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Design and Synthesis of Pyrano[3,2-*b*]indolones Showing Antimycobacterial Activity

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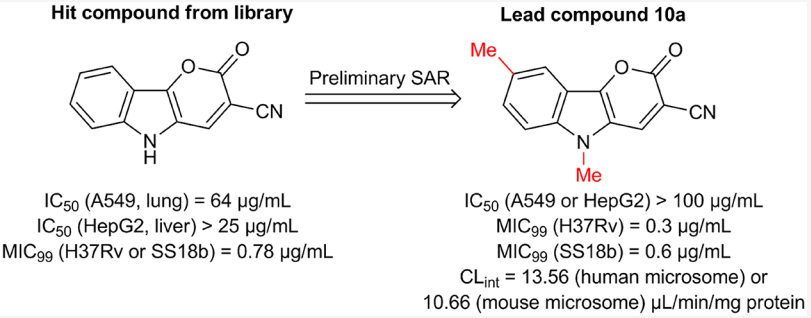

ABSTRACT: Latent *Mycobacterium tuberculosis* infection presents one of the largest challenges for tuberculosis control and novel antimycobacterial drug development. A series of pyrano[3,2-*b*]indolone-based compounds was designed and synthesized via an original eight-step scheme. The synthesized compounds were evaluated for their *in vitro* activity against *M. tuberculosis* strains H37Rv and streptomycin-starved 18b (SS18b), representing models for replicating and nonreplicating mycobacteria, respectively. Compound **10a** exhibited good activity with MIC_{99} values of 0.3 and 0.4 μg/mL against H37Rv and SS18b, respectively, as well as low toxicity, acceptable intracellular activity, and satisfactory metabolic stability and was selected as the lead compound for further studies. An analysis of **10a**-resistant *M. bovis* mutants disclosed a cross-resistance with pretomanid and altered relative amounts of different forms of cofactor F_{420} in these strains. Complementation experiments showed that F_{420}-dependent glucose-6-phosphate dehydrogenase and the synthesis of mature F_{420} were important for **10a** activity. Overall these studies revealed **10a** to be a prodrug that is activated by an unknown F_{420}-dependent enzyme in mycobacteria.

KEYWORDS: *pyrano[3,2-b]indolone, antimycobacterial activity, latent tuberculosis*

In the 21st century, tuberculosis (TB) remains a major public health problem; it is not only one of the top 10 causes of death globally but also the leading cause of death from a single infectious agent (ranking above HIV). According to the WHO estimates, about 10 million people fell ill with tuberculosis and 1.5 million people died from its active form in 2018 worldwide.[1] Even though TB treatment with available drugs is effective in many cases, drug-resistant (particularly with respect to first-line drug rifampicin) and multi-drug-resistant TB constitute a major health threat. Nevertheless, it is only one of the problems of global TB management. An additional challenge concerns latent TB: about 1.7 billion people have this form,[2] which can convert to active TB disease under adverse social or health conditions.[1] The treatment of people with latent TB infection should be an important part of TB course control.

It thus seems appropriate to develop drugs with potent efficacy against both active and latent forms of TB. To date, there are three new molecules (Figure 1), which have good proven *in vitro* activity against actively replicating and dormant *Mycobacterium tuberculosis*, the infectious agent causing the disease: bedaquiline, a first-in-class diarylquinoline compound (FDA/2012) and two molecules from the 5-nitroimidazole class, delamanid, and pretomanid (EMA/2014 and FDA/2019, respectively).[3−7] However, these drugs were approved only to treat active multi-drug-resistant TB in combination therapy,

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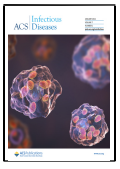

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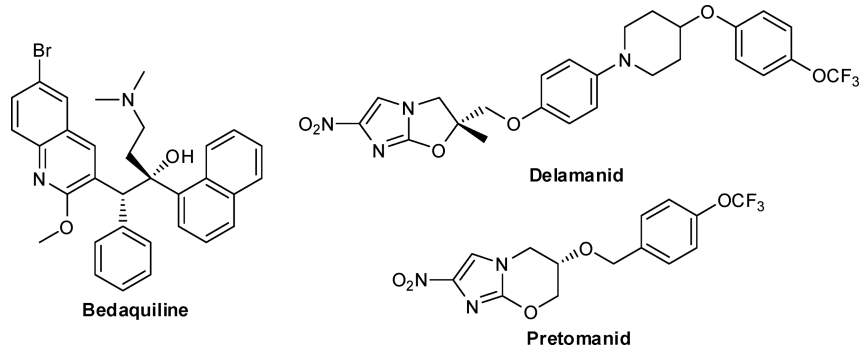
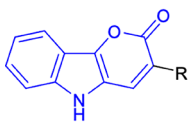


Figure 1. New approved antituberculosis drugs acting *in vitro* on both active and dormant *Mycobacterium tuberculosis*.

and there are no clinical data on their use to treat latent TB infection.[8−10] Despite the unprecedented value of these drugs for treatment of the most complicated forms of TB, it should be noted that they have certain limitations. For example, treatment with bedaquiline or delamanid is associated with QT prolongation and an increased risk of death; the FDA recommends the use of bedaquiline or a combination of these drugs only as salvage therapy.[8,9] Furthermore, delamanid is also one of the most expensive anti-TB drugs, making it unattractive for use in low-income countries with high rates of TB (although the price has been reduced for South Africa).[11] Pretomanid was approved only for limited use in combination with bedaquiline and linezolid for the treatment of a specific type of highly treatment-resistant tuberculosis of the lungs.[10] Taken together, the pipeline for the discovery of safe and inexpensive antituberculosis drugs with efficiency against both replicating and dormant *M. tuberculosis* cells remains empty.

During a routine screening of our chemical library, we found that compounds with a pyrano[3,2-*b*]indolone core[12] have moderate inhibitory activity against both replicating (H37Rv) and nonreplicating [streptomycin-starved 18b (SS18b)] *M. tuberculosis* cells (Figure 2, our unpublished data).



11126066 R = CN
TD_{50} (A549, lung / HepG2, liver) = 64 or > 25 μg/mL
MIC_{99} (H37Rv) = 0.78 μg/mL
MIC_{99} (SS18b) = 0.78 μg/mL
11126067 R = COOEt
TD_{50} (A549, lung / HepG2, liver) > 100 or > 25 μg/mL
MIC_{99} (H37Rv) = 25 μg/mL
MIC_{99} (SS18b) = 25 μg/mL
11326191 R = $CONH_2$
MIC_{99} (H37Rv) > 25 μg/mL

Figure 2. Compounds based on the pyrano[3,2-*b*]indolone core with moderate activity against *M. tuberculosis* strains found during the screening of the chemical library.

