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Small molecules with structural similarities to siderophores as novel antimicrobials against *Mycobacterium tuberculosis* and *Yersinia pestis*

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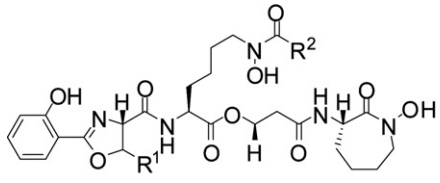
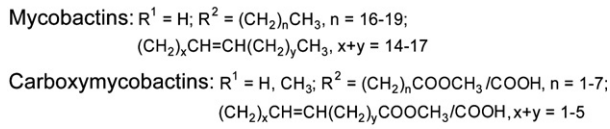
Abstract—Drugs inhibiting the iron scarcity-induced, siderophore-mediated iron-scavenging systems of *Mycobacterium tuberculosis* (*Mtb*) and *Yersinia pestis* (*Yp*) may provide new therapeutic lines of defense. Compounds with structural similarities to siderophores were synthesized and evaluated as antimicrobials against *Mtb* and *Yp* under iron-limiting conditions, which mimic the iron scarcity these pathogens encounter and must adapt to in the host, and under standard iron-rich conditions for comparison. New antimicrobials were identified, some of which warrant exploration as initial leads against potentially novel targets and small-molecule tools to assist in the elucidation of targets specific to iron-scarcity adapted *Mtb* and *Yp*.

Mycobacterium tuberculosis (*Mtb*), the etiologic agent of tuberculosis and *Yersinia pestis* (*Yp*), the causative agent of plague and a potential agent of biowarfare and bioterrorism, are pathogens with serious impacts on global public health. Multidrug-resistant (MDR) tuberculosis is an emerging pandemic and the surfacing of extensive drug-resistant (XDR) tuberculosis poses a new global threat.[1,2] Plague is a re-emerging disease and the occurrence of MDR *Yp* strains and self-transferable *Yp* plasmids conferring antibiotic resistance raises concerns about future plague control.[3,4] These scenarios underscore the need for expanding the anti-tuberculosis and anti-plague drug repertoires. Anti-infective drugs against in vivo *conditionally essential targets* may offer novel therapeutic possibilities, help the fight against MDR/XDR strains and the prevention of their selection and dissemination, and increase biodefense preparedness.[5]

Anti-infective drugs inhibiting the siderophore-mediated, iron-scavenging systems of *Mtb* and *Yp* may provide lines of defense against tuberculosis and plague, respectively. The *Mtb* siderophores (mycobactins and carboxymycobactins) and the *Yp* siderophore (yersiniabactin) (Fig. 1) have high affinity for Fe^{3+}





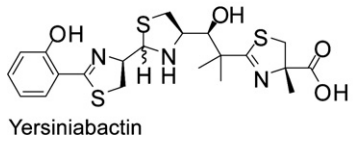


Figure 1. Structures of *M. tuberculosis* and *Y. pestis* siderophores.

Keywords: Siderophore; Antimicrobial; Drug target; *Mycobacterium tuberculosis*; *Yersinia pestis*; Diaryl-carbothioamide-pyrazolines.
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($K_d < 10^{-25}$ M), their production is induced under iron scarcity, and they are believed to be required for scavenging iron inside the host, where free iron is scarce (10^{-25}–10^{-15} M) and pathogens experience iron-limiting conditions.[6,7] The *Mtb* siderophore-deficient mutant is impaired for growth in macrophages and iron-limiting culture medium.[8] The *Mtb* mutant lacking the IrtAB ferri-siderophore uptake system is impaired for multiplication in macrophages, mouse lung, and iron-limiting medium.[9] Siderophore system-deficient *Yp* strains are avirulent in mice infected subcutaneously (a route imitating the fleabite transmission of *Yp*) and unable to multiply in iron-limiting medium.[10,11] Siderophore system-deficient mutants of enteropathogenic *Yersinia* spp. are also attenuated in mice.[12–14]

