40% EtOAc/hexanes). The resultant oil (42.1 mg, 0.12 mmol) was added a 1:1 mixture of TFA and DCM (1.0 mL). Following workup: 67% yield (29.2 mg, 0.12 mmol). Colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.18 (t, J = 7.6 Hz, 1H), 7.10 (d, J = 7.6 Hz, 2H), 2.77−2.68 (m, 2H), 2.64−2.57 (m, 4H), 1.22−1.11 (m, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 159.66, 158.09, 142.42, 133.11, 127.75, 126.32, 27.57, 24.93, 14.61. HRMS (LTQ FT): calcd for $C_{13}H_{22}N_4O$ $[M + H]^+$, 249.1710; found, 249.1701. Purity (LC): 97%.



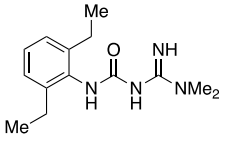
*N,N′-Bis(tert-butoxycarbonyl)-N″,N″-dimethylguanidine (**7c**).* Using general procedure B, 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (250 mg, 0.86 mmol, 1.0 equiv) and dimethylamine (40% in water, 160 μL, 1.29 mmol, 1.50 equiv) in THF (6.98 mL). Following MPLC (100% hexanes to 40% EtOAc/hexanes): 83% yield (205 mg, 0.72 mmol). White solid. mp 113.5−122.8 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.43 (s, 2H), 2.89 (s, 6H), 1.41 (s, 9H), 1.36 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 159.84, 152.22, 151.07, 80.02, 76.87, 37.77, 28.06, 27.92. HRMS (LTQ FT): calcd for $C_{13}H_{26}N_3O_4$ $[M + H]^+$, 288.1918; found, 288.1910. ^{1}H NMR data previously reported.[39]



*1-Propylguanidine Sulfate (**6b**).* Using general procedure A: 91% yield (579 mg, 2.91 mmol). Crystalline white solid. mp 246.3−247.4 °C. ^{1}H NMR (600 MHz, D_2O): δ 3.14 (t, J = 7.0 Hz, 2H), 1.59 (h, J = 7.3 Hz, 2H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, D_2O): δ 156.7, 42.8, 21.4, 10.3. HRMS (LTQ FT): calcd for $C_4H_{12}N_3$ $[M + H]^+$, 102.1026; found, 102.1032. These data are consistent with those previously reported.[40]



*N-(2,6-Diethylphenyl)-N′-[imino(propylamino)methyl]-urea (**4c**).*[41] Using general procedure C, with 1,3-diethyl-2-isocyanatobenzene (143 μL, 0.828 mmol, 1.1 equiv), 1-propylguanidine sulfate **6b** (150 mg, 0.753 mmol, 1.0 equiv), 10% KOH (aq, 0.50 mL, 0.828 mmol, 1.1 equiv), and THF (3.7 mL) and following MPLC (100% DCM to 10% MeOH/DCM): 72% yield (151 mg, 0.546 mmol). White solid. mp 119.4−124.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.22−7.14 (m, 1H), 7.10 (d, J = 7.5 Hz, 2H), 6.56 (s, 1H), 3.06 (t, J = 7.1 Hz, 2H), 2.67 (q, J = 7.6 Hz, 4H), 1.58 (q, J = 7.3 Hz, 2H), 1.19 (t, J = 7.6 Hz, 6H), 0.96 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.8, 160.6, 142.4, 134.0, 127.3, 126.2, 43.2, 25.0, 22.5, 14.7, 11.6. HRMS (LTQ FT): calcd for $C_{15}H_{25}N_4O$ $[M + H]^+$, 277.2027; found, 277.2023. Purity (LC): 96.7%.



*N-(2,6-Diethylphenyl)-N′,N′-[(dimethylamino)iminomethyl]-urea (**4d**).* Using general procedure F, with *N,N′*-bis(*tert*-butoxycarbonyl)-*N″,N″*-dimethylguanidine **7c** (82.7 mg, 0.29 mmol, 1.2 equiv), 2,6-diethylaniline (39.5 μL, 0.24 mmol, 1.0 equiv) and TEA (134 μL, 0.96 mmol, 4.0 equiv) in anhydrous THF (1.95 mL) were heated to reflux, followed by MPLC purification (100% hexanes to 50% EtOAc/hexanes and then 100% basic water to 100% MeCN): the resultant mixture (10.0 mg, 0.03 mmol) was added with TFA (0.5 mL) and DCM (0.5 mL). Following RP MPLC (100% basic water to 100% MeCN): 11% yield (6.5 mg, 0.02 mmol). White solid residue. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.15 (d, J = 7.5 Hz, 1H), 7.10 (d, J = 7.6 Hz, 3H), 6.30 (s, 1H), 2.68 (q, J = 7.6 Hz, 4H), 2.61 (d, J = 0.5 Hz, 6H), 1.20 (t, J = 7.5 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.76, 160.62, 142.21, 134.27, 127.06, 126.10, 41.16, 25.00, 14.67. HRMS (LTQ FT): calcd for $C_{14}H_{23}N_4O$ $[M + H]^+$, 263.1866; found, 263.1862. Purity (LC): 98%.

*N-(2-Ethylphenyl)-N′-[(ethylamino)iminomethyl]-urea (**4e**).* Using general procedure C, with 1-ethyl-2-isocyanatobenzene (126

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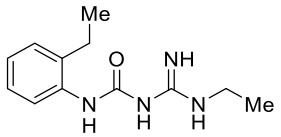
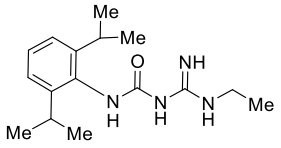
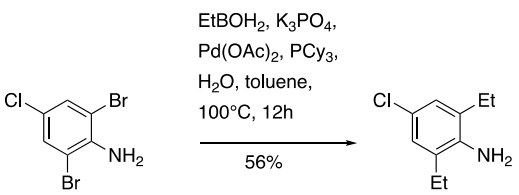


μL, 0.89 mmol, 1.1 equiv), 1-ethylguanidine sulfate **6a** (150 mg, 0.81 mmol, 1.0 equiv), 10% KOH (aq) (0.5 mL, 0.81 mmol, 1.1 equiv), and THF (6.6 mL) and following MPLC (100% DCM to 10% MeOH/DCM): 62% yield (118 mg, 0.47 mmol). White solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.91−7.80 (m, 1H), 7.23−7.12 (m, 2H), 7.01 (td, J = 7.5, 1.3 Hz, 1H), 6.69 (s, 1H), 3.19 (q, J = 7.2 Hz, 2H), 2.61 (q, J = 7.6 Hz, 2H), 1.24 (dt, J = 10.4, 7.4 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 163.91, 160.39, 136.88, 133.96, 128.37, 126.54, 123.62, 122.59, 36.15, 24.48, 14.57, 14.13. HRMS (ESI-TOF): calcd for $C_{12}H_{19}N_4O$ $[M + H]^+$, 235.1554; found, 235.1543. Purity (LC): >99%.



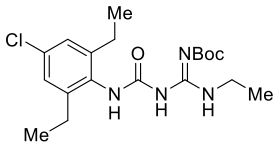
*N-(2,6-Diisopropylphenyl)-N′-[(ethylamino)iminomethyl]-urea (**4f**).* Using general procedure C, with 2-isocyanato-1,3-diisopropylbenzene (199 μL, 0.93 mmol, 1.1 equiv), 1-ethylguanidine sulfate **6a** (157 mg, 0.85 mmol, 1.0 equiv), 10% KOH (aq) (0.5 mL, 0.93 mmol, 1.1 equiv), and THF (6.9 mL) and following MPLC (100% DCM to 10% MeOH/DCM): 94% yield (232 mg, 0.80 mmol). White solid. mp 158.6−164.1 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.24 (t, J = 7.6 Hz, 1H), 7.15 (d, J = 7.7 Hz, 2H), 3.25 (p, J = 6.9 Hz, 2H), 3.13 (q, J = 7.2 Hz, 2H), 1.19 (m, 15H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 165.11, 160.44, 147.17, 132.50, 127.74, 123.34, 123.16, 36.01, 28.75, 24.28, 24.18, 23.89, 23.33, 14.45. HRMS (ESI-TOF): calcd for $C_{16}H_{27}N_4O$ $[M + H]^+$, 291.2180; found, 291.2152. Purity (LC): 97%.



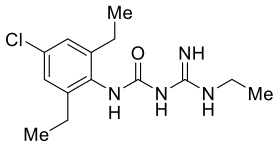
17

*4-Chloro-2,6-diethylaniline (**17**).* Following a literature procedure,[31] a solution of 2,6-dibromo-4-chloroaniline (500 mg, 1.75 mmol, 1.0 equiv), ethyl boronic acid (388 mg, 5.26 mmol, 3.0 equiv), and K_3PO_4 (1.49 g, 7.01 mmol, 4.0 equiv) in H_2O (4 mL) and toluene (15 mL) were stirred at rt under Ar. Ar was used to purge the solution for 15 min, and then $Pd(OAc)_2$ (39.3 mg, 0.175 mmol) and tricyclohexyl phosphine (49.1 mg, 0.175 mmol) were added. The mixture was purged with Ar for another 10 min. The resulting mixture was stirred at 100 °C for 12 h and monitored by UPLC for product formation. The mixture was cooled, diluted with water (25 mL), and extracted with EtOAc (2 × 25 mL). The combined organic extracts were washed with H_2O (1 × 25 mL), brine (1 × 25 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo* to afford the crude product as a dark-brown oil. The crude product was purified using MPLC (100% hexanes to 10:90 EtOAc/hexanes) to afford the product as a light brown oil in 56% yield (182 mg, 0.99 mmol). ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.93 (s, 2H), 3.60 (s, 2H), 2.49 (q, J = 7.5 Hz, 4H), 1.25 (t, J = 7.5 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 140.2, 129.3, 125.7, 123.1, 24.3, 12.9. HRMS (LTQ FT): calcd for $C_{10}H_{15}ClN$ $[M + H]^+$, 184.0888; found, 184.0883. These data are consistent with those previously reported.[31]

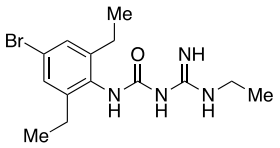
*N-(4-Chloro-2,6-diethylphenyl)-N′-[(ethylamino)N″-(tert-butoxycarbonyl)iminomethyl]-urea (**8a**).* Using general procedure D, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (220 mg, 0.76 mmol, 1.0 equiv), 4-chloro-2,6-diethylaniline **17** (154 mg, 0.84



mmol, 1.1 equiv) and TEA (320 μL, 2.29 mmol, 3.0 equiv) in anhydrous THF (12.4 mL) were heated to reflux, and the mixture was cooled to rt and the solvents were removed. Following MPLC (100% hexanes to 100% EtOAc): 53% yield (163 mg, 0.41 mmol). Off-white solid residue. ^{1}H NMR (600 MHz, $CDCl_3$): δ 12.06 (s, 1H), 8.11 (d, J = 5.8 Hz, 1H), 7.09 (s, 2H), 6.32 (s, 1H), 3.44 (qd, J = 7.2, 5.2 Hz, 2H), 2.63 (q, J = 7.6 Hz, 5H), 1.44 (s, 13H), 1.33−1.10 (m, 13H), 0.87 (q, J = 7.6 Hz, 1H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.13, 155.10, 153.56, 144.09, 132.96, 132.17, 126.17, 125.77, 125.75, 82.55, 35.78, 28.22, 28.18, 25.05, 24.88, 24.28, 14.65, 14.39, 14.33, 14.25, 12.89. *N*-Boc rotamers are present in NMR spectra. HRMS (LTQ FT): calcd for $C_{19}H_{30}ClN_4O_3$ $[M + H]^+$, 397.2001; found, 397.1996.



*N-(4-Chloro-2,6-diethylphenyl)-N′-[(ethylamino)iminomethyl]-urea (**5a**).* Using general procedure E, with *N*-(4-chloro-2,6-diethylphenyl)-*N*′-[(ethylamino)*N*″-(*tert*-butoxycarbonyl) iminomethyl]-urea **8a** (224 mg, 0.56 mmol) was added a 1:1 mixture of TFA and DCM (5.7 mL). The reaction mixture was stirred at rt and monitored by UPLC for the disappearance of the starting material. After 30 min, the solvents were removed, and to the crude sample was added EtOAc and sat. aq. $NaHCO_3$, and the aqueous mixture was extracted with EtOAc (3 × 10 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. Following MPLC (100% DCM to 10% MeOH/DCM and then 100% hexanes to 100% EtOAc): 17% yield (29 mg, 0.098 mmol). White solid residue. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.11 (s, 2H), 3.31 (d, J = 7.3 Hz, 2H), 2.58 (q, J = 7.6 Hz, 4H), 1.35 (t, J = 7.3 Hz, 3H), 1.20 (t, J = 7.6 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 155.07, 143.93, 134.15, 129.69, 126.54, 117.51, 115.58, 24.84, 14.16, 13.59. HRMS (LTQ FT): calcd for $C_{14}H_{22}ClN_4O$ $[M + H]^+$, 297.1477; found, 297.1487. Purity (LC): 94%.



*N-(4-Bromo-2,6-diethylphenyl)-N′-[(ethylamino)iminomethyl]-urea (**5b**).* Using general procedure F, *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (150 mg, 0.52 mmol, 1.0 equiv), 4-bromo-2,6-diethylaniline (143 mg, 0.63 mmol, 1.2 equiv), and TEA (291 μL, 2.1 mmol, 4.0 equiv) in anhydrous THF (4.23 mL) were heated to reflux, followed by MPLC purification (10% DCM/hexanes to 35% DCM/hexanes and then 100% hexanes to 5% EtOAc/hexanes): The resultant mixture (150 mg, 0.34 mmol) was added TFA (1.73 mL) and DCM (1.73 mL). Following MPLC (100% hexanes to 100% EtOAc/hexanes): 32% yield (37 mg, 0.11 mmol). Colorless solid residue. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (s, 2H), 3.18 (t, J = 7.2 Hz, 2H), 2.63 (q, J = 7.6 Hz, 4H), 1.30−1.22 (m, 4H), 1.18 (t, J = 7.6 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 160.62, 157.85, 144.79, 133.39, 121.22, 36.36, 36.32, 30.06, 25.10. HRMS (LTQ FT): calcd for $C_{14}H_{22}BrN_4O$ $[M + H]^+$, 341.0972; found, 341.0976. Purity (LC): 95%.