We decided to continue this work, and in this article, we report the synthesis, biological evaluation against replicating and nonreplicating *M. tuberculosis* cells, and structure−activity relationship studies of novel pyrano[3,2-*b*]indolone-based compounds containing different substitutions at the 4-, 5-, and 8-positions of the scaffold. Moreover, we present an evaluation of activity in two models of dormant *M. tuberculosis*, intracellular activity, *in vitro* metabolic stability, and our efforts toward the identification of the mechanism of action of lead compound **10a**.

RESULTS AND DISCUSSION

Synthesis of Pyrano[3,2-*b*]indolone Derivatives. We have developed an eight-step method for the synthesis of novel pyrano[3,2-*b*]indolones according to Scheme 1. The starting 1*H*-indole-3-carbaldehydes (**2**) were obtained from the corresponding commercially available 5-substituted 1*H*-indoles (**1**) under the conditions of the Vilsmeier−Haack reaction.[13] Compounds **2** were treated with excess acetic anhydride and triethylamine as a catalyst to protect the indole nitrogen atom and to obtain corresponding intermediates **3**. Mild oxidation of the formyl group of *N*-protected indoles **3** was successfully carried out using *meta*-chloroperoxybenzoic acid (*m*CPBA) in a dichloromethane medium.[14] 5-Substituted 1-acetyl-1*H*-indole-3-yl esters of formic acid **4** thus obtained were reacted with dimethoxymethylpiperidine to introduce the piperidin-1-ylmethylene fragment at the 2-position of indole[15] and then with triethylamine in methanol to synthesize the corresponding compounds, **5**. In the next stage, the acetylation of indoles **5** at the 3-position with acetyl chloride led to acetates **6**. The treatment of 2-formyl-1*H*-indoles (**6**) by malononitrile in the presence of triethylamine provided the major intermediates: 2-(2,2-dicyanovinyl)-1*H*-indoles (**7**).[16] The key process—cyclization and pyrano[3,2-*b*]indolone formation—was conducted using concentrated hydrochloric acid in refluxing acetonitrile to yield target compounds **8**.[12] Finally, *N*-alkylated and *N*-acylated target compounds **9**−**11** were prepared by the reaction of 2-oxo-2,5-dihydropyrano[3,2-*b*]indole-3-carbonitriles (**8**) with different alkyl and acyl halides in the presence of cesium carbonate in dimethylformamide (DMF).

In addition, we synthesized two 5,8-dimodified pyrano[3,2-*b*]indolones (**12a**,**b**) from **8d** as shown in Scheme 2, using methyl chloride (**12a**) or acetyl chloride (**12b**) in the presence of cesium carbonate.

Another synthesis scheme was developed for the synthesis of pyrano[3,2-*b*]indolones **17a**−**e** with a 4-substitution pattern (Scheme 3). The reaction of commercially available 1-acetylindolin-3-one (**13**) with methyl 2-cyano-3,3-bis(methylthio)acrylate **14** in the presence of sodium hydride as a strong base gave hydroxyindole **15**. Subsequent treatment of **15** with HCl in a MeOH/dioxane medium provided cyclization into pyrano[3,2-*b*]indolone (**16**). Finally, aminolysis of the methylthio group of **16** with various amines led to the target 4-modified pyrano[3,2-*b*]indolones (**17a**−**e**).

***In Vitro* Antimicrobial Activity and Cytotoxicity Studies of Novel Pyrano[3,2-*b*]indolones.** The dormant forms of *M. tuberculosis* present in latent TB infection (i.e., nonreplicating cells with greatly reduced metabolism) pose a challenge in developing novel antitubercular drugs.[17] Cur-

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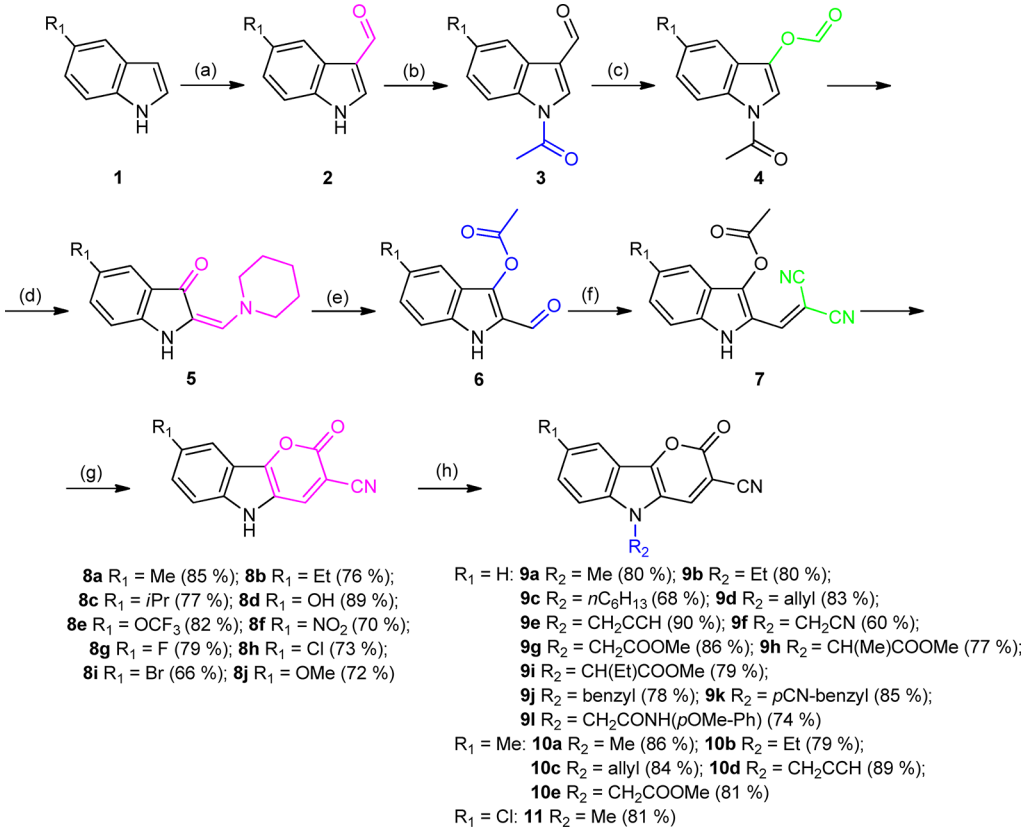
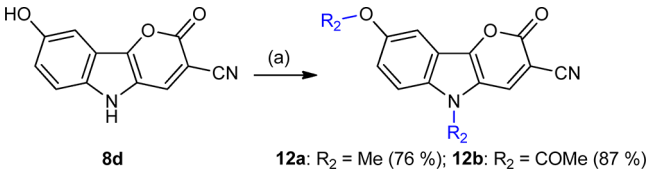
Scheme 1. Synthesis of 5-Substituted Pyrano[3,2-*b*]indolones (8−10)[a]



[a]Reagents and conditions: (a) $POCl_3$, DMF, 5 °C; (b) Ac_2O, Et_3N, Δ; (c) *m*CPBA, CH_2Cl_2; (d) (1) 1-(dimethoxymethyl)piperidine, piperidine, Δ and (2) Et_3N, MeOH, Δ; (e) CH_3COCl, DMF; (f) $CH_2(CN)_2$, Et_3N, benzene; (g) HCl/H_2O, acetonitrile, Δ; (h) Alk-Hal or Ac-Hal, Cs_2CO_3, DMF.