We recently developed the first antibacterial targeting siderophore biosynthesis, a salicyl-AMP biosynthetic intermediate analog called salicyl-AMS (Fig. 2).[15] Salicyl-AMS is a potent inhibitor of salicylic acid adenylation domains involved in biosynthesis of salicylate-derived siderophores, blocks production of *Mtb* and *Yp* siderophores, and inhibits *Mtb* and *Yp* growth with greater potency in iron-limiting media, where siderophores are crucial for uptake of essential iron.[15] More recently, others have independently reported the activity of salicyl-AMS.[16] Continuing the line of our previous work, we hypothesized that compounds with structural features resembling *Mtb* and *Yp* siderophores may also impair the siderophore system by, for example, inhibiting biosynthetic enzymes or (ferri-)siderophore transport systems, and halt bacterial growth under iron-limiting conditions. To begin testing this hypothesis, we synthesized a 32-member pilot library of 3,5-diaryl-1-carbothioamide-pyrazoline derivatives (compounds **1**–**32**, Fig. 3) with structural features resembling the hydroxyphenyl-oxazoline/thiazoline containing half of the siderophores and tested these compounds as *Mtb* and *Yp* growth inhibitors in iron-limiting media, which mimic the iron-scarcity condition that the pathogens encounter in the host, and in standard iron-rich media for comparison.[17] We also assessed whether selected compounds were bactericidal or bacteriostatic in iron-limiting media.[18] The ability of the compounds to inhibit YbtE (the *Yp* salicylation enzyme that is the intended target of salicyl-AMS[15]) in vitro was also examined.[19] Lastly, cytotoxicity toward mammalian cells was evaluated using a HeLa cell-based assay.[20]

Compounds **1**–**32** were synthesized from 2′-hydroxy chalcone derivatives[21] (Scheme 1). Hydroxy chalcones were prepared through Claisen–Schmidt condensation. 2′-Hydroxy acetophenone-derived chalcones were prepared by adding 60% aqueous solution of sodium hydroxide or potassium hydroxide to the mixture of ketone and aldehydes in methanol at 0 °C and stirring the reaction mixture for 4 h. Adjusting the pH of the reaction mixture to 2 using 6 N hydrochloric acid precipitated hydroxy chalcones. 2′,4′-Dihydroxy acetophenone-derived chalcones required 2 days with occasional stirring.[22] Pyrazoline derivatives were obtained by condensing 2′-hydroxy chalcones with 80% hydrazine hydrate in ethanol.[23] Hydrazine hydrate was used in excess and after 3 h of reflux, pyrazolines were precipitated out upon cooling. 2′,4′-Dihydroxy chalcone-derived pyrazolines were extracted using chloroform from the concentrated reaction mixture. The final products (**1**–**31**) were obtained by the reaction of pyrazoline derivatives with phenyl isothiocyanates.[23] Most of the thiocarboxamide derivatives precipitated out while the reaction mixture was hot, few upon cooling, and the rest upon concentration. Compound **32** was obtained by the reaction of chalcone with thiosemicarbazide in alkaline medium.[24] After 8 h of reflux, the reaction mixture was diluted with cooled water and acidified to precipitate it out. All the intermediates were characterized by IR spectroscopic analysis and elemental analysis for CHNS. In the elemental analysis, the observed values were within ±0.4% of the calculated values. Final compounds were characterized by ^{1}H NMR and FAB-MS.[21]