*4-Amino-3,5-diethylbenzonitrile (**21**).* Following a modified literature procedure,[33] a microwave vial charged with cyanocopper (400 mg, 5.0 mmol, 1.1 equiv) dissolved in DMSO (10 mL) was

https://doi.org/10.1021/acs.jmedchem.1c01565
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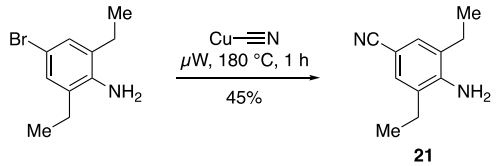

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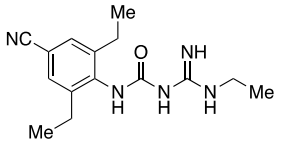
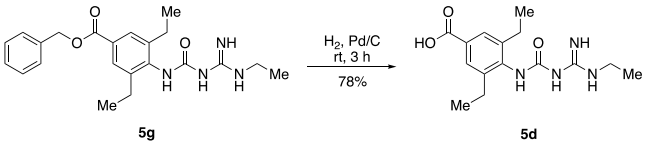
added 4-bromo-2,6-diethylaniline (0.80 mL, 4.0 mmol, 1.0 equiv). The solution was flushed with Ar and irradiated in the microwave at 180 °C for 1 h. Upon reaction completion, ice was added to the solution, and the desired product precipitated out as a sticky brown solid, which was used without further purification. 45% yield (377 mg, 2.1 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.24 (s, 2H), 4.12 (s, 2H), 2.50 (q, J = 7.5 Hz, 4H), 1.27 (t, J = 7.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.07, 130.04, 127.35, 120.92, 100.09, 23.88, 12.46. HRMS (ESI-TOF): calcd for $C_{11}H_{15}N_2$ $[M + H]^+$, 175.1230; found, 175.1233.



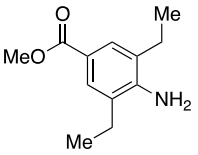
*N-(4-Cyano-2,6-diethylphenyl)-N′-[(ethylamino)iminomethyl]-urea (**5c**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (463 mg, 1.61 mmol, 1.1 equiv), 4-amino-3,5-diethylbenzonitrile **21** (255 mg, 1.47 mmol, 1.0 equiv) and DIPEA (1.0 mL, 5.86 mmol, 4.0 equiv) in anhydrous THF (3.6 mL) were heated in a microwave reactor for 10 min at 120 °C, followed by MPLC purification (100% hexanes to 40% EtOAc/hexanes) and then RP MPLC (100% MeCN to 100% water). The resultant mixture (256 mg, 0.44 mmol) was added TFA (384 μL, 5.01 mmol, 25 equiv) and DCM (5 mL). After stirring for 3 h, the solvents were removed, and to the crude sample was added DCM and sat. $NaHCO_3$ (aq), and the aqueous mixture was extracted three times with DCM. The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. Following MPLC (100% DCM to 5% MeOH/DCM): 24% yield over two steps (48 mg, 0.16 mmol). White solid. mp 137.6−142.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41 (s, 2H), 3.36−3.12 (m, 2H), 2.67 (q, J = 7.6 Hz, 4H), 1.31−1.17 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 155.07, 153.89, 143.45, 135.92, 130.18, 118.92, 112.07, 36.60, 24.72, 13.93, 13.66. HRMS (ESI-TOF): calcd for $C_{15}H_{22}N_5O$ $[M + H]^+$, 288.1819; found, 288.1825. Purity (LC): 97%.



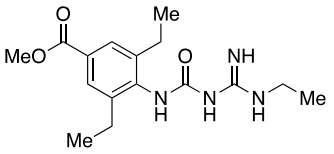
5g 5d

*3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)benzoic Acid (**5d**).* A solution of benzyl-3,5-diethyl-4-(3-(*N*-ethylcarbamimidoyl)-ureido)benzoate **5g** (8.6 mg, 0.02 mmol, 1.0 equiv) in ethanol (2 mL) was purged with Ar for 5 min, then added palladium on carbon (2.3 mg, 0.002 mmol, 0.1 equiv), and subsequently stirred under a hydrogen atmosphere for 3 h. The reaction was monitored by UPLC for the consumption of the starting material. After 3 h, the solution was filtered through a syringe filter, and the solvents were removed. The product was then used without further purification. 90% yield (6.4 mg, 0.02 mmol). White solid residue. ^{1}H NMR (400 MHz, DMSO-d_6 and D_2O): δ 7.56 (s, 2H), 2.53 (q, J = 7.6 Hz, 4H), 1.08 (t, J = 7.6 Hz, 9H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 175.71, 168.44, 150.19, 142.79, 141.28, 130.79, 127.69, 36.49, 24.79, 14.96, 14.85. Purity (LC): 94%. HRMS (ESI-TOF): calcd for $C_{15}H_{23}N_4O_3$ $[M + H]^+$, 307.1765; found, 307.1760.

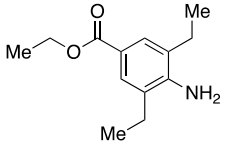
*Methyl-4-amino-3,5-diethylbenzoate (**9a**).* Following general procedure G, a 20 mL microwave vial was added DMAP (964 mg, 7.89 mmol, 2.0 equiv), xantphos (228 mg, 395 μmol, 0.1 equiv), $Pd(OAc)_2$ (44.3 mg, 197 μmol, 0.05 equiv), and 4-bromo-2,6-



diethylaniline (677 μL, 3.95 mmol, 1.0 equiv). The solid mixture was purged with Ar, followed by the addition of toluene (11.8 mL), MeOH (3.95 mL) and dicobalt octacarbonyl (337 mg, 986 μmol. 0.25 equiv) were added. The mixture was heated at 90 °C under microwave irradiation for 30 min. The solvents were removed under vacuum, and the residue was purified via NP SGC (100% hexanes to 10% EtOAc/hexanes) to afford the target (569 mg, 70%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.68 (s, 2H), 4.08 (s, 2H), 3.86 (t, J = 1.5 Hz, 3H), 2.52 (q, J = 7.6 Hz, 4H), 1.28 (t, J = 7.6 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 167.8, 146.4, 127.9, 126.5, 119.1, 51.6, 24.1, 12.8.



*Methyl-3,5-diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)benzoate (**5e**).* Following general procedure F, a mixture of methyl-4-amino-3,5-diethylbenzoate **9a** (449.5 mg, 2.169 mmol), *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (747.8 mg, 2.602 mmol), and Et_3N (1.20 mL, 8.675 mmol) in THF (3.10 mL) was heated at 120 °C under microwave irradiation for 10 min. The solvents were removed under vacuum, and the residue was dissolved in DCM, filtered, and purified via MPLC (100% hexanes to 10% EtOAc/hexanes) to afford the impure Boc-protected target as a colorless film, which was directly added TFA (0.8 mL) and DCM (8.0 mL). After stirring for 16 h, the solvents were removed by a stream of N_2, and the residue was purified via RP MPLC. One portion was purified using neutral conditions (H_2O/MeCN) to afford methyl 3,5-diethyl-4-(3-(*N*-ethylcarbamimidoyl)ureido)benzoate 2,2,2-trifluoroacetate (66.4 mg, 19%) as a TFA salt; the other portion was purified using basic conditions (H_2O/NH3·H_2O/MeCN, pH = 9) to afford methyl 3,5-diethyl-4-(3-(*N*-ethylcarbamimidoyl)ureido)benzoate (56.8 mg, 22%) as a neutral compound. TFA salt: ^{1}H NMR (600 MHz, $CDCl_3$): δ 13.21 (s, br, 1H), 9.41 (s, br, 1H), 7.82 (s, 2H), 7.72 (s, br, 1H), 3.92 (s, 3H), 3.35−3.31 (m, 2H), 2.65 (q, J = 7.6 Hz, 4H), 1.33 (t, J = 7.3 Hz, 3H), 1.24 (t, J = 7.6 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 166.9, 163.9, 163.6, 163.4, 163.2, 154.7, 153.5, 142.1, 135.3, 129.8, 127.7, 119.3, 117.4, 115.4, 113.5, 52.2, 36.4, 24.8, 14.1, 13.5. HRMS (LTQ FT): calcd for $C_{16}H_{25}N_4O_3$ $[M + H]^+$, 321.1921; found, 321.1917. Purity (LC): 98%. Neutral form: ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.77 (s, 2H), 7.12−6.71 (s, br, 1H), 3.89 (s, 3H), 3.09 (s, br, 2H), 2.68 (q, J = 7.6 Hz, 4H), 1.20 (t, J = 7.6 Hz, 6H), 1.17−1.11 (m, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 167.3, 164.2, 160.4, 142.2, 138.7, 128.2, 127.4, 52.0, 35.8, 24.8, 14.3, 14.2. HRMS (LTQ FT): calcd for $C_{16}H_{25}N_4O_3$ $[M + H]^+$, 321.1921; found, 321.1916. Purity (LC): 90%.



*Ethyl-4-amino-3,5-diethylbenzoate (**9b**).* Using general procedure G, with 4-bromo-2,6-diethylaniline (0.376 mL, 2.19 mmol, 1.0 equiv), diacetoxypalladium (24.6 mg, 0.11 mmol, 0.05 equiv), xantphos (127 mg, 0.22 mmol, 0.1 equiv), DMAP (536 mg, 4.38 mmol, 2.0 equiv), and dicobalt octacarbonyl (187 mg, 0.55 mmol, 0.25 equiv) in ethanol (2.0 mL) and toluene (6.6 mL). Following MPLC (100% hexanes to 100% EtOAc): 64% yield (308 mg, 1.4 mmol). Colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68 (s, 2H), 4.33 (q, J = 7.1 Hz, 2H),

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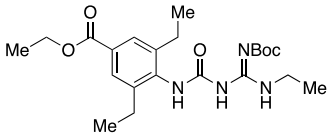
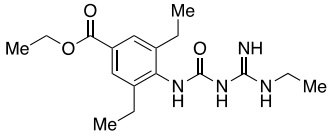
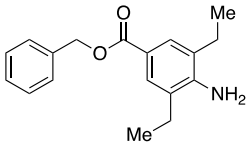
4.04 (s, 2H), 2.54 (q, J = 7.5 Hz, 4H), 1.37 (t, J = 7.1 Hz, 3H), 1.29 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.40, 146.29, 128.01, 126.54, 119.59, 60.31, 24.22, 14.60, 12.84. HRMS (ESI-TOF): calcd for $C_{13}H_{20}NO_2$ $[M + H]^+$, 222.1489; found, 222.1482.



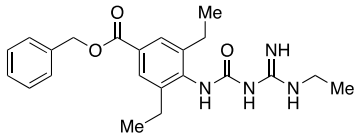
*Ethyl-(E)-4-(3-(N′-(tert-butoxycarbonyl)-N-ethylcarbamimidoyl)-ureido)-3,5-diethylbenzoate (**S4m**).* Using general procedure D, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (439 mg, 1.53 mmol, 1.2 equiv), ethyl-4-amino-3,5-diethylbenzoate **9b** (282 mg, 1.27 mmol, 1.0 equiv) and TEA (890 μL, 5.10 mmol, 4.0 equiv) in anhydrous THF (9 mL) were heated in a microwave at 120 °C for 10 min. The mixture was cooled to rt and the solvents were removed. Following MPLC (100% hexanes to 20% EtOAc/hexanes) and then repurification by RP MPLC (100% water to 100% MeCN): 44% yield (246 mg, 0.57 mmol). White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.03 (s, 1H), 8.13 (d, J = 6.3 Hz, 1H), 7.79 (s, 2H), 6.53 (s, 1H), 4.36 (q, J = 7.1 Hz, 2H), 3.52−3.29 (m, 2H), 2.69 (q, J = 7.5 Hz, 4H), 1.40 (m, 12H), 1.22 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.81, 163.77, 155.10, 153.48, 142.01, 137.99, 129.04, 127.54, 82.54, 60.89, 35.74, 28.12, 24.98, 14.58, 14.47, 14.42. HRMS (LTQ FT): calcd for $C_{22}H_{35}N_4O_5$ $[M + H]^+$, 435.2602; found, 435.2596. Purity (LC): 96%.



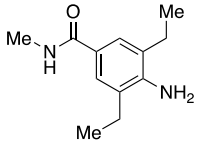
*Ethyl 3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)benzoate (**5f**).* Using general procedure E, with ethyl-(*E*)-4-(3-(*N*′-(*tert*-butoxycarbonyl)-*N*-ethylcarbamimidoyl)ureido)-3,5-diethylbenzoate **S4m** (110 mg, 0.25 mmol, 1.0 equiv) was added a mixture of TFA (0.48 mL, 6.3 mmol, 25 equiv) and DCM (5.0 mL). The reaction mixture was stirred at rt overnight and monitored by UPLC for the disappearance of the starting material. The solvents were removed, and the crude sample was neutralized with aqueous NaOH. Following purification by RP MPLC (100% water to 100% MeOH) and then by normal-phase MPLC (100% DCM to 10% MeOH/DCM): 73% yield (62 mg, 0.19 mmol). White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (s, 2H), 4.36 (q, J = 7.1 Hz, 2H), 3.19 (br s, 2H), 2.70 (q, J = 7.6 Hz, 4H), 1.39 (t, J = 7.1 Hz, 3H), 1.23 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.80, 160.41, 142.14, 128.59, 127.88, 127.38, 60.78, 35.84, 24.89, 24.10, 14.36, 14.33, 14.24, 12.72. HRMS (LTQ FT): calcd for $C_{17}H_{27}N_4O_3$ $[M + H]^+$, 335.2078; found, 335.2070. Purity (LC): 95%.