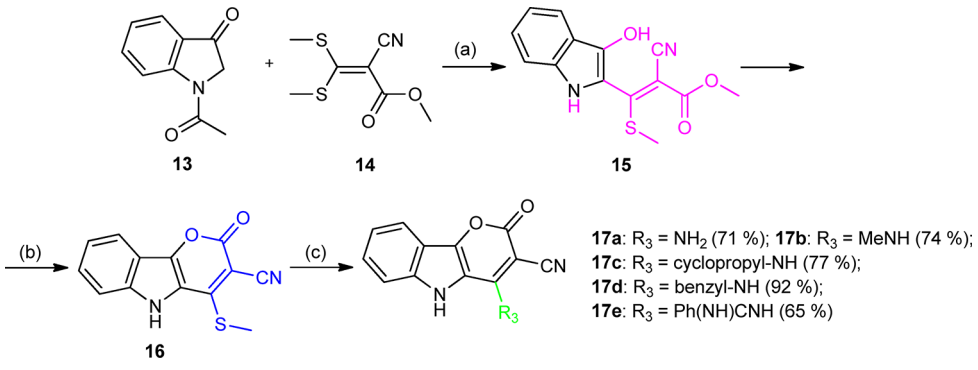
Scheme 2. Synthesis of 5,8-Dialkylated/Acylated Pyrano[3,2-*b*]indolones 12a,b from 8d[a]



[a]Reagents and conditions: (a) methyl chloride or acetyl chloride, Cs_2CO_3, DMF.

rently, there are several *in vitro* models of *M. tuberculosis* dormancy available.[18] In our work, we used a previously validated streptomycin-starved *M. tuberculosis* 18b model (SS18b).[19] In the absence of streptomycin, a viable but nonreplicating state of *M. tuberculosis* 18b is achieved, corresponding to a dormancy model for high-throughput drug susceptibility screening.[20] The synthesized compounds were evaluated for their activity against *M. tuberculosis* H37Rv and SS18b strains using the resazurin reduction microplate assay (REMA) as previously described.[21] A change in the color of resazurin from blue to pink indicates its reduction to resorufin, which directly correlates with bacterial growth and is

Scheme 3. Synthesis of 4-Amino and 4-Amino-Modified Pyrano[3,2-*b*]indolones 17a−e[a]



[a]Reagents and conditions: (a) NaH, MeOH; (b) HCl, MeOH/dioxane; (c) corresponding amine, MeOH, Δ.

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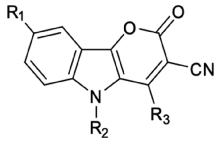
Table 1. Cytotoxicity (TD_{50}), *in Vitro* Activity against *M. tuberculosis* (MIC_{99}), and Selectivity Indexes (SI; Ratio of MIC_{99} to TD_{50}) of Pyrano[3,2-*b*]indolones 8−12 and 17



				MIC_{99} (μg/mL)		TD_{50} (μg/mL)		SI (H37Rv)	
no.	R_1	R_2	R_3	H37Rv	SS18b	A549, lung	HepG2, liver	A549	HepG2
8a	Me	H	H	0.3	0.4		0.8		
8b	Et	H	H	1.6	1.6	32.0	8.0	20	5
8c	*i*Pr	H	H	1.5	1.5	32.0	8.0	21.33	5.33
8d	OH	H	H	25.0	>25.0	32.0	8.0	1.28	0.32
8e	OCF_3	H	H	3.6	3.6	32.0	8.0	8.89	2.22
8f	NO_2	H	H	25.0	>25.0	>100.0	>100.0	>4	4
8g	F	H	H	1.6	3.2	32.0	8.0	20	5
8h	Cl	H	H	0.4	0.8				
8i	Br	H	H	>100.0	>100.0				
8j	OMe	H	H	0.3	<0.2	9.4	2.4	13.4	3.4
9a	H	Me	H	1.6	3.2				
9b	H	Et	H	6.2	6.2	32.0	16.0	5.16	2.58
9c	H	nC_6H_{13}	H	25.0	>25.0	>100.0	>100.0	>4	>4
9d	H	allyl	H	25.0	>25.0	>100.0	>100.0	>4	>4
9e	H	CH_2CCH	H	25.0	25.0	>100.0	>100.0	>4	>4
9f	H	CH_2CN	H	25.0	25.0	>100.0	>100.0	>4	>4
9g	H	CH_2COOMe	H	6.2	12.5	>100.0	>100.0	16.13	16.13
9h	H	CH(Me) COOMe	H	25.0	>25.0	>100.0	>100.0	>4	4
9i	H	CH(Et) COOMe	H	12.5	12.5	32.0	8.0	2.56	0.64
9j	H	benzyl	H	25.0	25.0	>100.0	>100.0	4	4
9k	H	*p*CN-benzyl	H	6.2	6.2	32.0	16.0	5.16	2.58
9l	H	CH_2CONH (*p*OMe-Ph)	H	25.0	>25.0	64.0	32.0	2.56	1.28
10a	Me	Me	H	0.3	0.4	>100.0	>100.0	>333	>333
10b	Me	Et	H	25.0	25.0	>100.0	>100.0	>4	4
10c	Me	allyl	H	25.0	>25.0	>100.0	>100.0	>4	4
10d	Me	CH_2CCH	H	25.0	25.0	>100.0	>100.0	>4	4
10e	Me	CH_2COOMe	H	3.1	3.1	64.0	32.0	20.56	10.32
11	Cl	Me	H	25.0	25.0	>100.0	>100.0	>4	4
12a	OMe	Me	H	12.5	12.5	>100.0	>100.0	>8	8
12b	OCOMe	COMe	H	25.0	25.0	>100.0	>100.0	>4	4
17a	H	H	NH_2	0.8	1.5				
17b	H	H	MeNH	25	>100.0	>100.0	>100.0	>4	>4
17c	H	H	cyclopropyl-NH	25	>100.0	>100.0	>100.0	>4	>4
17d	H	H	benzyl-NH	>100.0	>100.0				
17e	H	H	Ph(NH)CNH	>100.0	>100.0				
isoniazid (INH)				0.02	>100.0	>100.0	>100.0	>5000	>5000
bedaquiline (BDQ)				0.1	0.156	75.0	50.0	750	500

measured by fluorescence. Thus, the minimal inhibitory concentration (MIC_{99}) was defined as the lowest compound concentration that prevented this change in color. The cytotoxicity of novel pyrano[3,2-*b*]indolone derivatives was tested against two human cell lines, namely, HepG2 (human hepatocellular carcinoma cells) and A549 (lung epithelial cells). Their cytotoxic effects [50% toxic doses (TD_{50})] were measured as described in the experimental section and were defined as the compound concentration reducing the mean cell viability by 50%.