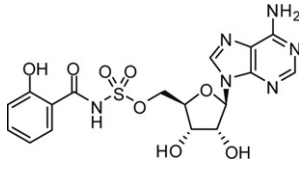
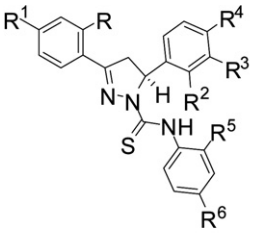
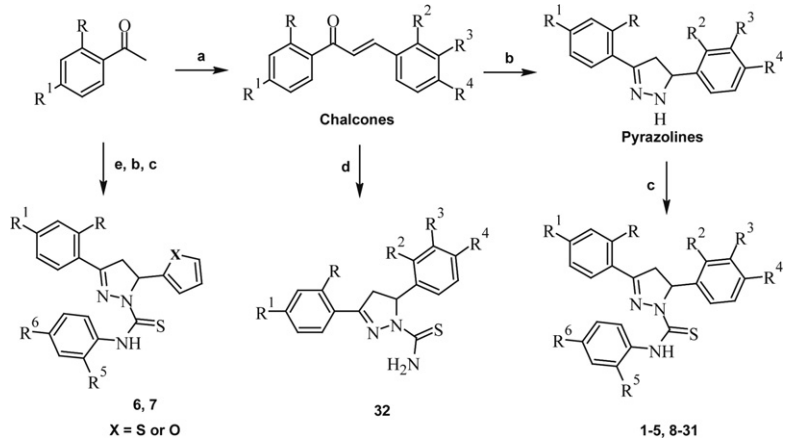


Figure 2. Reaction intermediate mimic 5′-*O*-[*N*-(salicyl)-sulfamoyl]-adenosine (salicyl-AMS).



Compound	R	R^1	R^2	R^3	R^4	R^5	R^6
1	OH	H	H	H	H	H	H
2	OH	H	Cl	H	H	H	H
3	OH	H	H	H	Cl	H	H
4	OH	H	OMe	H	H	H	H
5	OH	H	H	H	OMe	H	H
6	OH	H	------ 2-thiophenyl ------			H	H
7	OH	H	---------- 2-furyl --------			H	H
8	OH	H	OH	H	H	H	H
9	OH	H	OH	H	H	OMe	H
10	OH	H	OH	H	H	H	OMe
11	OH	H	OH	H	H	Me	H
12	OH	H	OH	H	H	H	Me
13	OH	H	H	H	OH	H	H
14	OH	H	H	H	OH	OMe	H
15	OH	H	H	H	OH	H	OMe
16	OH	H	H	H	OH	Me	H
17	OH	H	H	H	OH	H	Me
18	OH	H	H	OMe	OH	H	H
19	OH	H	H	OMe	OH	OMe	H
20	OH	H	H	OMe	OH	H	OMe
21	OH	H	H	OMe	OH	Me	H
22	OH	H	H	OMe	OH	H	Me
23	OH	OH	H	H	OH	H	H
24	OH	OH	H	H	OH	OMe	H
25	OH	OH	H	H	OH	H	OMe
26	OH	OH	H	OMe	OH	H	H
27	OH	OH	H	OMe	OH	OMe	H
28	OH	OH	H	OMe	OH	H	OMe
29	H	OH	H	H	OH	H	H
30	H	H	OH	H	H	H	H
31	H	H	H	H	OH	H	H
32	OH	H	H	H	OH	------ H -----	

Figure 3. Structures of compounds **1**–**32**. In **6** and **7**, 2-thiophenyl and 2-furyl groups replace, respectively, the R^{1-3}-bearing phenyl group. In **32**, H replaces the R^{5-6}-bearing phenyl group.



Scheme 1. Synthesis of compounds 1–32. Conditions: (a) (i) R^2, R^3, R^4 —C_6H_2—CHO, aq. NaOH (60%), stirring at rt 4–48 h, (ii) ice cold HCl (6N), pH adjusted to 2; (b) $NH_2NH_2{\cdot}H_2O$ (80%) excess, C_2H_5OH, reflux 3–6 h; (c) R^5, R^6 —C_6H_3—NCS, C_2H_5OH or CH_3OH, reflux 15–30 min.; (d) (i) $NH_2NHC(S)NH_2$, NaOH excess, CH_3OH, reflux 8h, (ii) ice cold HCl (3N), pH adjusted between 2–4; (e) thiophene-2-carboxaldehyde or furfuraldehyde followed by step (b) and (c).