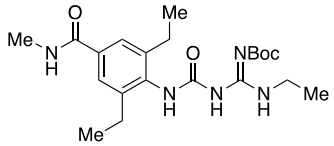
*Benzyl-4-amino-3,5-diethylbenzoate (**9c**).* Using general procedure G, with 4-bromo-2,6-diethylaniline (0.376 mL, 2.19 mmol, 1.0 equiv), diacetoxypalladium (24.6 mg, 0.11 mmol, 0.05 equiv), xantphos (127 mg, 0.22 mmol, 0.1 equiv), DMAP (536 mg, 4.38 mmol, 2.0 equiv), dicobalt octacarbonyl (187 mg, 0.55 mmol, 0.25 equiv), and benzyl alcohol (1.14 mL, 11.0 mmol, 5.0 equiv) in toluene (9.0 mL). Following MPLC (100% hexanes to 20% EtOAc/hexanes): 67% yield (415 mg, 1.47 mmol). Yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (s, 2H), 7.47−7.42 (m, 2H), 7.40−7.28 (m, 3H), 4.06 (s, 2H), 2.52 (q, J = 7.5 Hz, 4H), 1.27 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.09, 146.41, 136.82, 128.49, 128.15, 128.00, 127.92, 126.48, 119.03, 65.94, 24.11, 12.73. HRMS (ESI-TOF): calcd for $C_{18}H_{22}NO_2$ $[M + H]^+$, 284.1645; found, 284.1631.



*Benzyl-3,5-diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)benzoate (**5g**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (426 mg, 1.5 mmol, 1.2 equiv), benzyl-4-amino-3,5-diethylbenzoate **9c** (350 mg, 1.2 mmol, 1.0 equiv) and TEA (860 μL, 4.9 mmol, 4.0 equiv) in anhydrous THF (8.0 mL) were heated in a microwave reactor for 10 min at 120 °C, followed by MPLC purification (100% hexanes to 20% EtOAc/hexanes) and then RP MPLC (100% water to 100% MeOH). The resultant mixture (127 mg, 0.26 mmol) was added a mixture of TFA (1.0 mL) and DCM (5.0 mL). After stirring overnight, the solvents were removed, and the crude sample was neutralized with NaOH. Following MPLC (100% DCM to 10% MeOH/DCM): 18% yield (89 mg, 0.22 mmol). Off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (s, 2H), 7.51−7.26 (m, 4H), 5.34 (s, 2H), 3.34 (s, 1H), 2.62 (q, J = 7.6 Hz, 4H), 1.30 (t, J = 7.2 Hz, 3H), 1.18 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.40, 155.63, 142.35, 136.30, 136.16, 129.56, 128.69, 128.33, 128.20, 128.20, 127.84, 66.82, 36.84, 24.93, 14.38, 13.96. HRMS (ESI-TOF): calcd for $C_{22}H_{29}N_4O_3$ $[M + H]^+$, 397.2234; found, 397.2226. Purity (LC): 95%.



*4-Amino-3,5-diethyl-N-methylbenzamide (**9d**).* Using general procedure G, with 4-bromo-2,6-diethylaniline (0.150 mL, 0.88 mmol, 1.0 equiv), diacetoxypalladium (19.7 mg, 0.88 mmol, 0.1 equiv), xantphos (101 mg, 0.18 mmol, 0.2 equiv), DMAP (214 mg, 1.75 mmol, 2.0 equiv), dicobalt octacarbonyl (74.9 mg, 0.22 mmol, 0.25 equiv), and methanamine (2.0 M, 1.30 mL, 2.63 mmol, 3.0 equiv) in toluene (2.63 mL). Following MPLC (100% hexanes to 50% EtOAc/hexanes): 87% yield (158 mg, 0.77 mmol). Colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (s, 2H), 5.95 (s, 1H), 3.89 (s, 2H), 2.96 (d, J = 4.8 Hz, 3H), 2.51 (q, J = 7.5 Hz, 4H), 1.25 (t, J = 7.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.74, 144.87, 126.99, 125.17, 124.03, 26.85, 24.41, 12.96. HRMS (ESI-TOF): calcd for $C_{12}H_{19}N_2O$ $[M + H]^+$, 207.1492; found, 207.1488.



*3,5-Diethyl-4-(3-(N′-(tert-butoxycarbonyl)-N-ethylcarbamimidoyl)ureido)-N-methylbenzamide (**S4n**).* Using general procedure D, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (84 mg, 0.29 mmol, 1.2 equiv), 4-amino-3,5-diethyl-*N*-methylbenzamide **9d** (50 mg, 0.24 mmol, 1.0 equiv) and DIPEA (170 μL, 0.97 mmol, 4.0 equiv) in anhydrous THF were heated in a microwave at 120 °C for 10 min. The mixture was cooled to rt, and the solvents were removed. Following MPLC (100% hexanes to 100% EtOAc) and then repurification by RP MPLC (100% water to 100% MeOH): 46% yield (47 mg, 0.11 mmol). White sticky solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.03 (s, 1H), 8.12 (s, 1H), 7.49 (s, 2H), 6.45 (s, 1H), 6.10 (s, 1H), 3.51−3.35 (m, 2H), 3.00 (d, J = 4.8 Hz, 3H), 2.69 (q, J = 7.5 Hz, 4H), 1.44 (s, 9H), 1.26−1.20 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.63, 163.94, 155.12, 153.53, 142.40, 136.56, 133.61, 124.82, 82.58, 35.80, 28.17, 26.97, 25.08, 14.64, 14.48. HRMS

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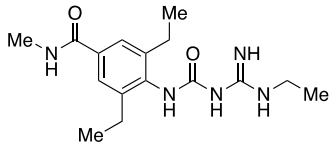
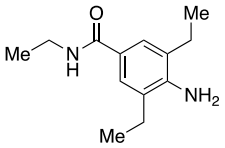
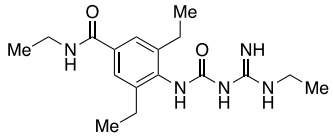
(LTQ FT): calcd for $C_{21}H_{34}N_5O_4$ [M + H]$^+$, 420.2605; found, 420.2598. Purity (LC): 96%.



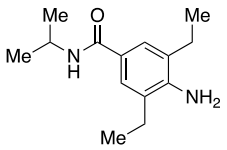
*3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)-N-methylbenzamide (**5h**).* Using general procedure E, with 3,5-diethyl-4-(3-(*N*′-(*tert*-butoxycarbonyl)-*N*-ethylcarbamimidoyl)ureido)-*N*-methylbenzamide **S4n** (70 mg, 0.17 mmol, 1.0 equiv) was added a 1:1 mixture of TFA (0.32 mL, 4.25 mmol, 25 equiv) and DCM (0.32 mL). The reaction mixture was stirred at rt overnight and monitored by UPLC for the disappearance of the starting material. The solvents were removed, and the crude sample was neutralized with aqueous NaOH. Following purification by RP MPLC (100% water to 100% MeOH): 79% yield (42 mg, 0.13 mmol). Beige sticky solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (s, 2H), 6.24 (s, 1H), 3.23−3.05 (m, 2H), 2.95 (d, J = 4.8 Hz, 3H), 2.68 (q, J = 7.6 Hz, 4H), 1.20 (t, J = 7.6 Hz, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.82, 160.53, 142.54, 137.11, 133.40, 124.82, 36.05, 26.92, 25.12, 14.51, 14.48. HRMS (LTQ FT): calcd for $C_{16}H_{26}N_5O_2$ [M + H]$^+$, 320.2081; found, 320.2076. Purity (LC): 98%.



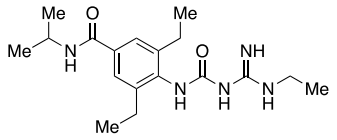
*4-Amino-N-3,5-triethylbenzamide (**9e**).* Using general procedure G, with 4-bromo-2,6-diethylaniline (0.376 mL, 2.19 mmol, 1.0 equiv), diacetoxypalladium (49.2 mg, 0.22 mmol, 0.1 equiv), xantphos (254 mg, 0.44 mmol, 0.2 equiv), DMAP (536 mg, 4.38 mmol, 2.0 equiv), dicobalt octacarbonyl (187 mg, 0.55 mmol, 0.25 equiv), and ethanamine (2.0 M, 3.29 mL, 6.58 mmol, 3.0 equiv) in toluene (6.64 mL). Following MPLC (100% hexanes to 40% EtOAc/hexanes): 89% yield (294 mg, 1.18 mmol). Light brown solid. mp 132.8−139.6. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (s, 2H), 5.95 (s, 1H), 3.92 (s, 2H), 3.48 (qd, J = 7.3, 5.6 Hz, 2H), 2.55 (q, J = 7.5 Hz, 4H), 1.26 (dt, J = 16.4, 7.4 Hz, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.91, 144.84, 126.95, 125.19, 124.06, 34.82, 24.39, 15.20, 12.94. HRMS (ESI-TOF): calcd for $C_{13}H_{21}N_2O$ [M + H]$^+$, 221.1648; found, 221.1651.



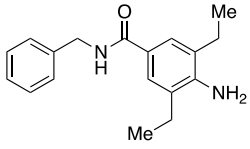
*N,3,5-Triethyl-4-(3-(N-ethylcarbamimidoyl)ureido)benzamide (**5i**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (405 mg, 1.41 mmol, 1.2 equiv), 4-amino-*N*-3,5-triethylbenzamide **9e** (259 mg, 1.18 mmol, 1.0 equiv) and DIPEA (820 μL, 4.70 mmol, 4.0 equiv) in anhydrous THF (4.2 mL) were heated in a microwave reactor for 10 min at 120 °C, followed by MPLC purification (100% hexanes to 100% EtOAc/hexanes). The resultant mixture (182 mg, 0.42 mmol) was added a mixture of TFA (1.5 mL, 10.5 mmol, 25 equiv) and DCM (10 mL). The reaction mixture was stirred at rt overnight and monitored by UPLC for the disappearance of the starting material. After stirring overnight, the solvents were removed, and to the crude sample was added DCM and sat. aq. $NaHCO_3$, and the aqueous mixture was extracted three times with DCM. The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. Following RP MPLC (100% water to 100% MeOH): 16% yield (45 mg, 0.13 mmol). Off-white solid residue. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (s, 2H), 6.13 (s, 1H), 3.55−3.38 (m, 2H), 3.15 (s, 2H), 2.69 (q, J = 7.6 Hz, 4H), 1.24−1.18 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.11, 164.48, 160.56, 142.63, 137.17, 133.60, 124.81, 35.93, 34.99, 25.11, 15.04, 14.51, 14.45. HRMS (ESI-TOF): calcd for $C_{17}H_{28}N_5O_2$ [M + H]$^+$, 334.2238; found, 334.2242. Purity (LC): 99%.



*4-Amino-3,5-diethyl-N-isopropylbenzamide (**9f**).* Using general procedure G, with 4-bromo-2,6-diethylaniline (0.376 mL, 2.19 mmol, 1.0 equiv), diacetoxypalladium (49.2 mg, 0.22 mmol, 0.1 equiv), xantphos (254 mg, 0.44 mmol, 0.2 equiv), DMAP (536 mg, 4.38 mmol, 2.0 equiv), dicobalt octacarbonyl (187 mg, 0.55 mmol, 0.25 equiv), and isopropylamine (2.0 M, 3.29 mL, 6.58 mmol, 3.0 equiv) in toluene (6.64 mL). Following MPLC (100% hexanes to 40% EtOAc/hexanes): 86% yield (358 mg, 1.30 mmol). Light brown solid. mp 124.5−131.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38 (s, 2H), 4.37−4.18 (m, 1H), 3.91 (s, 2H), 2.55 (q, J = 7.5 Hz, 4H), 1.34−1.17 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.17, 144.81, 127.02, 125.21, 124.24, 41.69, 24.46, 23.13, 13.00. HRMS (ESI-TOF): calcd for $C_{14}H_{23}N_2O$ [M + H]$^+$, 235.1805; found, 235.1808.



*3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)-N-isopropylbenzamide (**5j**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (377 mg, 1.31 mmol, 1.2 equiv), 4-amino-3,5-diethyl-*N*-isopropylbenzamide **9f** (256 mg, 1.09 mmol, 1.0 equiv) and DIPEA (760 μL, 4.38 mmol, 4.0 equiv) in anhydrous THF (6.2 mL) were heated in a microwave reactor for 10 min at 120 °C, followed by RP MPLC purification (100% water to 100% MeOH). The resultant mixture (122 mg, 0.27 mmol) was added TFA (520 μL, 6.80 mmol, 25 equiv) and DCM (7 mL). The reaction mixture was stirred at rt overnight and monitored by UPLC for the disappearance of the starting material. After stirring overnight, the solvents were removed, and to the crude sample was added DCM and sat. $NaHCO_3$ (aq), and the aqueous mixture was extracted three times with DCM. The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. Following RP MPLC (100% water to 100% MeOH): 18% yield (50 mg, 0.13 mmol). White solid residue. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41 (s, 2H), 6.10 (d, J = 7.9 Hz, 1H), 4.42−4.09 (m, 1H), 3.06 (br s, 2H), 2.64 (q, J = 7.5 Hz, 4H), 1.30−1.10 (m, 12H), 1.07 (t, J = 7.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.38, 164.51, 160.57, 142.68, 137.25, 133.63, 124.75, 41.94, 35.83, 25.07, 22.85, 14.50, 14.42. HRMS (ESI-TOF): calcd for $C_{18}H_{30}N_5O_2$ [M + H]$^+$, 348.2394; found, 348.2400. Purity (LC): 97%.