New pyrano[3,2-*b*]indolone-based compounds were synthesized with substituents displaying electronic, steric, or hydrophilic/hydrophobic properties in the phenyl fragment of the indole ring, at the nitrogen atom, and at the 4-position of pyrano[3,2-*b*]indolone in order to preliminarily identify the crucial positions of the scaffold and the necessary substituents at these positions for an increase in antituberculosis activity. The *in vitro* antimycobacterial activity and cytotoxicity of the novel compounds are summarized in Table 1.

During the structure−activity relationship (SAR) investigation, it was found that an increase in the length and volume of the alkyl chain at the 8-position of the scaffold from the hydrogen atom to the isopropyl group slightly reduced the activity of the compounds against both *M. tuberculosis* H37Rv

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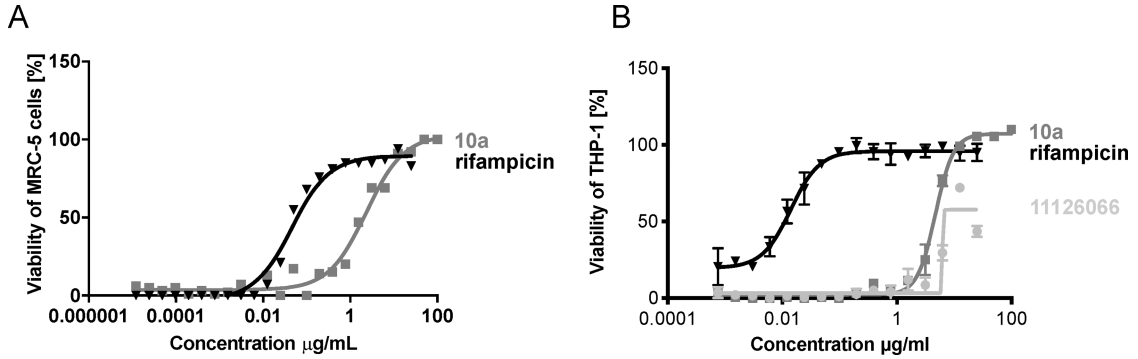

Figure 3. Intracellular activity of compound **10a** and hit compound **11126066** in (A) MRC-5 cells and (B) THP-1 cells.

and SS18b. Unsubstituted 2-oxo-2,5-dihydropyrano[3,2-*b*]-indole-3-carbonitrile **11126066**, our hit compound (Figure 2), has an MIC_{99} value of 0.78 μg/mL toward H37Rv and SS18b strains, whereas derivatives **8b,c** with alkyl groups had similar MIC_{99} values among themselves (1.5−1.6 μg/mL). However, compound **8a** with a small methyl group inhibited H37Rv and SS18b metabolism with MIC_{99} values of 0.3−0.4 μg/mL but was significantly cytotoxic toward liver cells (TD_{50} = 0.8 μg/mL). The introduction of the 8-hydroxyl group led to a great reduction in activity (**8d**, MIC_{99}s ≥ 25 μg/mL). The same antituberculosis activity was observed in the case of the nitro group (**8f**), but the compound did not show toxicity (TD_{50} > 100 μg/mL toward A459 and HepG2 cells). It is interesting to note that the activity of compound **8e**, with a strongly electron-withdrawing trifluoromethoxy group, was higher than that of **8f** but was about 2 times lower than that of **8b−c** (MIC_{99}s 3.6 μg/mL). The structure−activity relationship in a series of compounds with halogen atoms (**8g−i**) is unclear. It was found that **8g** with a fluorine atom had MIC values of 1.6 and 3.2 μg/mL, and the compound with chlorine atom **8h** was more active with MIC values of 0.4 and 0.8 μg/mL against H37Rv and SS18b, respectively. At the same time, **8i** with a bromine atom was completely inactive against *M. tuberculosis* H37Rv and SS18b. Compound **8j** with the 8-OMe group was more active (MIC_{99} values of 0.3 and <0.2 μg/mL against strains tested) than related compound **8d**, but it was also more cytotoxic (TD_{50} values of 2.4−9.4 μg/mL) and thus does not meet the criteria of a "lead compound".

It was observed that pyrano[3,2-*b*]indolones with the free 8-position and with bulky alkyl substituents at the 5-position were inactive against both *M. tuberculosis* strains (**9c−e**, MIC_{99} ≥ 25 μg/mL), while compounds **9a** and **9b** with *N*-methyl or *N*-ethyl chains, respectively, had moderate activity with MIC_{99} values of 1.6 μg/mL (H37Rv) and 3.2 μg/mL (SS18b) for the former compound and 6.2 μg/mL (both strains) for the latter. The replacement of a hydrogen atom in the *N*-methyl group by a cyano group (**9f**) resulted in a large activity decrease. The same replacement by a methoxycarbonyl group (**9g**) slightly increased the antituberculosis activity, but the replacement of the second hydrogen atom in the *N*-methyl group by the alkyl side chain (**9h,i**) greatly reduced the activity. It is interesting that a change in just one CH_2 group (**9h** → **9i**) not only improved the activity but at the same time increased the toxicity. Nitrogen modification by the benzyl group indicated the same reduction in the activity (**9j**), but interestingly, a compound with *p*-cyanobenzyl group **9k** was slightly more active and exhibited an MIC_{99} value of 6.2 μg/mL but was also more toxic toward cells tested than **9j**.

Among the pyrano[3,2-*b*]indolones with a methyl group at the C8 position and different substituents at the C5 position, the introduction of alkyl substituents, such as ethyl, allyl, and propargyl decreased the activity (**10b−d**, MIC_{99} ≥ 25 μg/mL), except for compound **10a** with a 5-methyl group, which was the most active compound in the series against *M. tuberculosis* H37Rv and SS18b strains with MIC_{99} values of 0.3 and 0.4 μg/mL, respectively. Moreover, derivatives **10a−d** with this type of substitution pattern are not cytotoxic. The introduction of the CH_2COOMe group at the nitrogen atom position resulted in a reduction of activity and an increase in toxicity: compound **10e** had an MIC_{99} value of 3.1 μg/mL for the tested strains of *M. tuberculosis* and TD_{50} values of 32.0 and 64.0 μg/mL for both cell lines. Another modification of the 5,8-positions of the scaffold simultaneously (**12a,b**) did not improve the activity overall.