Testing against *Mtb* revealed that 15 compounds had IC_{50}s and MICs (3–500 μM range; Table 1) within the concentration series tested in the iron-limiting medium, GAST-D. Nine of these compounds also had determinable IC_{50}s and MICs (4–500 μM range) in the iron-rich medium, GAST-D-Fe. Interestingly, **10**, **12**, **16**, **25**, **26**, and **32** were ⩾3-fold more potent against *Mtb* cultured under iron scarcity (Table 1). Compound **32**, with 16-fold higher potency in GAST-D, stood out in this group. This compound, along with **13** and **16**, was the only bactericidal compound (>99% inoculum killing) among those examined for mode of action against *Mtb* (**10**, **12**, and **16**, tested at 2× $MIC_{GAST\text{-}D}$; **13** and **32**, tested at 5× $MIC_{GAST\text{-}D}$). These bactericidal compounds had CD_{50}s against eukaryotic cells in the 21–398 μM range and were among those with the lowest cytotoxicity in the library, in which compounds had 10- to 3980-fold lower cytotoxicity than the reference cytotoxic compound cycloheximide[25] (CD_{50} = 0.1 μM; Table 3). Compound **13**, with the highest activity against *Mtb* (IC_{50} = 3–4 μM, MIC = 12–13 μM; Table 1) and the second best selectivity index relative to *Mtb* (SI_{Mtb} = 14; Table 3), and **16** were 420- and 210-fold less cytotoxic than cycloheximide, respectively, but 5- and 9-fold more cytotoxic, respectively, than the reference anti-tuberculosis drug rifampicin used in our assay. Rifampicin had a CD_{50} of 190 μM (1900-fold lower cytotoxicity than cycloheximide) and the same antimicrobial potency

Table 1. Antimicrobial activity against *M. tuberculosis*

Compound	IC_{50}[a] (μM)		Ratio	MIC_{90}[b] (μM)		Ratio	Mode of action
	GAST-D-Fe	GAST-D		GAST-D-Fe	GAST-D		
1–3, **5**, **9**, **14**, **15**, **17–20**, **22**, **31**	⩾500	⩾500	nd	⩾500	⩾500	nd	nd
4	>250	>250	nd	>250	>250	nd	nd
6	500	250	2	>500	>500	nd	nd
7	72	73	1	208	250	1	nd
8	65	29	2	100	83	1	nd
10	167	28	6	>250	63	>4	BS
11	42	28	2	>500	104	>5	nd
12	208	27	8	>250	63	>4	BS
13	4	3	1	12	13	1	BC
16	83	29	3	417	63	7	BC
21	96	101	1	>500	417	>1	nd
23	>250	167	>2	>250	>250	nd	nd
24	156	80	2	250	125	2	nd
25	250	63	4	>500	167	>3	nd
26	333	84	4	>500	125	>4	nd
27	55	51	1	125	125	1	nd
28	167	125	1	>250	>250	nd	nd
29	105	83	1	250	125	2	nd
30	42	24	2	167	125	1	nd
32	125	8	16	333	21	16	BC
RIF	nd	nd	nd	0.008	0.008	1	nd

All values were rounded to the nearest non-fractional number. RIF, rifampicin; nd, not determined; BS, bacteriostatic; BC, bactericidal.
[a] IC_{50}s were calculated from sigmoidal curves fitted to triplicate sets of dose–response data.
[b] MIC_{90}s are means of triplicates.

in GAST-D and GAST-D-Fe (MIC = 8 nM). Notably, **32**, with one of the strongest antitubercular activity (Table 1), was 2-fold and 3980-fold less cytotoxic than the rifampicin and cycloheximide references, respectively, and had the best selectivity index relative to *Mtb* (SI_{Mtb} = 50; Table 3).