*4-Amino-N-benzyl-3,5-diethylbenzamide (**9g**).* Using general procedure G, with 4-bromo-2,6-diethylaniline (0.376 mL, 2.19 mmol, 1.0 equiv), diacetoxypalladium (49.2 mg, 0.22 mmol, 0.1 equiv), xantphos (254 mg, 0.44 mmol, 0.2 equiv), DMAP (536 mg, 4.38 mmol, 2.0 equiv), dicobalt octacarbonyl (187 mg, 0.55 mmol, 0.25 equiv), and benzylamine (0.958 mL, 8.77 mmol, 4.0 equiv) in toluene (6.6 mL). Following MPLC (100% hexanes to 100% EtOAc/hexanes): 23% yield (150 mg, 0.50 mmol). Brown oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44 (s, 2H), 7.39−7.27 (m, 4H), 6.31 (s, 1H), 4.64

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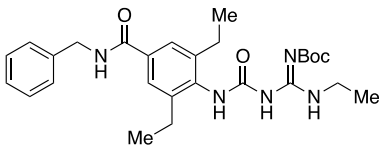
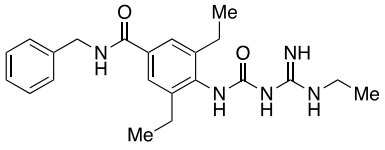
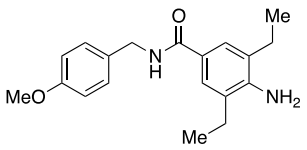
(d, J = 5.7 Hz, 2H), 3.95 (s, 2H), 2.54 (q, J = 7.6 Hz, 4H), 1.27 (t, J = 7.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.81, 145.11, 138.98, 128.83, 128.06, 127.54, 127.04, 125.39, 123.56, 44.08, 24.44, 12.96. HRMS (ESI-TOF): calcd for $C_{18}H_{23}N_2O$ $[M + H]^+$, 283.1805; found, 283.1810.



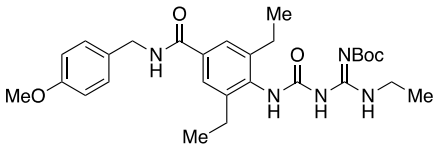
*3,5-Diethyl-4-(3-(N′-(tert-butoxycarbonyl)-N-ethylcarbamimidoyl)ureido)-N-methylbenzamide (**S4o**).* Using general procedure D, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (440 mg, 1.53 mmol, 1.2 equiv), 4-amino-*N*-benzyl-3,5-diethylbenzamide **9g** (360 mg, 1.28 mmol, 1.0 equiv) and DIPEA (890 μL, 5.10 mmol, 4.0 equiv) in anhydrous THF (3.1 mL) were heated in a chemical microwave at 120 °C for 10 min. The mixture was cooled to rt, and the solvents were removed. Following MPLC (100% hexanes to 100% EtOAc) and then repurification by RP MPLC (100% water to 100% MeOH): 26% yield (173 mg, 0.33 mmol). Yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.03 (s, 1H), 8.13 (s, 1H), 7.53 (s, 2H), 7.40−7.29 (m, 5H), 6.45 (s, 1H), 6.33 (s, 1H), 4.66 (d, J = 5.7 Hz, 2H), 3.62−3.34 (m, 2H), 2.69 (q, J = 7.6 Hz, 4H), 1.44 (s, 9H), 1.29−1.04 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.01, 164.21, 155.40, 153.79, 142.77, 138.80, 137.08, 133.51, 129.15, 128.23, 127.91, 125.28, 82.86, 44.44, 36.06, 28.43, 25.38, 14.90, 14.79. HRMS (LTQ FT): calcd for $C_{27}H_{38}N_5O_4$ $[M + H]^+$, 496.2918; found, 496.2927. Purity (LC): 94%.



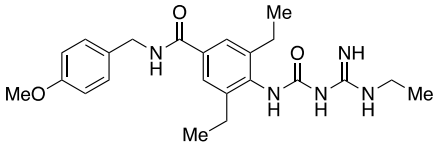
*N-Benzyl-3,5-diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)-benzamide (**5k**).* Using general procedure E, with 3,5-diethyl-4-(3-(*N*′-(*tert*-butoxycarbonyl)-*N*-ethylcarbamimidoyl)ureido)-*N*-methylbenzamide **S4o** (116 mg, 0.23 mmol, 1.0 equiv) was added a mixture of TFA (448 μL, 5.86 mmol, 25 equiv) and DCM (6 mL). The reaction mixture was stirred at rt and monitored by UPLC for the disappearance of the starting material. After stirring overnight, the solvents were removed, and to the crude sample was added DCM and sat. $NaHCO_3$ (aq), and the aqueous mixture was extracted three times with DCM. The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. Following MPLC (100% DCM to 10% MeOH/DCM): 71% yield (67.8 mg, 0.17 mmol). Colorless solid residue. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52 (s, 2H), 7.37−7.32 (m, 5H), 4.64 (d, J = 5.7 Hz, 2H), 3.30 (d, J = 7.3 Hz, 2H), 2.64 (q, J = 7.5 Hz, 4H), 1.31 (t, J = 7.2 Hz, 3H), 1.21 (t, J = 7.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.84, 155.57, 147.71, 142.69, 138.28, 134.87, 134.06, 128.89, 127.89, 127.71, 125.22, 44.22, 36.68, 29.83, 25.02, 14.41, 13.89. HRMS (LTQ FT): calcd for $C_{22}H_{30}N_5O_2$ $[M + H]^+$, 396.2394; found, 396.2403. Purity (LC): 98%.



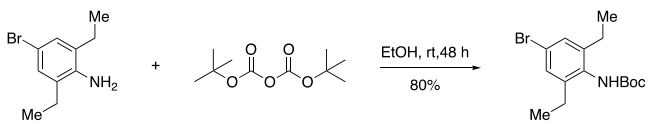
*4-Amino-3,5-diethyl-N-(4-methoxybenzyl)benzamide (**9h**).* Using general procedure G, with 4-bromo-2,6-diethylaniline (0.376 mL, 2.19 mmol, 1.0 equiv), diacetoxypalladium (49.2 mg, 0.22 mmol, 0.1 equiv), xantphos (127 mg, 0.22 mmol, 0.1 equiv), DMAP (536 mg, 4.38 mmol, 2.0 equiv), dicobalt octacarbonyl (187 mg, 0.55 mmol, 0.25 equiv), and 4-methoxybenzylamine (1.43 mL, 11.0 mmol, 5.0 equiv) in toluene (9.3 mL). Following MPLC (100% hexanes to 20% EtOAc/hexanes): 50% yield (345 mg, 1.1 mmol). Yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42 (s, 2H), 7.33−7.27 (m, 2H), 6.88 (d, J = 8.7 Hz, 2H), 6.20 (s, 1H), 4.57 (d, J = 5.6 Hz, 2H), 3.93 (s, 2H), 3.80 (s, 3H), 2.54 (q, J = 7.5 Hz, 4H), 1.27 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.73, 159.15, 145.06, 131.05, 129.45, 127.05, 125.38, 123.68, 114.23, 55.47, 43.60, 24.46, 12.98. HRMS (ESI-TOF): calcd for $C_{19}H_{25}N_2O_2$ $[M + H]^+$, 313.1911; found, 313.1903.



*3,5-Diethyl-4-(3-(N′-(tert-butoxycarbonyl)-N-ethylcarbamimidoyl)ureido)-N-(4-methoxybenzyl)benzamide (**S4p**).* Using general procedure D, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (153 mg, 0.53 mmol, 1.0 equiv), 4-amino-3,5-diethyl-*N*-(4-methoxybenzyl)benzamide **9h** (200 mg, 0.64 mmol, 1.2 equiv) and TEA (297 μL, 53.3 mmol, 4.0 equiv) in anhydrous THF (4.3 mL) were heated to reflux for 16 h. The mixture was cooled to rt, and the solvents were removed. Following MPLC (100% hexanes to 60% EtOAc): 76% yield (214 mg, 0.40 mmol). White solid. mp 94.7−101.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.04 (s, 1H), 8.20−8.06 (m, 1H), 7.52 (s, 2H), 7.28 (d, J = 8.5 Hz, 2H), 6.95−6.81 (m, 2H), 6.49 (s, 1H), 6.37 (s, 1H), 4.57 (d, J = 5.6 Hz, 2H), 3.80 (s, 3H), 3.44 (dd, J = 7.4, 5.4 Hz, 2H), 2.68 (q, J = 7.5 Hz, 4H), 1.44 (s, 9H), 1.33−1.08 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.60, 163.91, 159.17, 155.10, 153.50, 142.46, 136.72, 133.29, 130.55, 129.32, 124.95, 114.24, 82.55, 55.42, 43.67, 35.76, 28.13, 25.09, 14.50. HRMS (LTQ FT): calcd for $C_{28}H_{40}N_5O_5$ $[M + H]^+$, 526.3024; found, 526.3005. Purity (LC): 99%.



*3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)-N-(4-methoxybenzyl)benzamide (**5l**).* Using general procedure E, with 3,5-diethyl-4-(3-(*N*′-(*tert*-butoxycarbonyl)-*N*-ethylcarbamimidoyl)-ureido)-*N*-(4-methoxybenzyl)benzamide **S4p** (100 mg, 0.19 mmol, 1.0 equiv) was added a 1:1 mixture of TFA (0.5 mL) and DCM (0.5 mL). The reaction mixture was stirred at rt and monitored by UPLC for the disappearance of the starting material. After stirring for 4 h, the solvents were removed, and to the crude sample was added EtOAc and sat. $NaHCO_3$ (aq), and the aqueous mixture was extracted five times with EtOAc. The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as a white solid. Following RP MPLC (100% basic water to 100% MeCN): 59% yield (47.4 mg, 0.11 mmol). Sticky white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.49 (s, 2H), 7.30−7.24 (m, 2H), 6.98−6.85 (m, 2H), 6.37 (t, J = 5.5 Hz, 1H), 4.55 (d, J = 5.5 Hz, 2H), 3.80 (s, 3H), 3.15 (s, 2H), 2.68 (q, J = 7.6 Hz, 4H), 1.25−1.08 (m, 9H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 167.63, 164.40, 160.43, 159.06, 142.45, 132.92, 130.46, 129.23, 124.81, 114.13, 55.32, 43.55, 35.94, 25.04, 14.43, 14.32. HRMS (LTQ FT): calcd for $C_{23}H_{32}N_5O_3$ $[M + H]^+$, 426.2500; found, 426.2495. Purity (LC): 98%.



*tert-Butyl(4-bromo-2,6-diethylphenyl)carbamate (**10**).* Using a modified literature procedure,[42] di-*tert*-butyl dicarbonate (1.0 g, 4.6 mmol, 1.5 equiv) was added to a solution of 4-bromo-2,6-diethylaniline (700 mg, 3.1 mmol, 1.0 equiv) in anhydrous ethanol

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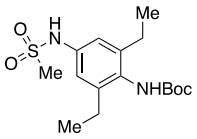
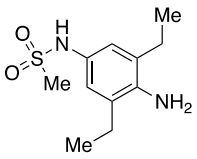
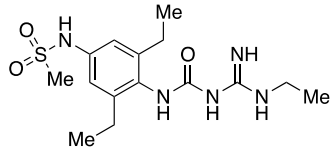
(6.0 mL). The reaction mixture was stirred at room temperature for 48 h under an Ar atmosphere. The reaction was monitored via UPLC, and upon completion of the reaction, the solvent was removed, and the crude material was purified via normal-phase MPLC (100% hexanes to 20% EtOAc/hexanes): 80% yield (809 mg, 2.5 mmol). Light pink solid. mp 125.8−126.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (s, 2H), 2.60 (q, J = 7.6 Hz, 4H), 1.53 (s, 9H), 1.19 (t, J = 7.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.85, 146.90, 144.42, 129.38, 121.47, 85.33, 28.43, 27.57, 24.80, 14.34. HRMS (LTQ FT): calcd for $C_{15}H_{23}BrNO_2$ $[M + H]^+$, 328.0912; found, 328.0895. Purity (LC): 99%.



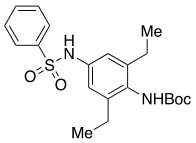
*tert-Butyl(2,6-diethyl-4-(methylsulfonamido)phenyl)carbamate (**11a**).* Using general procedure H, with *tert*-Butyl(4-bromo-2,6-diethylphenyl)carbamate **10** (200 mg, 0.61 mmol, 1.0 equiv), methanesulfonamide (86.9 mg, 0.91 mmol, 1.5 equiv), Tris-(dibenzylideneacetone)dipalladium(0) (61.4 mg, 0.07 mmol, 0.11 equiv), DBTTBP (46.9 mg, 0.10 mmol, 0.16 equiv), and cesium carbonate (298 mg, 0.91 mmol, 2.5 equiv) in 1,4-dioxane (8.0 mL). Following MPLC (100% hexanes to 100% EtOAc): 48% yield (100 mg, 0.29 mmol). Brown foamy solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.93 (s, 2H), 3.00 (s, 3H), 2.62 (q, J = 7.6 Hz, 4H), 1.51 (s, 9H), 1.19 (t, J = 7.5 Hz, 5H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 154.59, 144.03, 135.81, 129.91, 118.29, 80.17, 39.31, 28.31, 24.91, 14.32. HRMS (ESI-TOF): calcd for $C_{16}H_{25}N_2O_4S$ $[M - H]^-$, 341.1541; found, 341.1551.



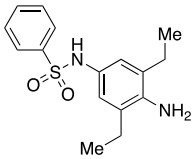
*N-(4-Amino-3,5-diethylphenyl)methanesulfonamide (**12a**).* Using general procedure J, with *tert*-butyl(2,6-diethyl-4-(methylsulfonamido)phenyl)carbamate **11a** (71.2 mg, 0.21 mmol) in a 1:1 mixture of TFA/DCM (2 mL). Following MPLC (100% DCM to 10% MeOH/DCM): 78% yield (39 mg, 0.16 mmol). White solid residue. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.88 (s, 2H), 6.28 (s, 1H), 3.68 (d, J = 15.4 Hz, 2H), 2.94 (s, 3H), 2.51 (q, J = 7.5 Hz, 4H), 1.25 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 140.66, 128.80, 126.69, 122.20, 38.82, 24.36, 12.97. HRMS (ESI-TOF): calcd for $C_{11}H_{19}N_2O_2S$ $[M + H]^+$, 243.1162; found, 243.1151.