Structure−activity relationship studies have also shown that large groups at position 4 reduced the antimycobacterial activity of the compounds. Among derivatives **17a−e**, only **17a** with a small amino group had moderate activity with MIC values of 0.8 and 1.5 μg/mL against H37Rv and SS18b strains, respectively, whereas other amino-modified compounds **17b−e** are almost or completely inactive.

An evaluation of the antimicrobial activity and cytotoxicity of the novel pyrano[3,2-*b*]indolone-based compounds pointed to 5,8-dimethyl-2-oxo-2,5-dihydropyrano[3,2-*b*]indole-3-carbonitrile (**10a**) as the most promising derivative with an acceptable selectivity index of >333. We thus chose this molecule as a lead compound and decided to examine its activity in another *in vitro* dormancy model, the recently developed "Salina model".[22,23] In this model, dormancy is achieved by cultivating *M. tuberculosis* cells under potassium limiting conditions, and the survival of the resulting non-culturable mycobacteria following the drug treatment is evaluated after their resuscitation in ADC-supplemented Sauton's medium.[22] It was found that compound **10a** decreased the viability of nonculturable cells in a dose-dependent manner, similar to that of replicating cells (Figure S1). It efficiently killed bacteria at 5 μg/mL (a 1 log decrease in colony-forming units), and the incubation of nonculturable cells with 50 μg/mL compound resulted in an approximately 3 log killing effect (Figure S1), which further underlines the potential of **10a** to target dormant mycobacteria.

Intracellular Activity and Microsomal Stability of Lead Compound 10a. To assess the activity of **10a** *ex vivo*, we tested its ability to protect fibroblast cells (MRC-5) against cytotoxicity mediated by the Erdman strain of *M. tuberculosis*. Treatment with pyrano[3,2-*b*]indolone **10a** protected MRC-5

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Table 2. Metabolic Stability of 10a in Human and Mouse Microsomes

microsome origin	human				mouse			
cmpd	**10a**	PBTZ 169	NIF	CBZ	**10a**	PBTZ 169	NIF	CBZ
CL_{int}, μL/min/mg protein	13.56	28[24]	219.50	3.59	10.66	36.7[20]	117.10	7.84

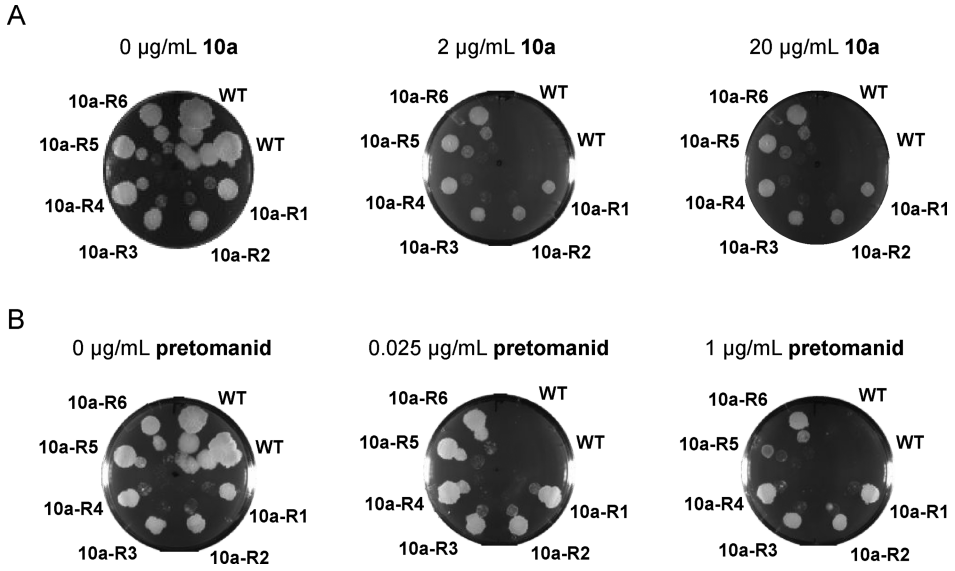

Figure 4. Evaluation of the resistance of *M. bovis* BCG mutant strains 10a-R1−10a-R6 against (A) compound **10a** and (B) pretomanid. The standard *M. bovis* BCG strain and selected mutants 10a-R1−10a-R6 were diluted to OD_{600} 0.5, and from these cultures dilutions of 10^0, 10^{-1}, and 10^{-2} were applied to the solid medium as 4 μL drops. The plates were incubated for (A) 24 or (B) 30 days at 37 °C. WT, standard strain; 10a-R1−10a-R6, resistant strains of *M. bovis* BCG.

cells from cell death induced by *M. tuberculosis* (Figure 3A) and seemed to reduce the intracellular bacterial load by protecting fibroblasts from killing induced by *M. tuberculosis*. These data were confirmed in another *ex vivo* model of intracellular infection employing THP-1 macrophages. When compared with hit compound **11126066**, lead compound **10a** was more efficient at protecting macrophages against *M. tuberculosis* (Figure 3B).

To evaluate the potential of pyrano[3,2-*b*]indolone derivative **10a** for future *in vivo* studies, we tested its metabolic stability in human or mouse CD-1 microsomes (Table 2). The intrinsic clearance (CL_{int}) of **10a** was established and compared to the CL_{int} of carbamazepine (CBZ) and nifedipine (NIF), representing the controls for low and high CL_{int}, respectively. Lead compound **10a** is metabolically stable in the presence of both human and mouse microsomal enzymes and has a CL_{int} considered to be medium but superior to benzothiazinone PBTZ169 (macozinone),[24] which is currently in phase II of clinical development.[25] These data suggest that metabolic stability should not present a liability for the further development of this compound.