Most compounds were more active against *Yp* than against *Mtb*, which has a thick, waxy cell envelope that makes penetration of many drugs difficult. Twenty-five compounds had considerable activity against *Yp* in the iron-limiting medium, PMH-D (IC_{50}s = 0.01–17 μM range; Table 2). Notably, 17 compounds had both IC_{50}s and MICs that were at least >3-fold higher in PMH–D–Fe (iron-rich medium) than in PMH-D (Table 2). In this group were **18**, **20**, and **23**–**26**, each of which had >30-fold more potent IC_{50}s and MICs against *Yp* cultured under iron scarcity. These compounds were 130- to 630-fold less cytotoxic than cycloheximide, but have over 250- to 1250-fold greater cytotoxicity than the reference anti-plague drug streptomycin. Streptomycin had a CD_{50} >500 μM (>5000-fold less cytotoxic than cycloheximide) and the same antimicrobial activity in PMH-D and PMH-D–Fe (MIC = 0.2 μM). Compound **25**, along with **6** and **28**, had the best selectivity indexes relative to *Yp* (SI_{Yp}; Table 3). Twenty-two compounds evaluated for mode of action against *Yp* were bacteriostatic when tested at up to the maximum multiple of the $MIC_{PMH\text{-}D}$ permitted by solubility, which ranged from 1–38 × $MIC_{PMH\text{-}D}$ (Table 2).

Compounds **1**–**32** were not specifically designed to inhibit a particular enzyme in the siderophore biosynthetic pathways. However, the compounds were tested as inhibitors of YbtE, which is the intended target of salicyl-AMS (Fig. 2).[15] None of the compounds was as potent as the bona fide inhibitor salicyl-AMS (Table 4). The compounds had IC_{50}s in the 0.2- to >12.5-μM range and were 29- to >1786-fold less potent than salicyl-AMS (IC_{50}s = 0.007 μM). Moreover, no clear structural–activity relationships emerged from these data. Interestingly, however, the three library compounds (**29**–**31**) lacking the hydroxyl *ortho* to the 5-membered ring as seen in the siderophores were among the four compounds with drastically increased IC_{50}s (>12.5 μM; Table 4) compared with salicyl-AMS. No meaningful correlation trend between the IC_{50}s in the YbtE assay and the IC_{50}s in the *Yp* growth assay was observed.

Table 2. Antimicrobial activity against *Y. pestis*

Compound	IC_{50}[a] (μM)		Ratio	MIC_{90}[a] (μM)		Ratio	Mode of action
	PMH-D-Fe	PMH-D		PMH-D-Fe	PMH-D		
1	>9	>9	nd	>9	>9	nd	nd
2	>5	>5	nd	>5	>5	nd	nd
3	>5	0.5	>10	>5	1	>5	BS
4	>5	0.4	>13	>5	>5	nd	nd
5	>19	0.7	>27	>19	>19	nd	nd
6	>9	0.01	>900	>9	>9	nd	nd
7	>9	1	>9	>9	2	>5	BS
8	>19	0.7	>27	>19	2	>10	BS
9	>5	2	>3	>5	5	>1	BS
10	>2	1	>2	>2	3	>0.7	BS
11	>19	2	>10	>19	5	>4	BS
12	>9	0.8	>11	>9	2	>5	BS
13	>19	>19	nd	>19	>19	nd	nd
14	>19	2	>10	>19	6	>3	BS
15	>19	0.7	>27	>19	2	>10	BS
16	>19	3	>6	>19	5	>4	BS
17	>9	0.9	>10	>9	3	>3	BS
18	>150	2	>75	>150	5	>30	BS
19	>9	1	>9	>9	6	>2	BS
20	>150	0.6	>250	>150	2	>75	BS
21	>19	3	>6	>19	9	>2	BS
22	>19	1	>19	>19	2	>10	BS
23	>150	1	>150	>150	2	>75	BS
24	>150	0.7	>214	>150	3	>50	BS
25	>150	0.4	>375	>150	5	>30	BS
26	>150	0.6	>375	>150	2	>75	BS
27	>38	17	>2	>38	>38	nd	BS
28	>9	0.2	>45	>9	1	>9	BS
29	>75	>75	nd	>75	>75	nd	nd
30	>19	>19	nd	>19	>19	nd	nd
31	>38	>38	nd	>38	>38	nd	nd
32	>38	>38	nd	>38	>38	nd	nd
STR	nd	nd	nd	0.2	0.2	1	nd

All values <1 and values >1 were rounded to one significant digit and to the nearest non-fractional number, respectively.
STR, streptomycin; nd, not determined; BS, bacteriostatic.
[a] IC_{50}s were calculated from sigmoidal curves fitted to triplicate sets of dose–response data.