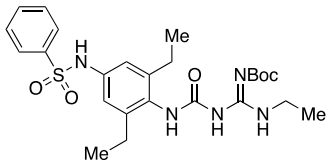
*N-(3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)phenyl)-methanesulfonamide (**5m**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (34 mg, 0.12 mmol, 1.0 equiv), *N*-(4-amino-3,5-diethylphenyl)methanesulfonamide **12a** (29 mg, 0.12 mmol, 1.0 equiv) and TEA (67 μL, 0.48 mmol, 4.0 equiv) in anhydrous THF (1.0 mL) were heated to reflux for 16 h, followed by MPLC purification (100% hexanes to 100% EtOAc/hexanes). The resultant mixture (35 mg, 0.08 mmol) was added TFA (0.5 mL) and DCM (0.5 mL). Following RP MPLC (100% water to 100% MeCN): 43% yield (15 mg, 0.04 mmol). White powder. ^{1}H NMR (400 MHz, CD_3OD): δ 7.01 (s, 2H), 3.26 (q, J = 7.2 Hz, 2H), 2.93 (s, 3H), 2.62 (q, J = 7.6 Hz, 4H), 1.19 (t, J = 7.5 Hz, 9H). ^{13}C NMR (101 MHz, CD_3OD): δ 166.36, 162.16, 145.30, 138.54, 132.01, 119.48, 38.86, 36.64, 26.06, 14.96, 14.89. Purity (LC): 98%. HRMS (ESI-TOF): calcd for $C_{15}H_{26}N_5O_3S$ $[M + H]^+$, 356.1751; found, 356.1738.



*tert-Butyl-(2,6-diethyl-4-(phenylsulfonamido)phenyl)carbamate (**11b**).* Using general procedure H, with *tert*-Butyl(4-bromo-2,6-diethylphenyl)carbamate **10** (200 mg, 0.61 mmol, 1.0 equiv), benzenesulfonamide (144 mg, 0.91 mmol, 1.5 equiv), Tris-(dibenzylideneacetone)dipalladium(0) (61.4 mg, 0.07 mmol, 0.11 equiv), DBTTBP (46.9 mg, 0.10 mmol, 0.16 equiv), and cesium carbonate (298 mg, 0.91 mmol, 2.5 equiv) in 1,4-dioxane (8.0 mL). Following MPLC (100% hexanes to 80% EtOAc/hexanes): 77% yield (191 mg, 0.47 mmol). White solid. mp 148.5−169.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, J = 7.5 Hz, 2H), 7.50 (t, J = 7.4 Hz, 1H), 7.41 (t, J = 7.6 Hz, 2H), 6.68 (s, 2H), 5.77 (s, 1H), 2.50 (q, J = 7.6 Hz, 4H), 1.51 (s, 9H), 1.07 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 155.03, 143.68, 139.82, 136.03, 133.18, 129.31, 127.72, 120.26, 80.62, 28.77, 25.12, 14.62. HRMS (ESI-TOF): calcd for $C_{21}H_{27}N_2O_4S$ $[M - H]^-$, 403.1697; found, 403.1705.



*N-(4-Amino-3,5-diethylphenyl)benzenesulfonamide (**12b**).* Using general procedure J, with *tert*-Butyl-(2,6-diethyl-4-(phenylsulfonamido)phenyl)carbamate **11b** (186 mg, 0.46 mmol) in a 1:1 mixture of TFA/DCM (2 mL). Following MPLC (100% DCM to 10% MeOH/DCM): 88% yield (124 mg, 0.41 mmol). White solid. mp 150.6−155.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72−7.63 (m, 2H), 7.57−7.47 (m, 1H), 7.42 (dd, J = 8.3, 6.9 Hz, 2H), 6.59 (s, 2H), 6.05 (s, 1H), 3.60 (s, 2H), 2.41 (q, J = 7.5 Hz, 4H), 1.13 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 140.48, 139.23, 132.54, 128.68, 128.22, 127.45, 126.00, 123.20, 23.98, 12.71. HRMS (ESI-TOF): calcd for $C_{16}H_{21}N_2O_2S$ $[M + H]^+$, 305.1318; found, 305.1305.



*N-(3,5-Diethyl-4-(3-(N′-(tert-butoxycarbonyl)-(N-ethylcarbamimidoyl)ureido)phenyl)benzenesulfonamide (**S4q**).* Using general procedure D, *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (94.4 mg, 0.33 mmol, 1.0 equiv), *N*-(4-amino-3,5-diethylphenyl)benzenesulfonamide **12b** (100 mg, 0.33 mmol, 1.0 equiv), and TEA (183 μL, 1.31 mmol, 4.0 equiv) in anhydrous THF (2.7 mL) were heated to reflux for 16 h. The mixture was cooled to rt, and the solvents were removed. Following MPLC (100% hexanes to 70% EtOAc) and then RP MPLC (100% water to 100% MeCN): 50% yield (85.6 mg, 0.17 mmol). White powder. mp 160.4−164.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.96 (s, 1H), 8.12 (s, 1H), 7.78 (d, J = 7.8 Hz, 2H), 7.52 (t, J = 7.7 Hz, 1H), 7.42 (t, J = 7.5 Hz, 2H), 6.71 (s, 2H), 6.28 (s, 1H), 3.42 (p, J = 6.6 Hz, 2H), 2.54 (q, J = 7.4 Hz, 4H), 1.42 (s, 9H), 1.23 (t, J = 7.3 Hz, 3H), 1.08 (t, J = 7.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 164.14, 153.57, 143.50, 132.99, 132.71, 129.08, 128.86, 127.62, 127.49, 123.34, 119.54, 82.57, 35.78, 28.17, 24.88, 14.62, 14.36. HRMS (LTQ FT): calcd for $C_{25}H_{36}N_5O_5S$ $[M + H]^+$, 518.2432; found, 518.2416. Purity (LC): 99%.

*N-(3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)phenyl)-benzenesulfonamide (**5n**).* Using general procedure E, *N*-(3,5-

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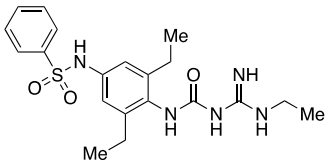
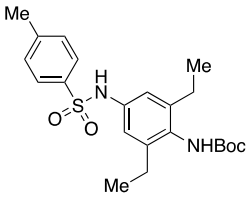
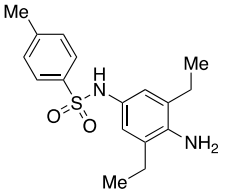


diethyl-4-(3-(*N*′-(*tert*-butoxycarbonyl)-(*N*-ethylcarbamimidoyl)-ureido)phenyl)benzenesulfonamide **S4q** (100 mg, 0.19 mmol, 1.0 equiv) was added a 1:1 mixture of TFA (0.5 mL) and DCM (0.5 mL). The reaction mixture was stirred at rt overnight, the solvents were removed, and to the crude sample was added EtOAc and sat. $NaHCO_3$ (aq), and the aqueous mixture was extracted five times with EtOAc. The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as a white solid. Following MPLC (100% DCM to 10% MeOH/DCM): 51% yield (21.5 mg, 0.05 mmol). White solid residue. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.06 (s, 1H), 7.77 (d, J = 7.6 Hz, 2H), 7.56 (dt, J = 14.9, 7.2 Hz, 3H), 7.37 (s, 1H), 6.73 (s, 2H), 3.13 (s, 2H), 2.39 (q, J = 7.5 Hz, 4H), 1.05 (t, J = 7.2 Hz, 3H), 0.97 (t, J = 7.5 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 164.48, 156.60, 142.62, 132.61, 129.22, 129.18, 129.13, 126.73, 121.43, 117.39, 35.68, 24.38, 14.91, 14.16. HRMS (LTQ FT): calcd for $C_{20}H_{28}N_5O_3S$ $[M + H]^+$, 418.1907; found, 418.1891. Purity (LC): 98%.

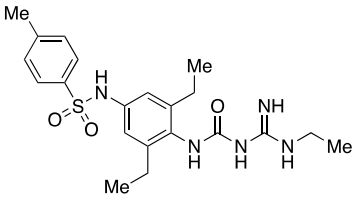


*tert-Butyl-(2,6-diethyl-4-((4-methylphenyl)sulfonamido)phenyl)-carbamate (**11c**).* Using general procedure H, with *tert*-butyl(4-bromo-2,6-diethylphenyl)carbamate **10** (250 mg, 0.76 mmol, 1.0 equiv), toluenesulfonamide (196 mg, 1.14 mmol, 1.5 equiv), Tris(dibenzylideneacetone)dipalladium(0) (76.7 mg, 0.84 mmol, 0.11 equiv), DBTTBP (58.6 mg, 0.12 mmol, 0.16 equiv), and cesium carbonate (372 mg, 1.14 mmol, 1.5 equiv) in 1,4-dioxane (8.0 mL). Following MPLC (100% hexanes to 100% EtOAc/hexanes): 85% yield (270 mg, 0.64 mmol). Light yellow solid. mp 151.6−157.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.65 (d, J = 8.0 Hz, 2H), 7.22 (d, J = 7.9 Hz, 2H), 6.74 (s, 2H), 2.52 (q, J = 7.6 Hz, 4H), 2.37 (s, 3H), 1.49 (s, 9H), 1.09 (t, J = 7.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 152.57, 143.78, 143.39, 135.58, 132.61, 129.57, 129.51, 127.36, 119.15, 80.05, 76.70, 28.31, 24.73, 21.51, 14.21. HRMS (ESI-TOF): calcd for $C_{22}H_{29}N_2O_4S$ $[M - H]^-$, 417.1854; found, 417.1866.

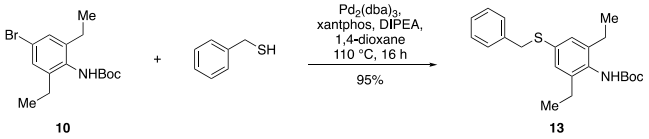


*N-(4-Amino-3,5-diethylphenyl)-4-methylbenzenesulfonamide (**12c**).* Using general procedure J, with *tert*-butyl-(2,6-diethyl-4-((4-methylphenyl)sulfonamido)phenyl)carbamate **11c** (200 mg, 0.48 mmol) in a 1:1 mixture of TFA/DCM (2 mL). Following MPLC (100% hexanes to 100% EtOAc/hexanes): 92% yield (140 mg, 0.44 mmol). White solid. mp 170.5−175.6 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 7.53 (d, J = 8.3 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 6.57 (s, 2H), 2.44 (q, J = 7.5 Hz, 4H), 2.39 (s, 3H), 1.12 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, CD_3OD): δ 144.56, 141.30, 138.05, 130.24, 129.88, 128.65, 128.52, 123.34, 25.00, 21.39, 13.50. HRMS (ESI-TOF): calcd for $C_{17}H_{23}N_2O_2S$ $[M + H]^+$, 319.1475; found, 319.1463.

*N-(3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)phenyl)-4-methylbenzenesulfonamide (**5o**).* Using general procedure F, with

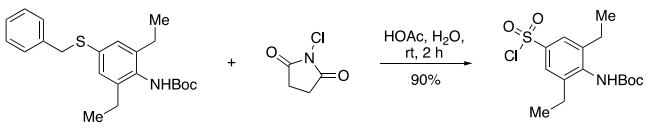


N,N′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (68 mg, 0.24 mmol, 1.0 equiv), *N*-(4-amino-3,5-diethylphenyl)-4-methylbenzenesulfonamide **12c** (75 mg, 0.24 mmol, 1.0 equiv) and TEA (130 μL, 0.94 mmol, 4.0 equiv) in anhydrous THF (1.9 mL) were heated to reflux for 16 h, followed by MPLC purification (100% hexanes to 100% EtOAc/hexanes). The resultant mixture (20 mg, 0.04 mmol) was added TFA (0.5 mL) and DCM (0.5 mL). Following RP MPLC (100% water to 100% MeCN): 57% yield (14 mg, 0.03 mmol). White solid residue. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.66 (d, J = 7.9 Hz, 2H), 7.33 (d, J = 7.9 Hz, 3H), 6.73 (s, 2H), 3.13 (s, 2H), 2.46−2.35 (m, 4H), 2.33 (s, 3H), 1.05 (t, J = 7.2 Hz, 3H), 0.98 (t, J = 7.6 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 164.53, 160.11, 143.02, 142.61, 137.04, 131.65, 129.57, 126.79, 116.93, 116.83, 28.98, 24.37, 20.95, 14.14, 14.12. HRMS (ESI-TOF): calcd for $C_{21}H_{30}N_5O_3S$ $[M + H]^+$, 432.2064; found, 432.2049. Purity (LC): 94%.



10 13

*tert-Butyl-(4-(benzylthio)-2,6-diethylphenyl)carbamate (**13**).* Using a modified literature procedure,[30] tris(dibenzylideneacetone)-dipalladium(0) (100 mg, 0.2 mmol, 0.05 equiv), xantphos (200 mg, 0.3 mmol, 0.1 equiv), and *N*-ethyl-*N*-isopropylpropan-2-amine (800 mg, 6.0 mmol, 2.1 equiv) were added to a stirred solution of *tert*-butyl(4-bromo-2,6-diethylphenyl)carbamate **10** (1.0 g, 3.0 mmol, 1.0 equiv) in 1,4-dioxane (20 mL). The mixture was degassed, and phenylmethanethiol (400 mg, 3.0 mmol, 1.0 equiv) was subsequently added. The reaction was heated at 110 °C for 16 h and was monitored via UPLC. Upon completion of the reaction, the crude reaction mixture was filtered over Celite and the solvent was removed. Following purification via normal-phase MPLC: (100% hexanes to 100% DCM/hexanes): 95% yield (951 mg, 2.6 mmol). Yellow solid. mp 67.7−71.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36−7.18 (m, 5H), 7.02 (s, 2H), 5.76 (s, 1H), 4.09 (s, 2H), 2.56 (q, J = 7.6 Hz, 4H), 1.49 (s, 9H), 1.14 (t, J = 7.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 154.35, 142.74, 137.77, 135.34, 130.58, 129.03, 128.62, 127.93, 127.27, 80.07, 77.48, 39.38, 28.45, 24.89, 24.83, 14.45. HRMS (LTQ FT): calcd for $C_{22}H_{30}NO_2S$ $[M + H]^+$, 372.1997; found, 372.1979. Purity (LC): 94%.