Search for Mechanism of Action of 10a. Numerous attempts to isolate **10a**-resistant strains of *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra failed. Finally, we were able to select six *M. bovis* BCG mutants resistant to **10a**. The resistance of these mutants was confirmed by the evaluation of their growth on a solid medium in the presence of **10a** at the MIC established for the parent strain under the same conditions (2 μg/mL) as well as at a 10 times higher concentration (20 μg/mL). Although *M. bovis* BCG mutants 10a-R1−10a-R6 appeared to grow slightly worse compared to the wild type, all of them were resistant to **10a** at both concentrations (Figure 4A). Subsequently, we tested the sensitivity of strains 10a-R1−10a-R2 against isoniazid, ethambutol, benzothiazinone BTZ043, pretomanid, bedaquiline, rifampicin, and ethionamide by REMA assay. We observed that sensitivity is comparable to the parent strain for all of the tested drugs except for pretomanid, which was not active against the mutant strains. The resistance to pretomanid was subsequently confirmed for all of the mutant strains (Figure 4B, Figure S2). Several studies have shown that pretomanid-resistant strains of *M. tuberculosis* H37Rv and *M. bovis* BCG carry mutations in the *ddn* gene, encoding an F_{420}-dependent nitroreductase involved in the activation of the prodrug, or in the genes involved in the synthesis or reduction of a mature active form of cofactor F_{420}.[26] From a structural point of view, this cofactor is a deazaflavin derivative with functions comparable to those of nicotinamide cofactors.[27] The synthesis of F_{420} is initiated by the production of the deazariboflavin precursor, fluorescently active Fo-chromophore 7,8-didemethyl-8-hydroxy-5-deazariboflavin, by the catalytic action of the Fo-synthase encoded by the *fbiC* gene.[28,29] Subsequently, according to a recently revised pathway for the F_{420} cofactor in prokaryotes, intermediate Fo reacts with enolpyruvyl-diphospho-5′-guanosine by the action of *fbiA*-encoded transferase enzyme to form dehydro F_{420}-0.[30] The final steps in the production of an active form of F_{420} are the reduction of dehydro F_{420}-0 to F_{420}-0 and the addition of two to eight glutamyl residues, catalyzed by a bifunctional enzyme encoded by *fbiB*.[30,31] The reduction of the mature F_{420} cofactor is catalyzed by the *fgd1* gene product, F_{420}-dependent glucose-6-phosphate dehydrogenase.[32]

Since we did not expect nitroreductase Ddn to be involved in the metabolism of the drug due to the lack of a nitro group

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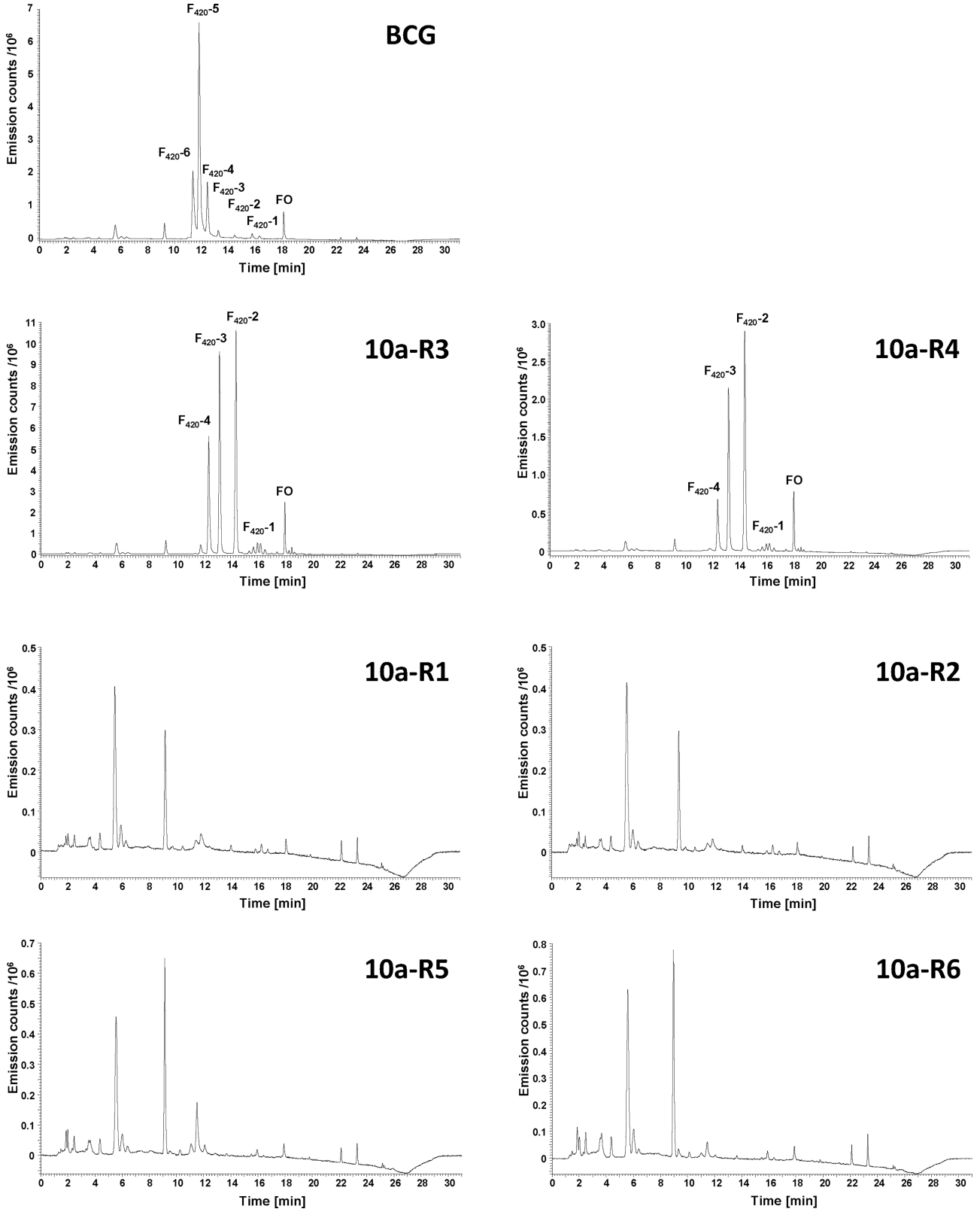

Figure 5. HPLC-FLD chromatograms of cell extracts prepared from *M. bovis* BCG and the corresponding 10a-R1−10a-R6 strains.

in the structure of **10a**, we focused on the analysis of the genes involved in the production (*fbiA*, *fbiB*, and *fbiC*) and reduction (*fgd1*) of the F_{420} cofactor. We amplified the target genes along with their promoter regions by PCR, using chromosomal DNA isolated from control strain *M. bovis* BCG and the corresponding 10a-R1−10a-R6 strains as templates. Sequencing of the isolated PCR products revealed that strains 10a-R1, 10a-R2, 10a-R5, and 10a-R6 carry mutations in the *fbiC* gene and 10a-R4 contains a mutation in the *fgd1* gene (Figure S3). Since PCR amplification of the *fgd1* gene in strain

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10a-R3 was not successful and *fbiA*, *fbiB*, or *fbiC* did not carry any mutation, we decided to perform whole-genome sequencing of this strain. *In silico* analysis of the obtained data revealed a 460-bp-long deletion of the chromosomal DNA comprising the 83- and 300-bp-long 5′ regions of the genes encoding hypothetical β-lactamase-like hydrolase (*Mb0414c*) and F_{420}-dependent glucose-6-phosphate dehydrogenase (*fgd1*), respectively, as well as a 77-bp-long intergenic sequence (Figure S3).