Table 3. Cytotoxicity and selectivity assessment

Compound	CD_{50}[a] (μM)	SI_{Mtb} ($CD_{50}/IC_{50GAST\text{-}D}$)	SI_{Yp} ($CD_{50}/IC_{50PMH\text{-}D}$)
1	3	<0.006	<0.3
2	5	<0.01	<1
3	5	<0.01	10
4	6	<0.02	15
5	5	<0.01	7
6	3	0.01	300
7	3	0.04	3
8	3	0.1	4
9	15	<0.03	8
10	1	0.04	1
11	17	0.6	9
12	11	0.4	14
13	42	14	<2
14	1	0.002	0.5
15	4	<0.008	6
16	21	0.7	7
17	3	0.007	3
18	13	<0.03	7
19	11	0.02	11
20	25	<0.05	42
21	15	0.1	5
22	16	<0.03	16
23	63	0.4	63
24	23	0.3	33
25	62	1.0	155
26	30	0.4	50
27	26	0.5	2
28	31	0.2	155
29	248	3	<3
30	19	0.8	<1
31	29	0.06	<0.8
32	398	50	<10
RIF	190	nd	nd
STR	>500	nd	nd
CHX	0.1	nd	nd

SI_{Mtb} and SI_{Yp}, selectivity index relative to activity against *M. tuberculosis* and *Y. pestis*, respectively. CD_{50} and SI values <1 and values >1 were rounded to one significant digit and to the nearest non-fractional number, respectively. RIF, rifampicin; STR, streptomycin; CHX, cycloheximide; nd, not determined.

[a] CD_{50}s were calculated from sigmoidal curves fitted to triplicate sets of dose–response data.

Table 4. Inhibition of YbtE by compounds **1**–**32**

Compound	IC_{50} (μM)	IC_{50} **1**–**32** / IC_{50} SAMS
1	0.5	71
2	2	286
3	0.7	100
4	3	429
5	1	143
6	2	286
7	0.3	43
8	0.6	86
9	0.8	114
10	0.2	29
11	1	143
12	0.5	71
13	0.4	57
14	0.3	43
15	7	1000
16	0.7	100
17	0.4	57
18	0.2	29
19	0.8	114
20	0.5	71
21	1	143
22	0.5	71
23	1	143
24	0.6	86
25	>12.5	1786
26	0.7	100
27	0.2	29
28	1	143
29	>12.5	1786
30	>12.5	1786
31	>12.5	1786
32	0.5	71
SAMS	0.007	1

All values <1 and values >1 were rounded to one significant digit and to the nearest non-fractional number, respectively. The maximum compound concentration tested in the assay was 12.5 μM. SAMS, salicyl-AMS.

Considering that the compounds active against *Yp* had IC_{50}s in the high nanomolar–low micromolar range in the cellular assay under the iron-scarcity condition (Table 2) and that the activity of these compounds is likely to be drastically reduced in the cellular assay (due to factors such as penetration/efflux, intracellular stability, and off-target binding) compared with the enzymatic assay, it is unlikely that YbtE inhibition plays a major role in the antimicrobial activity of these compounds.

In sum, 30 compounds of our pilot library had detectable antimicrobial activity. To our knowledge, these are novel scaffolds not previously shown to have this property. In line with our aforementioned hypothesis, several compounds had higher potency under iron-limiting conditions. Under this condition bacteria depend on siderophores for iron scavenging and engage an adaptive response to tailor their physiology to iron scarcity, thus exposing novel potential in vivo conditional targets.[5] Some of these antimicrobials may impair siderophore system functioning as discussed above, a property that would result in bacteriostatic activity conditional to environmental iron scarcity (e.g., as seen with **25** against *Yp*). Others may inhibit functions conditionally essential to the iron scarcity-associated physiology, which is expected to be adopted by the pathogens in the iron-limiting environments of the host, and could have bactericidal activity (e.g., as seen with **32** against *Mtb*). Compounds with antimicrobial activity that was independent from the iron content of the media were also identified (e.g., **13** against *Mtb*). These antimicrobials may target essential bacterial functions required under both low and high iron conditions.