13 14

*tert-Butyl-(4-(chlorosulfonyl)-2,6-diethylphenyl)carbamate (**14**).* Using a modified literature procedure,[30] *tert*-butyl (4-(benzylthio)-2,6-diethylphenyl)carbamate **13** (700 mg, 1.9 mmol, 1.0 equiv) was dissolved in water (3 mL) and acetic acid (9 mL). To the solution was added *N*-chlorosuccinimide (755 mg, 5.7 mmol, 3.0 equiv), and the reaction mixture was stirred at room temperature for 2 h. The mixture was diluted with ca. 6 mL of water to yield a white precipitate, which was filtered to yield the product (591 mg, 1.7 mmol) as a white solid in 90% yield, which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (s, 2H), 2.72 (q, J = 7.6 Hz, 4H), 1.48 (s, 9H), 1.29−1.21 (m, 6H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 154.34, 146.52, 141.22, 134.10, 123.20, 78.08, 28.22, 24.37, 14.36. HRMS

https://doi.org/10.1021/acs.jmedchem.1c01565
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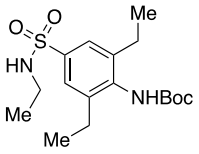
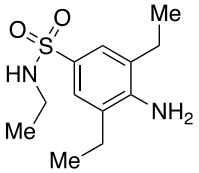
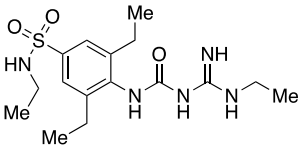
(LTQ FT): calcd for $C_{15}H_{21}NO_4SCl$ [M - H]$^-$: 346.0880; found, 346.0897. Purity (LC): 94%.



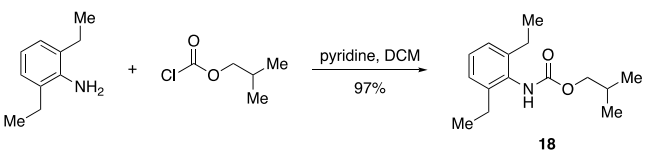
*tert-Butyl-(2,6-diethyl-4-(N-ethylsulfamoyl)phenyl)carbamate (**15a**).* Using general procedure I, with *tert*-butyl-(4-(chlorosulfonyl)-2,6-diethylphenyl)carbamate **14** (145 mg, 0.42 mmol, 1.0 equiv), ethanamine (2.0 M in THF, 229 μL, 0.46 mmol, 1.1 equiv), triethylamine (120 μL, 0.84 mmol, 2.0 equiv) in DCM (3 mL). Following MPLC (100% hexanes to 50% EtOAc/hexanes): 99% yield (149 mg, 0.42 mmol). White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.60 (s, 2H), 4.20 (s, 1H), 3.02 (dd, *J* = 7.4, 6.2 Hz, 2H), 2.69 (q, *J* = 7.6 Hz, 4H), 1.50 (s, 9H), 1.23 (t, *J* = 7.6 Hz, 6H), 1.12 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 151.60, 143.28, 138.51, 135.24, 125.08, 80.84, 28.40, 15.28, 14.21. HRMS (LTQ FT): calcd for $C_{17}H_{29}N_2O_4S$ [M − H]$^-$, 355.1697; found, 355.1712.



*4-Amino-N,3,5-triethylbenzenesulfonamide (**16a**).* Using general procedure J, with *tert*-butyl(2,6-diethyl-4-(*N*-ethylsulfamoyl)phenyl)-carbamate **15a** (120 mg, 0.34 mmol, 1.0 equiv) in a 1:1 mixture of TFA and DCM (2 mL). Following MPLC (100% hexanes to 100% EtOAc/hexanes): 96% yield (82.7 mg, 0.32 mmol). White solid. mp 133.4−136.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (s, 2H), 4.21−3.97 (m, 3H), 3.06−2.90 (m, 2H), 2.54 (q, *J* = 7.5 Hz, 4H), 1.29 (t, *J* = 7.5 Hz, 6H), 1.10 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 145.99, 127.67, 127.13, 125.41, 38.32, 24.24, 15.23, 12.63. HRMS (ESI-TOF): calcd for $C_{12}H_{21}N_2O_2S$ [M + H]$^+$, 257.1318; found, 257.1307.

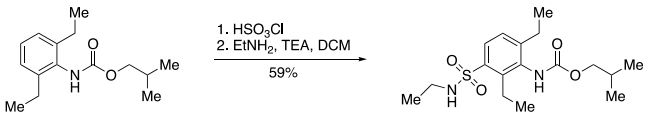


*N,3,5-Triethyl-4-(3-(N-ethylcarbamimidoyl)ureido)-benzenesulfonamide (**5p**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (78 mg, 0.27 mmol, 1.0 equiv), 4-amino-*N*,3,5-triethylbenzenesulfonamide **16a** (70 mg, 0.27 mmol, 1.0 equiv) and TEA (150 μL, 1.1 mmol, 4.0 equiv) in anhydrous THF (2.2 mL) were heated to reflux for 16 h, followed by MPLC purification (100% hexanes to 100% EtOAc/hexanes). The resultant mixture (20 mg, 0.04 mmol) was added TFA (0.5 mL) and DCM (0.5 mL). Following MPLC (100% DCM to 10% MeOH/DCM): 27% yield (11 mg, 0.03 mmol). Clear residue. ^{1}H NMR (600 MHz, CD_3OD): δ 7.59 (s, 2H), 3.27 (br s, 2H), 2.89 (q, *J* = 7.3 Hz, 2H), 2.73 (q, *J* = 7.6 Hz, 4H), 1.23 (t, *J* = 7.6 Hz, 9H), 1.08 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (151 MHz, CD_3OD): δ 162.11, 156.10, 145.06, 139.70, 125.69, 125.57, 39.02, 36.65, 26.00, 15.22, 15.22, 14.87, 14.63. Purity (LC): 98%. HRMS (ESI-TOF): calcd for $C_{16}H_{28}N_5O_3S$ [M + H]$^+$, 370.1907; found, 370.1893.



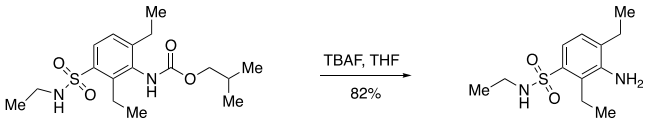
18

*Isobutyl-(2,6-diethylphenyl)carbamate (**18**).* Following a modified literature procedure,[43] isobutyl chloroformate (392 μL, 3.02 mmol, 1.5 equiv) was added dropwise to a stirring solution of 2,6-diethylaniline (300 mg, 2.01 mmol, 1.0 equiv) and pyridine (160 μL, 2.01 mmol, 1.0 equiv) in DCM (3 mL). The reaction mixture was stirred at room temperature for 3 h and monitored by TLC (20% EtOAc/hexanes). After completion of the reaction, the reaction mixture was diluted with DCM (3 mL) and washed with water (6 mL) three times. The organic layer was dried over sodium sulfate, filtered, and concentrated in vacuo to afford the crude product. Following MPLC (100% hexanes to 30% EtOAc/Hex): 97% yield (484 mg, 1.94 mmol). Pale pink solid. mp 75.8−77.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.21 (dd, *J* = 8.3, 6.8 Hz, 1H), 7.11 (d, *J* = 7.6 Hz, 2H), 5.97 (s, 1H), 3.90 (d, *J* = 39.2 Hz, 2H), 2.64 (q, *J* = 7.6 Hz, 4H), 2.02 (d, *J* = 22.3 Hz, 1H), 1.20 (t, *J* = 7.6 Hz, 6H), 1.05−0.68 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.73, 143.22, 142.54, 128.32, 126.82, 71.80, 28.60, 25.21, 19.45, 14.93. HRMS (ESI-TOF): calcd for $C_{15}H_{24}NO_2$ [M + H]$^+$, 250.1802; found, 250.1798.



18 19

*Isobutyl (2,6-Diethyl-3-(N-ethylsulfamoyl)phenyl)carbamate (**19**).* A round-bottom flask chilled to 0 °C was charged with isobutyl (2,6-diethylphenyl)carbamate **18** (150 mg, 0.60 mmol, 1.0 equiv) and carefully added chlorosulfonic acid (400 μL, 5.97 mmol, 9.9 equiv) and stirred for 3 h at room temperature. The reaction mixture was then pipetted onto ice dropwise to yield isobutyl (3-(chlorosulfonyl)-2,6-diethylphenyl)carbamate as a white solid, which was filtered, rinsed with water, concentrated in vacuo, and immediately dissolved in DCM (5 mL); then added ethylamine (2.0 M in THF, 308 μL, 0.62 mmol, 1.1 equiv) and triethylamine (160 μL, 1.12 mmol, 2.0 equiv); and stirred at room temperature overnight. After completion of the reaction, to the solution was added brine (3 mL) and extracted three times with DCM (5 mL). The organic layers were dried over sodium sulfate, filtered, and concentrated in vacuo. Following MPLC (100% hexanes to 100% EtOAc/hexanes): 62.6% yield (125 mg, 0.35 mmol). Colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90 (d, *J* = 8.3 Hz, 1H), 7.24 (d, *J* = 8.3 Hz, 1H), 6.58 (s, 1H), 6.19 (s, 1H), 4.81 (t, *J* = 6.0 Hz, 2H), 3.97 (d, *J* = 6.7 Hz, 2H), 3.01 (q, *J* = 6.9 Hz, 2H), 2.68 (q, *J* = 7.6 Hz, 2H), 1.99 (d, *J* = 9.2 Hz, 1H), 1.24−1.16 (m, 6H), 1.10 (t, *J* = 7.3 Hz, 3H), 0.98−0.86 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 155.26, 148.35, 141.92, 136.45, 135.06, 129.26, 126.27, 71.77, 38.29, 28.20, 24.97, 22.55, 19.06, 15.29, 14.68, 13.82. HRMS (ESI-TOF): calcd for $C_{17}H_{29}N_2O_4S$ [M + H]$^+$, 357.1843; found, 357.1833.



19 20

*3-Amino-N,2,4-triethylbenzenesulfonamide (**20**).* Following a modified literature procedure,[32] isobutyl (2,6-diethyl-3-(*N*-ethylsulfamoyl)phenyl)carbamate **19** (123 mg, 0.35 mmol, 1.0 equiv) was dissolved in THF (4 mL) and added TBAF (1.0 M, 1.73 mL, 1.73 mmol, 5.0 equiv). The reaction was refluxed overnight and monitored by TLC (30% EtOAc/hexanes). Upon completion of the reaction, the solvent was removed, and the crude product was dissolved in ca. 4 mL of EtOAc and extracted three times with 5 mL of brine. The organic layer was dried over sodium sulfate, filtered, and concentrated to afford the crude product as a red oil. Following MPLC (100% hexanes to 100% EtOAc/hexanes): 82% yield (72.4 mg, 0.28 mmol. Yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (d, *J* = 8.1 Hz, 1H), 7.03 (d, *J* = 8.1 Hz, 1H), 4.68 (t, *J* = 6.0 Hz, 1H),

https://doi.org/10.1021/acs.jmedchem.1c01565
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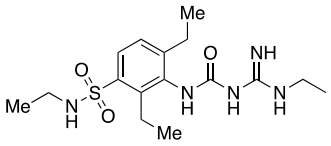
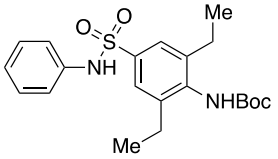
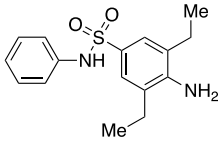
4.03−3.76 (m, 2H), 3.08−2.92 (m, 4H), 2.54 (q, J = 7.5 Hz, 2H), 1.26 (q, J = 7.7 Hz, 6H), 1.12 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 143.71, 135.96, 132.91, 125.52, 125.26, 119.39, 38.27, 24.58, 21.23, 15.26, 12.37. HRMS (ESI-TOF): calcd for $C_{12}H_{21}N_2O_2S$ $[M + H]^+$, 257.1383; found, 257.1313.



*N,2,4-Triethyl-3-(3-(N-ethylcarbamimidoyl)ureido)-benzenesulfonamide (**5q**).* Using general procedure F, with 3-amino-*N*,2,4-triethylbenzenesulfonamide **20** (30.0 mg, 0.12 mmol, 1.0 equiv), *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (34.0 mg, 0.12 mmol, 1.0 equiv), and TEA (65.0 μL, 0.47 mmol, 4.0 equiv) in THF (0.95 mL). The reaction mixture was heated to reflux overnight and purified via MPLC (100% hexanes to 100% EtOAc/hexanes) to yield a mixture containing the Boc-protected product, which was added a 1:1 mixture of TFA/DCM (1.0 mL). Following RP MPLC (100% basic water to 100% MeCN): 60% yield (5.1 mg, 0.01 mmol). Yellow oil. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.62 (d, J = 8.2 Hz, 1H), 7.21 (d, J = 8.3 Hz, 1H), 3.17 (s, 2H), 2.95−2.86 (m, 2H), 2.83 (q, J = 6.6, 6.1 Hz, 2H), 2.63−2.52 (m, 2H), 1.34−0.57 (m, 12H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 164.56, 147.55, 141.58, 138.39, 136.89, 126.20, 125.16, 124.74, 37.44, 34.77, 24.53, 21.70, 15.11, 14.39, 13.81. HRMS (LTQ FT): calcd for $C_{16}H_{28}N_5O_3S$ $[M + H]^+$, 370.1907; found, 370.1899. Purity (LC): >99%.