These findings were confirmed by the assessment of the relative amounts of different forms of cofactor F_{420} in the cell extracts of the control strain and mutants 10a-R1−10a-R6 by high-performance liquid chromatography with fluorescence detection (HPLC-FLD) (Figure 5).

The profile of cofactor F_{420} species in the wild-type *M. bovis* BCG revealed the presence of Fo and the predominant amount of F_{420}-5, containing five L-glutamic acid residues.[33] Strains 10a-R3 and 10a-R4 with mutations in the *fgd1* gene lost the capacity to synthesize longer, active forms of the cofactor, F_{420}-5, and F_{420}-6 (Figure 5). This profile is comparable to that obtained for delamanid-resistant strains of *M. bovis* BCG with mutations found in the *fgd1* gene.[34,35] Mutations in the *fbiC* genes in strains 10a-R1, 10a-R2, 10a-R5, and 10a-R6 led to a complete loss of F_{420}, as observed before (Figure 5).[28]

To verify the critical role of the F_{420}-dependent redox system in the activation of **10a**, we tried to complement the mutant strains with the wild-type copies of the respective affected genes *fgd1* and *fbiC*. The wild-type control *M. bovis* BCG and mutant strains 10a-R1−10a-R6 were transformed with corresponding plasmids pVV2-$fgd1_{BCG}$ and pVV16-$fbiC_{BCG}$, respectively, designed for the constitutive production of the N-terminally or C-terminally His-tagged target proteins in mycobacteria.[36,37] While the complementation was not successful for the strains with the mutated *fbiC* gene, since the constitutive expression of *fbiC* using pVV16 expression system was toxic, the synthesis of recombinant Fgd1 in strains 10a-R3 and 10a-R4 led to their reversion to the sensitive phenotype, confirming the role of F_{420} (Figure S4). We thus concluded that **10a** interacts with an unknown F_{420}-dependent oxidoreductase in *M. bovis* BCG and *M. tuberculosis*, which is responsible for its activation.

In addition to the genetic approach, we further addressed the possible mechanism of action of **10a** for *M. tuberculosis* H37Rv by metabolic labeling studies using [^{14}C]-labeled acetate, leucine, or uracil (Figure S5). However, no significant effects were observed.

CONCLUSIONS

A series of novel pyrano[3,2-*b*]indolone-based compounds were synthesized, characterized physicochemically, and evaluated *in vitro* for activity against replicating and nonreplicating *M. tuberculosis*. Preliminary SAR investigation of the scaffold revealed that the hydrogen atom (i.e., unsubstituted position) or the methyl group at the 8 position of the pyrano[3,2-*b*]indolone core is optimal for antituberculosis activity. In addition, it was established that the introduction of a methyl group at the 5 position of the scaffold increases the activity. Thus, compound **10a** with 5,8-dimethyl groups exhibited the most promising antituberculosis activity against H37Rv and SS18b strains (MIC_{99} 0.3 and 0.6 μg/mL, respectively). Although the mechanism of action of **10a** in *M. tuberculosis* and close relatives such as *M. bovis* BCG is currently not known, it appears to be a prodrug requiring activation by an F_{420}-dependent enzyme. The favorable selectivity index, intracellular activity, and microsomal stability of lead compound **10a** all support additional in-depth structure−activity relationship studies of pyrano[3,2-*b*]indolones as a new class of potential antituberculosis agents.

METHODS

Chemistry. All reagents and solvents were purchased from commercial suppliers and used without further purification. ^{1}H and ^{13}C spectra were recorded with a Bruker AC-300 (300 MHz, ^{1}H) or a Bruker AC-200 (50 MHz, ^{13}C). Chemical shifts were measured in DMSO-d_6 using tetramethylsilane as an internal standard and reported as unit (ppm) values. The following abbreviations are used to indicate the multiplicity: s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets; brs, broad singlet; and brm, broad multiplet. Mass spectra were recorded on Finnigan MAT INCO 50 mass spectrometer (EI, 70 eV) with direct injection. The purity of the final compounds was analyzed on an Agilent 1290 Infinity II HPLC system coupled to an Agilent 6460 triple-quadrupole mass spectrometer equipped with an electrospray ionization source. All tested final compounds are >95% pure. Elemental analysis (% C, H, N) was carried out with a EURO EA elemental analyzer. Melting points were determined on an Electrothermal 9001 (10 °C per min) and are uncorrected. Merck KGaA silica gel 60 F_{254} plates were used for analytical thin-layer chromatography. Yields refer to purified products and are not optimized.

*General Procedure for the Synthesis of 5-Substituted 1H-Indole-3-carbaldehydes (**2**).* Dimethylformamide (DMF, 50 mmol) was cooled to 5 °C, and then phosphoryl chloride (12.0 mmol) was added dropwise with stirring. The corresponding commercially available 5-substituted 1*H*-indoles (10.0 mmol) were added to the solution in one step, and the resulting mixture was stirred for 12 h at room temperature. The reaction mixture was poured into ice-cold water (200 mL) and stirred for 2 h. The precipitate was filtered, washed with water, and dried.

*General Procedure for the Synthesis of 5-Substituted 1-Acetyl-1H-indole-3-carbaldehydes (**3**).* A mixture of the corresponding 5-substituted 1*H*-indole-3-carbaldehydes (**2**) (10 mmol), acetic anhydride (70 mmol), and triethylamine (3 mmol) was refluxed for 3 h. The cooled reaction mixture was concentrated *in vacuo*. The residue was treated with water and stirred for 1 to 2 h. Crystals were collected, washed with water, and dried.

*General Procedure for the Synthesis of 5-Substituted 1-Acetyl-1H-indole-3-yl Esters of Formic Acid (**4**).* To a solution of the corresponding carbaldehydes (**3**) (10 mmol) in dichloromethane (50 mL), *meta*-chloroperoxybenzoic acid (*m*CPBA, 15 mmol) was added, and the resulting mixture was stirred for 20 h at room temperature. The reaction solution was washed with an aqueous solution of sodium bicarbonate and water (three times). The organic phase was dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was treated with diethyl ether and stored in the refrigerator for 12 h. The precipitate was collected, washed with diethyl ether, and dried.

*General Procedure for the Synthesis of 5-Substituted 2-(Piperidin-1-ylmethylene)-1,2-dihydro-3H-indole-3-ones (**5**).* To a solution of the corresponding formates (**4**) (10.0 mmol) in benzene (40 mL), 1-(dimethoxymethyl)piperidine (20.0 mmol) and piperidine (1.1 mmol) were added, and the

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resulting solution was stirred for 30 min at room temperature and then refluxed for 3 h. The reaction mixture was concentrated *in vacuo*. Methanol (30 mL) and triethylamine (10.0 mmol) were added to the residue, and the resulting mixture was refluxed for 2 h. The cooled reaction solution was concentrated *in vacuo*, and then the residue was treated with diethyl ether and stored in the refrigerator for 12 h. The precipitated crystals were collected, washed with diethyl ether, and dried.