This study provides proof-of-principle for the effectiveness of screening compound libraries in iron-limiting conditions to identify antimicrobials that may selectively target iron scarcity-adapted bacteria. Our screening approach allowed us to identify compounds with antimicrobial activity that is conditional to iron scarcity and would not have been revealed in conventional screens, which are performed under iron-rich conditions. Some of the identified antimicrobials warrant exploration as

initial leads against potential in vivo conditionally essential targets and small-molecule tools to assist in the elucidation of targets and pathways critical to iron-scarcity adaptation in *Mtb* and *Yp*. Studies are underway to elucidate the molecular mechanisms of action of selected library compounds, a process that may lead to the discovery of novel mechanisms of antimicrobial activity and drug target candidates.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.025.

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17. Antimicrobial activity was tested in dose–response experiments using standard, 96-well plate-based, twofold-microdilution assays as reported previously.[15] *Mtb* (strain H37Rv)[26] was grown in iron-limiting GAST-D medium and GAST-D supplemented with 100 μM $FeCl_3$(GAST-D-Fe).[15] *Yp* (strain KIM6-2082.1+)[27] was grown in iron-limiting PMH-D medium and PMH-D with 100 μM $FeCl_3$(PMH-D-Fe).[15] This *Yp* strain is avirulent due to the lack of the Lcr virulence-conferring plasmid and excluded from the Select Agent Program (http://www.cdc.gov/od/sap/sap/exclusion.htm). Cultures were started with $\sim 5 \times 10^4$ colony-forming units (CFU)/mL. Growth was assessed as optical density after incubation at 37 °C (*Mtb*: 15 days, stationary condition; *Yp*: 26 h, 200 rpm) using a Spectra Max Plus plate reader (Molecular Dynamics). Compounds were evaluated at up to the highest concentration permitted by their solubility, with a 500 μM upper testing limit, and added as DMSO solutions. DMSO was kept at 0.5% in treated and DMSO-control cultures. IC_{50}s were calculated from sigmoidal curves fitted to triplicate dose–response data using KaleidaGraph (Synergy Software). MIC_{90}s were calculated as the lowest concentration tested that inhibited growth by $\geqslant$90% relative to DMSO controls.
18. After cultures were treated with compounds and incubated as above, the mode of action was evaluated by enumerating CFU/mL after plating serial dilutions of triplicate *Yp* and *Mtb* cultures on TBA plates (Difco) and Middlebrook 7H11 supplemented as reported,[28] respectively. Plates were incubated at 37 °C for 4 weeks for *Mtb* and at 30 °C for 3 days for *Yp* before colony counting.
19. The YbtE assay was performed in 96-well FlashPlate® PLUS plates (PerkinElmer) as reported[15] and described in the Supplementary material. Briefly, YbtE-catalyzed incorporation of the [^{3}H]-salicyl group into the well-bound aryl carrier protein domain of *Yp* protein HMWP2 in the absence or presence of test compounds at a range of concentrations was quantified using a plate counter and IC_{50}s were derived from the dose–response data.
20. HeLa cells were cultured using standard techniques.[29] Cells were seeded in opaque, white 96-well plates (Corning Life Sciences) at 2700 cells/well, in 90 μL of Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Ten microliters of each test compound solution and DMSO in controls were added 4 h later such that all wells contained 0.5% DMSO. Each compound was tested in triplicate. After plate incubation (24 h, 37 °C/5% CO_2), 100 μL of ATPLite™ reagent (Perkin-Elmer) prepared in accordance with the manufacturer's instructions was added to each well. Plates were incubated at room temperature for an additional 10 min, and the resulting luminescence signal was read in an EnVision plate reader (Perkin-Elmer).
21. Synthesis of **1**–**32** was carried out using reported methods. Synthesis, final yields, and physicochemical and spectral data of compounds are presented in the Supplementary material.
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