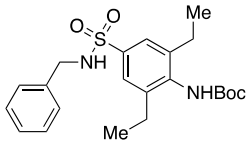


*tert-Butyl-(2,6-diethyl-4-(N-phenylsulfamoyl)phenyl)carbamate (**15b**).* Using general procedure I, with *tert*-butyl-(4-(chlorosulfonyl)-2,6-diethylphenyl)carbamate **14** (150 mg, 0.43 mmol, 1.0 equiv), aniline (44.2 mg, 0.47 mmol, 1.1 equiv), and TEA (120 μL, 0.86 mmol, 2.0 equiv) in DCM (2.77 mL). Following MPLC (100% hexanes to 50% EtOAc/hexanes): 70% yield (122 mg, 0.30 mmol). White solid. mp 177.9−182.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44 (s, 2H), 7.26−7.19 (m, 2H), 7.17−7.01 (m, 3H), 6.58 (s, 1H), 5.86 (s, 1H), 2.59 (q, J = 7.5 Hz, 4H), 1.47 (s, 9H), 1.11 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.69, 143.09, 137.56, 136.61, 129.47, 129.47, 125.79, 125.09, 122.31, 80.88, 28.37, 24.90, 14.04. HRMS (ESI-TOF): calcd for $C_{21}H_{27}N_2O_4S$ $[M - H]^-$: 403.1697; found, 403.1715.

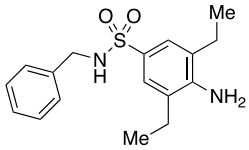


*4-Amino-3,5-diethyl-N-Phenylbenzenesulfonamide (**16b**).* Using general procedure J, with *tert*-butyl-(2,6-diethyl-4-(*N*-phenylsulfamoyl)phenyl)carbamate **15b** (100 mg, 0.25 mmol, 1.0 equiv) in a 1:1 mixture of TFA and DCM (1 mL). Crude yield 100% (75.3 mg, 0.25 mmol); used without purification. Beige solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.31 (s, 2H), 7.22 (d, J = 7.7 Hz, 2H), 7.13−7.01 (m, 3H), 6.25 (s, 1H), 4.05 (s, 2H), 2.45 (q, J = 7.5 Hz, 4H), 1.18 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 137.12, 131.83, 129.16, 126.83, 126.74, 125.33, 125.07, 121.77, 23.91, 12.33. HRMS (ESI-TOF): calcd for $C_{16}H_{21}N_2O_2S$ $[M + H]^+$, 305.1318; found, 305.1303.

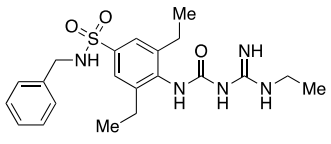
*3,5-Diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)-N-phenylbenzenesulfonamide (**5r**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (43 mg, 0.15 mmol, 1.0 equiv), 4-amino-3,5-diethyl-*N*-phenylbenzenesulfonamide **16b** (55 mg, 0.18 mmol, 1.2 equiv) and TEA (84.0 μL, 0.60 mmol, 4.0 equiv) in anhydrous THF (1.2 mL) were heated to reflux for 16 h, followed by MPLC purification (100% hexanes to 60% EtOAc/hexanes). The resultant mixture (30 mg, 0.06 mmol) was added TFA (0.5 mL) and DCM (0.5 mL). Following MPLC (100% DCM to 10% MeOH/DCM): 48% yield (19.4 mg, 0.05 mmol). Clear residue. ^{1}H NMR (600 MHz, CD_3OD): δ 7.48 (s, 2H), 7.29−7.17 (m, 2H), 7.12 (d, J = 7.6 Hz, 3H), 7.06 (t, J = 7.4 Hz, 2H), 3.30−3.13 (m, 2H), 2.64 (q, J = 7.6 Hz, 4H), 1.27−1.16 (m, 3H), 1.13 (t, J = 7.6 Hz, 6H). ^{13}C NMR (151 MHz, CD_3OD): δ 166.20, 159.80, 144.81, 139.22, 130.12, 130.09, 130.06, 125.79, 125.66, 122.35, 36.63, 25.86, 14.47, 14.47. Purity (LC): 98%. HRMS (ESI-TOF): calcd for $C_{20}H_{28}N_5O_3S$ $[M + H]^+$, 418.1907; found, 418.1891.



*tert-Butyl-(4-(N-benzylsulfamoyl)-2,6-diethylphenyl)carbamate (**15c**).* Using general procedure I, with *tert*-butyl-(4-(chlorosulfonyl)-2,6-diethylphenyl)carbamate **14** (150 mg, 0.43 mmol, 1.0 equiv), benzylamine (50.8 mg, 0.47 mmol, 1.1 equiv), and TEA (120 μL, 0.86 mmol, 2.0 equiv) in DCM (2.77 mL). Following MPLC (100% hexanes to 50% EtOAc/hexanes): 73% yield (132 mg, 0.31 mmol). White solid. mp 152.8−154.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (s, 2H), 7.34−7.26 (m, 3H), 7.25−7.17 (m, 2H), 5.92 (s, 1H), 4.60 (t, J = 6.0 Hz, 1H), 4.15 (d, J = 6.2 Hz, 2H), 2.69 (q, J = 7.6 Hz, 4H), 1.54−1.41 (m, 7H), 1.23 (t, J = 7.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 153.94, 143.35, 138.47, 137.32, 136.39, 128.89, 128.13, 128.07, 125.16, 80.86, 47.48, 28.41, 25.07, 14.22. HRMS (ESI-TOF): calcd for $C_{22}H_{29}N_2O_4S$ $[M - H]^-$: 417.1854; found, 417.1871.



*4-Amino-N-benzyl-3,5-diethylbenzenesulfonamide (**16c**).* Using general procedure J, with *tert*-butyl-(4-(*N*-benzylsulfamoyl)-2,6-diethylphenyl)carbamate **15c** (113 mg, 0.27 mmol, 1.0 equiv) in a 1:1 mixture of TFA and DCM (1 mL). Crude yield 100% (86.6 mg, 0.27 mmol); used without purification. White powder. mp 156.4−159.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48 (s, 2H), 7.33−7.17 (m, 5H), 4.11 (d, J = 6.2 Hz, 2H), 2.61−2.46 (m, 4H), 1.28 (t, J = 7.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 146.13, 136.84, 128.77, 128.05, 127.93, 127.43, 127.17, 125.56, 47.41, 24.24, 12.63. HRMS (ESI-TOF): calcd for $C_{17}H_{23}N_2O_2S$ $[M + H]^+$, 319.1475; found, 319.1459.



*N-Benzyl-3,5-diethyl-4-(3-(N-ethylcarbamimidoyl)ureido)-benzenesulfonamide (**5s**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (53 mg, 0.18 mmol, 1.0 equiv), 4-amino-*N*-benzyl-3,5-diethylbenzenesulfonamide **16c** (70 mg, 0.22 mmol, 1.2 equiv), and TEA (100 μL, 0.73 mmol, 4.0 equiv)

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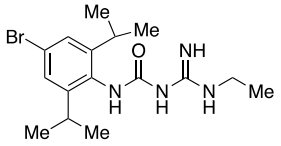
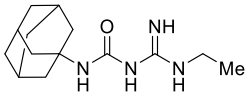
in anhydrous THF (1.5 mL) were heated to reflux for 16 h, followed by MPLC purification (100% hexanes to 60% EtOAc/hexanes). The resultant mixture (30 mg, 0.06 mmol) was added TFA (0.5 mL) and DCM (0.5 mL). Following MPLC (100% DCM to 10% MeOH/DCM): 71% yield (17.3 mg, 0.04 mmol). Clear residue. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.13 (s, 1H), 7.52 (s, 2H), 7.35−7.01 (m, 6H), 3.99 (d, J = 5.7 Hz, 2H), 3.28 (s, 2H), 2.60 (q, J = 7.5 Hz, 4H), 1.07−1.15 (m, 9H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 167.28, 156.61, 142.84, 137.69, 128.21, 128.10, 127.58, 127.14, 123.99, 123.96, 46.13, 35.66, 24.35, 14.18, 14.07. Purity (LC): 96% HRMS (ESI-TOF): calcd for $C_{21}H_{30}N_5O_3S$ $[M + H]^+$, 432.2064; found, 432.2047.



*N-(4-Bromo-2,6-diisopropylphenyl)-N′-[(ethylamino)-iminomethyl]-urea (**5t**).* Using general procedure F, with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-ethylguanidine **7a** (150 mg, 0.52 mmol, 1.0 equiv), 4-bromo-2,6-diisopropylaniline (160 mg, 0.63 mmol, 1.2 equiv) and TEA (291 μL, 2.1 mmol, 4.0 equiv) in anhydrous THF (4.23 mL) were heated to reflux, followed by MPLC purification (100% hexanes to 20% EtOAc/hexanes). The resultant mixture (140 mg, 0.30 mmol) was added a 1:1 mixture of TFA and DCM (3.0 mL). After 30 min, the solvents were removed, and to the crude sample was added EtOAc and sat. $NaHCO_3$ (aq), and the aqueous mixture was extracted with EtOAc (3 × 10 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to afford the crude product as an oil. Following MPLC (100% hexanes to 100% EtOAc/hexanes): 48% yield (53 mg, 0.14 mmol). Off-white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 7.28 (s, 2H), 3.36−3.23 (m, 3H), 3.21−3.02 (m, 2H), 1.20 (m, 15H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 157.09, 149.22, 126.80, 125.51, 122.54, 117.37, 114.95, 36.08, 28.78, 21.93, 13.50. HRMS (LTQ FT): calcd for $C_{16}H_{26}BrN_4O$ $[M + H]^+$, 369.1285; found, 369.1288. Purity (LC): 99%.



*N-Adamantyl-N′-[(ethylamino)iminomethyl]-urea (**5u**).* Using general procedure C, with 1-isocyanatoadamantane (158 mg, 0.89 mmol, 1.1 equiv), 1-ethylguanidine sulfate **6a** (150 mg, 0.81 mmol, 1.0 equiv), 10% KOH (aq) (0.5 mL, 0.89 mmol, 1.1 equiv), and THF (6.6 mL) and following MPLC (100% DCM to 5% MeOH/DCM): 85% yield (183 mg, 0.69 mmol). White solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.84 (s, 1H), 3.16 (q, J = 7.2 Hz, 2H), 2.10−2.02 (m, 3H), 1.98 (d, J = 2.9 Hz, 7H), 1.72−1.62 (m, 7H), 1.22 (t, J = 7.2 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 165.23, 159.77, 50.39, 42.28, 36.69, 36.06, 29.68, 14.74. HRMS (ESI-TOF): calcd for $C_{14}H_{25}N_4O$ $[M + H]^+$, 265.2023; found, 265.2045. Purity (LC): >99%.

Biology. *Purification of PptT.* *E. coli* Bl21 DE3 cells (Invitrogen) transformed with the pMCSG28 plasmid (containing the pptT gene with C-terminal 6x HISTAG) were grown to OD_{600} 0.6−0.8 and induced with 1 mM IPTG overnight at 16 °C after cold shock on ice for 20 min. After the overnight induction, the cells were pelleted and then resuspended in lysis buffer (50 mM MES pH 6.7, 250 mM NaCl, 50 mM L-Glu, 50 mM L-Arg, 10 mM $MgCl_2$, 5 mM imidazole, 1 mM DTT, and 10% glycerol) and lysed using a microfluidics cell disruptor. The resulting lysates were centrifuged at 35000 *g* for 45 min. The Ni-NTA resin was pre-equilibrated with lysis buffer, and the supernatant was loaded onto the column and agitated for 15 min via gentle mixing. Afterward, the supernatant was allowed to flow through the column, and the column was then washed with 1 L of lysis buffer. Afterward, the protein was eluted with an elution buffer (50 mM MES pH 6.7, 250 mM NaCl, 50 mM L-Glu, 50 mM L-Arg, 10 mM $MgCl_2$, 1 mM DTT 300 mM imidazole, and 10% glycerol). The resulting elutant was concentrated to 2 mL via the use of a 10K MWCO Centricon filter and loaded onto a S-75 Sephadex gel filtration column pre-equilibrated with gel filtration buffer (50 mM MES pH 5.8, 250 mM NaCl, 50 mM L-Glu, 50 mM L-Arg, 10 mM $MgCl_2$, 1 mM DTT, and 10% glycerol) and fractionated with an AKTA system. The resulting fractions containing monomeric PptT were pooled, concentrated, and either aliquoted and frozen with liquid N_2 for later use in enzyme assays or utilized immediately for crystallization. An image of the SDS-PAGE gel of purified PptT is provided in Figure S1 (Supporting Information).

Purification of BpsA. *E. coli* BL21 DE3 cells (Invitrogen) transformed with the pMCSG7 plasmid (containing the BpsA gene with N-terminal 6x HISTAG) were grown to OD_{600} 0.6−0.8 and induced with 1 mM IPTG overnight at 16 °C after cold shock on ice for 20 min. After the overnight induction, the cells were pelleted and then resuspended in lysis buffer (50 mM Tris pH 8, 500 mM NaCl, 5 mM imidazole, 1 mM DTT, and 12.5% glycerol) and lysed using a microfluidics cell disruptor. The resulting lysates were centrifuged at 35000 *g* for 45 min. The Ni-NTA resin was pre-equilibrated with lysis buffer, and the supernatant was loaded onto the column and agitated for 15 min via gentle mixing. Afterward, the supernatant was allowed to flow through the column, and the column was then washed with 1 L of lysis buffer. Afterward, the protein was eluted with elution buffer (50 mM Tris pH 8, 500 mM NaCl, 300 mM imidazole, 1 mM DTT, and 12.5% glycerol). The resulting elutant was then dialyzed in dialysis buffer (50 mM Tris pH 8, 50 mM NaCl, 1 mM DTT, and 12.5% glycerol) overnight. Afterward, the sample was concentrated, snap-frozen with liquid N_2, and stored at −80 °C for later use. An image of the SDS-PAGE gel of purified BpsA is provided in Figure S1 (Supporting Information).

BpsA-Coupled PptT Assay. In order to assess PptT's activity, the BpsA-coupled PptT assay was used, which relies on quantitating the rate of apo to holo conversion of BpsA via monitoring the formation of indigoidine production from ATP and glutamine spectroscopically, which holo-BpsA catalyzes.[44,45] The final reaction condition consisted of 100 mM Tris−HCl pH 7.5, 5 mM ATP, 5 mM L-glutamine, 5 mM $MgCl_2$, 0.5 mM DTT, 100 nM PPT, 2 μM CoA, and 2.5 μM BpsA. The compounds were serially diluted 2-fold from 200 μM over a span of 9 dilutions, yielding 10 different concentrations. The reactions were initiated with the addition of PptT, and the data were processed as described.[45]

PptT FP-Based Assay. Reaction conditions consisted of 100 mM MES pH 6.7, 200 mM NaCl, 20 mM $MgCl_2$, 0.01% Tween 20, 5% DMSO, 25 μM GST-PKS13 CP substrate, 2.5 μM bodipy-CoA, and 200 nM PptT. Reactions were initiated by the addition of enzyme, and the reaction was monitored by measuring the increase in fluorescence polarization (482 excitation/504 dichroic/530 emission at RT) using a BMG Labtech plate reader.

Crystallization, Data Collection, and Structure Determination. PptT was pre-incubated with excess AU and optimized/crystallized as reported for the PptT PAP (CoA) 8918 cocrystals.[22] Briefly, after incubation with AU, PptT/AU solution was mixed with variations of the crystallization condition (100 mM bis−tris propane pH 6.7, 1.8 M $MgSO_4$ featuring adjustments in the pH, and precipitation concentration) in a 1:1 ratio as 10 μL hanging drops (vapor diffusion), and the samples were allowed to incubate at 16 °C. Crystals generally formed within 2 weeks.

Prior to shipment to the synchrotron beamlines, the crystals were cryoprotected by submerging them in mother liquor solution featuring 30% glycerol and were subsequently stored in liquid N_2. X-ray diffraction data were collected at the 19-ID-D and 23-ID-D beamlines (APS Argonne National Labs). The on-site HKL3000 package[46] was used for the data reduction of the 8978B and 9016 structures, while the on-site automated XDS processing[47] was used for the data reduction of 9056.

The data sets were phased via Phenix Phaser (molecular replacement) using the PptT portion of the PptT PAP (CoA) 8918 complex as the search model (having removed all heteroatoms). Refinement initially involved cycles of rigid body, occupancy, reciprocal space, ADP refinement, and simulated annealing using

https://doi.org/10.1021/acs.jmedchem.1c01565
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phenix refine,[48] while COOT[49] was used for manual model building. Cycles of refinement using phenix refine and manual model building in COOT were utilized until each model was sufficiently refined. See Table S1 for data collection and refinement statistics.

The PDB depositions for the PptT PAP (CoA) complexes are 7N8M (for 8978B, compound **4b**), 7N8E (for 9016, compound **4c**), and 7N8L for 9056 (compound **4f**).

Activity Assays against a Panel of M. tuberculosis Strains. The activity of test agents against replicating *M. tuberculosis* was measured according to the published protocols.[22,50−52] The *M. tuberculosis* wild-type H37Rv, the PptT knockdown (DAS-PptT clone TetON-10), and the Δ*pptH* knock-out strains were passaged as described previously.[22] In brief, the wild-type *M. tuberculosis*, the PptT knockdown, and the PptH knockout strains were grown at 20% O_2 and 5% CO_2 in Middlebrook 7H9 medium containing 0.2% glycerol, 10% OADC (oleic acid, albumin, dextrose, and catalase), and 0.02% tyloxapol as a dispersal agent. *M. tuberculosis* mc^{2}6220 (Δ*panCD*Δ*lysA*)[50,53,54] was grown in Middlebrook 7H9 medium containing 0.2% glycerol, 10% OADC (oleic acid, albumin, dextrose, and catalase), and 0.02% tyloxapol, supplemented with additional glycerol (0.5% final), CAS amino acids[45] (0.05%), pantothenate (24 μg/mL), and lysine (240 μg/mL). Bacilli were exposed to test agents in tissue-culture-treated Greiner 384-well plates (reference 781091) for 6−7 days (WT and Δ*pptH*) or 9−10 days (DAS-PptT +/− ATc), after which OD_{580} was determined. Percent inhibition of growth was calculated relative to a rifampicin control or vehicle alone (DMSO).

Cytotoxicity in HepG2 Cells. Cytotoxicity was assessed in 96-well plates using 10^4 HepG2 cells per well in DMEM F-12 medium (Gibco) in the presence of 5% fetal bovine serum, of 0.1 mM non-essential amino acids (Gibco), of 1 mM Na pyruvate (Gibco), and of the compounds at various concentrations in 1% DMSO final. The viability of the cells was measured after 40 h incubation at 37 °C, under 5% CO_2, using the Celltiter Glo assay (Promega).

LogD Determination. LogD values at pH 7.4 were determined by a standardized HPLC method as derived from Genieser and co-workers.[55] The calculation of the logD value for measured compounds is performed by comparison of the retention times with standard compounds of known distribution coefficients between 1-octanol and water at pH 7.4.

Thermodynamic (Equilibrium) Solubility. The selected compound is suspended in phosphate buffer 50 mM pH7.4 at a target concentration of 1 mg/mL. After overnight stirring at room temperature protected from light, suspensions are filtered. An aliquot of the resulting supernatant is quantified using the UPLC UV method against a reference solution obtained by the preparation of a DMSO stock solution. The solubility value is expressed as μg per mL.

Caco-2 Apparent Permeability. Human Caco-2/TC7 cells are seeded onto transwell plates at 1×10^5 cells/cm^2. The cells were cultured in DMEM and media is changed every 2 or 3 days. On day 20, the permeability study is performed. Cell culture and assay incubations are carried out at 37 °C in an atmosphere of 5% CO_2 with a relative humidity of 95%. On the day of the assay, the monolayers are prepared by rinsing both apical and basolateral surfaces twice with Hanks balanced salt solution (HBSS) at pH 7.4 warmed to 37 °C. The cells are then incubated with HBSS at the desired pH in both apical and basolateral compartments for 40 min to stabilize the physiological parameters. The dosing solutions are prepared by diluting the test compound with assay buffer to afford the desired test compound incubation concentration. The compound is tested at 20 μM in assay buffer and its permeability is measured in the absence and presence of elacridar, a P-glycoprotein transporter inhibitor. For the assessment of apical → basal permeability, HBSS is removed from the apical compartment and replaced with the test compound dosing solution. The apical compartment insert is placed into a companion plate containing fresh buffer. After 120 min under agitation at 37 °C without CO_2, the apical compartment inserts and the companion plates are separated, and the apical and basolateral samples were diluted for analysis. The compounds are quantified by LC−MS/MS. The permeability coefficient (P_{app}) and recovery are calculated according to the following equations:

$$P_{app}(\text{nm/s}) = \frac{\text{basal amount at time 120}}{\text{time} \times \text{filter area} \times \text{apical concentration at time 0}}$$

$$\text{recovery (\%)} = \frac{(\text{basal amount at time 120} + \text{apical amount at time 120}) \times 100}{\text{apical amount at time 0}}$$

Metabolic Stabilities. The metabolic stabilities of the test compounds were determined essentially as described (1, 2). Microsomes (purchased from BD) are prepared at 0.5 mg/mL protein concentration and 0.1 M phosphate buffer pH 7.4, and test compound are pre-incubated at 37 °C prior to the addition of NADPH (final concentration 1 mM). A minus cofactor control incubation is included for each compound tested, where 0.1 M phosphate buffer pH 7.4 is added instead of NADPH (minus NADPH). Each compound is incubated for 0, 5, 15, 30, and 45 min. The control (minus NADPH) is incubated for 45 min only. The reactions are stopped by transferring the incubate into acetonitrile at the appropriate time points in a 1:3 ratio. The termination plates are centrifuged at 3000 rpm for 20 min at 4 °C to precipitate the protein. Following protein precipitation, the sample supernatant is analyzed using LC−MS/MS. Subsequently, half-life and intrinsic clearance are calculated as described in the literature.[56,57]

Plasma Stability. Mouse and human plasma stability are measured after 2 h of incubation of 1 μM test compounds at 37 °C; the test compounds were considered stable at 2 h if the percentage of stability was within the 80−120% range. Briefly, each compound is incubated for 0, 5, 15, 30, 60, and 120 min at 37 °C. The reactions are stopped by transferring 50 μL of the incubate to 150 μL of acetonitrile containing the internal standard at the appropriate time points. The termination plates are centrifuged at 3000 rpm for 45 min at 4 °C to precipitate the protein. Following protein precipitation, the sample samples are analyzed using LC−MS/MS. From a plot of ln peak area ratio (compound peak area/internal standard peak area) against time, the gradient of the line is determined. Subsequently, the elimination rate constant and the half-life ($t1/2$) (min) are calculated.

Inhibition Studies using Human Liver Microsomes. The test compound or vehicle is pre-incubated in 0.1 M phosphate buffer pH 7.4 with human liver microsomes (final concentration 0.1 mg/) either pre-incubated for 30 min in the absence and presence of NADPH or undergo a 0 min pre-incubation. At the end of the pre-incubation stage, a cytochrome P450 isoform-specific probe substrate (midazolam at 2.5 μM and testosterone at 50 μM) and NADPH (1 mM) were then added (final DMSO concentration 0.5%), and the samples were incubated for 5 min at 37 °C. Following termination of the reactions, the metabolites are monitored by LC−MS/MS, and a decrease in the formation of the metabolite formation of metabolites 1-hydroxymidazolam 6β-hydroxytestosterone is monitored by LC−MS/MS, respectively. Following the termination of the reactions and protein precipitation with acetonitrile, supernatants are collected to monitor metabolite 1-hydroxymidazolam or 6β-hydroxytestosterone by LCMS/MS. A decrease in metabolite formation compared to vehicle control is used to calculate an IC_{50} value. A decrease in the formation of the metabolite compared to vehicle control is used to calculate an IC_{50} value.

Inhibition of the hERG, hCav1.2, or hNav1.5 Channel in Transfected HEK Cells in an Automatic Patch Clamp Format. HEK cells stably expressing hERG, hCav1.2, or hNav1.5 are grown in minimum essential medium supplemented (Gibco) with 10% fetal bovine serum to 60−80% confluency. Before running the experiment, the culture media is removed, and the cells were washed with DPBS without calcium and magnesium. The rapid ICE system (QPatch HTX) is performed according to the manufacturer's instructions. The composition of the pipette solution is 130 mM KCl, 1.0 mM $MgCl_2$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM MgATP, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) 10 at pH 7.2. For the hERG assay, the composition of the bath solution (PAA Laboratories GmbH, Austria) is 137 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES at pH 7.4. For the

https://doi.org/10.1021/acs.jmedchem.1c01565
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hNav1.5 assay, the bath solution is 60 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES at pH 7.4. For the hCav1.2 assay, the composition of the bath solution is 120 mM *N*-methyl-D-glucamine (NMDG), 20 mM $BaCl_2$, 10 mM HEPES, and 7 mM D-glucose at pH 7.4.

eCiphrCardio Assay. CDI iCell cardiomyocytes (Fujifilm Cellular Dynamics) were plated onto fibronectin-coated 48-well microelectrode array plates at a density of 50,000 cells per well and differentiated for 3−5 days according to the manufacturer's instructions until a stable beating phenotype was achieved. The day of the assay, after media change, the cells were treated with vehicle (0.2% DMSO), quinidine (positive control), or test compounds. The activity of cells in a 48-well microelectrode array was recorded prior to treatment (baseline) and at 1 and 24 h post-treatment using the Axion Biosystems Maestro MEA system. The recording conditions were at 37 °C with 5% CO_2 using the standard Cardiac settings on the Axion Biosystems Maestro Axis software version 2.1.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c01565.

Additional analogues, additional synthesis schemes, experimental details, and characterization data (including representative IR data and HPLC traces) (PDF)

List of molecular formula strings and associated biological data (IC_{50}s in the BpsA and FP assays and MIC90s against H37Rv and Mtb++ strains (CSV)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the TB Drug Accelerator of the Bill and Melinda Gates Foundation, the Abby and Howard P. Milstein Program in Translational Medicine, the National Institute of General Medical Sciences (P50GM069663), an NIH TB Research Unit (U19 AI111143), and the National Institute of Allergy and Infectious Disease (AI155510). The Department of Microbiology and Immunology is supported by the William Randolph Hearst Foundation. The molecular graphics and analyses were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311. Schrödinger modeling efforts for this program were generously supported by the Bill & Melinda Gates Foundation (53118). Results shown in this report are derived from the work performed at Argonne National Laboratory, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE

Office of Science by Argonne National Laboratory. Data were collected from the Structural Biology Center (SBC) at the Advanced Photon Source (Argonne National Laboratory). SBC-CAT is operated by UChicago Argonne, LLC, for the U.S. Department of Energy, Office of Biological and Environmental Research under contract DE-AC02-06CH11357. We thank the staff at beamline 19-ID-D and 23-ID-D for assistance with data collection and the Midwest Center for Structural Genomics for contributing LIC vectors.

■ ABBREVIATIONS

AU, amidinourea; Mtb, *Mycobacterium tuberculosis*; PptT, phosphopantetheinyl phosphoryl transferase; PptH, phosphopantetheinyl hydrolase; BCG, bacillus Calmette−Guérin; PAP, phosphoadenosine phosphate; BpsA, blue pigment indigoidine synthetase; FP, fluorescence polarization; SspB, stringent starvation protein B; ATC, anhydrotetracycline; TC50, median toxic dose; MPLC, medium pressure liquid chromatography; LTQ FT, hybrid linear trap quadrupole Fourier Transform; UPLC, ultra-performance liquid chromatography; TEA, triethylamine; DIPEA, *N*,*N*-diisopropylethylamine; DBTTBP, di-*tert*-butyl(2′,4′,6′-triisopropyl-3,4,5,6-tetramethyl-[1,1′-biphenyl]-2-yl)phosphane; dba, dibenzylideneacetone; NP, normal-phase; RP, reversed-phase; IPTG, isopropyl *β*-D-1-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; MWCO, molecular weight cutoff; DMEM, Dulbecco's modified Eagle's medium; P_{app}, permeability coefficient; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; MEA, multielectrode array

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