*General Procedure for the Synthesis of 5-Substituted 2-Formyl-1H-indole-3-yl Acetates (**6**).* To a suspension of the corresponding indolones (**5**) (10 mmol) in DMF (40 mL) was added acetyl chloride (30 mmol) dropwise, and the resulting mixture was stirred for 2 h at room temperature. The reaction solution was concentrated *in vacuo*. The residue was treated with water and stirred for 2 h. The crystals were collected, washed with water, and dried.

*General Procedure for the Synthesis of 5-Substituted 2-(2,2-Dicyanovinyl)-1H-indole-3-yl Acetates (**7**).* To a suspension of the corresponding acetates **6** (10.0 mmol) in benzene (100 mL) were added malononitrile (12.5 mmol) and triethylamine (1.0 mmol), and the resulting mixture was stirred for 4 h at room temperature. The precipitated crystals were collected, washed with diethyl ether, and dried.

*General Procedure for the Synthesis of 8-Substituted 2-Oxo-2,5-dihydropyrano[3,2-b]indole-3-carbonitriles (**8**).* To a refluxing suspension of the corresponding acetates (**7**) (10 mmol) in acetonitrile (250 mL) was slowly added concentrated hydrochloric acid (30 mL) dropwise with stirring, and then the resulting mixture was refluxed for 3 h. The cooled reaction mixture was partially concentrated *in vacuo*. The precipitate was collected, washed with an acetonitrile/water (1:1) mixture, water, and methanol, and recrystallized from DMF−CH_3COOH, DMF−EtOH, or DMF−H_2O.

*General Procedure for the Synthesis of 5,8-Disubstituted 2-Oxo-2,5-dihydropyrano[3,2-b]indole-3-carbonitriles (**9**−**12**).* To a suspension of the corresponding carbonitriles (**8**) (1.00 mmol) and cesium carbonate (1.25 mmol) in DMF (5 mL) was added the corresponding alkyl halide or acyl halide (1.50 mmol) dropwise, and then the resulting mixture was stirred at room temperature. After 3 h of stirring, acetic acid (5.00 mmol) and water (10 mL) were added to the reaction mixture. The precipitated crystals were collected, washed with a DMF−water mixture, and recrystallized from acetonitrile.

*Synthesis of Methyl 2-Cyano-3-(3-hydroxy-1H-indol-2-yl)-3-(methylthio)acrylate (**15**).* To a suspension of NaH (0.01 mol) in methanol were added 1-acetylindolin-3-one **13** (0.01 mol) and methyl 2-cyano-3,3-bis(methylthio)acrylate **14** (0.01 mol) dropwise at room temperature, and then the resulting mixture was refluxed for 2 h. The mixture was concentrated *in vacuo* and treated with water and 1 to 2 drops of 10% HCl solution. The precipitate was collected and recrystallized from methanol.

*Synthesis of 4-(Methylthio)-2-oxo-2,5-dihydropyrano[3,2-b]indole-3-carbonitrile (**16**).* A mixture of **15** (0.01 mmol) and hydrogen chloride solution in dioxane (0.02 mmol) in methanol was refluxed for 2 h. The cooled reaction mixture was filtered, and the precipitate was recrystallized from a DMF−methanol mixture.

*General Procedure for the Synthesis of 4-Substituted 2-Oxo-2,5-dihydropyrano[3,2-b]indole-3-carbonitriles (**17a**−**e**).* To a suspension of **16** (1.00 mmol) in methanol was added the corresponding amine (1.50 mmol) dropwise, and then the resulting mixture was stirred at 65 °C for 2 h (**17d**, **17e** − reflux). The solid was collected, washed with methanol and diethyl ether, and recrystallized from a DMF−methanol mixture.

In Vitro Antimycobacterial Testing. *Bacterial Strains and Culture Conditions.* *M. tuberculosis* strains H37Rv and 18b were grown at 37 °C with shaking in Middlebrook 7H9 (Difco) broth supplemented with 10% albumin−dextrose−catalase (ADC) enrichment, 0.2% glycerol, 0.05% Tween 80, and, in the case of 18b, 50 μg/mL streptomycin (STR) or on a solid Middlebrook 7H10 medium (Difco) supplemented with 0.5% glycerol, 10% oleic acid−albumin−dextrose−catalase (OADC), and 50 μg/mL STR.

Nonreplicating SS18b was generated as follows. Strain 18b was grown to mid-log phase in STR-containing medium and washed three times in phosphate-buffered saline (PBS) containing 0.05% Tween 80 (PBST). The bacterial pellets were resuspended in STR-free medium. SS18b cultures were maintained at an optical density at 600 nm (OD_{600}) of between 0.2 and 0.5 for 2 weeks (with the addition of fresh medium if necessary), by which time cells had stopped replicating.

Resazurin Reduction Microplate Assay (REMA). To determine the *in vitro* activity of the compounds (MIC_{99}), a 2-week-old SS18b culture (OD_{600} = 0.2) and an H37Rv culture (OD_{600} = 0.0002) were used in the resazurin reduction microplate assays (REMA) as previously described.[21] Briefly, serial 2-fold dilutions (starting from 25 μg/mL (for H37Rv) or 10 μg/mL (for SS18b) of each drug were prepared in 96-well plates containing *M. tuberculosis* H37Rv or SS18b in 100 μL of 7H9 medium (the medium for SS18b contained streptomycin at 50 μg/mL). After 7 days of incubation at 37 °C, 10 μL of 0.025% resazurin was added to each well, and the plates were incubated overnight at 37 °C again. Then, the fluorescence level of resorufin was measured by using a TECAN Infinite M200 PRO microplate reader (excitation and emission at 560 and 590 nm, respectively). DMSO (1%) and rifampicin (0.5 mg/mL) were used as negative and positive controls, respectively. The minimal inhibitory concentration (MIC_{99}) was defined as the lowest compound concentration that prevented the change in color from blue to pink (reduction of resazurin to resorufin). The mean MIC_{99} values were calculated from at least three independent experiments.

Determination of Cytotoxicity. To determine the cytotoxicity of the compounds (TD_{50}), HepG2 human hepatocellular carcinoma cells and A549 lung epithelial cells were seeded in 96-well plates at a concentration of 4×10^4 cells/mL in DMEM (without phenol red) supplemented with 10% fetal calf serum (FCS). Cells were incubated in the presence of different concentrations of compounds in a humidified incubator with 5% CO_2 at 37 °C for 3 days, and then the cell viability was measured by fluorescence spectroscopy 6 h after adding resazurin. The cytotoxic concentration (TD_{50}) was defined as the compound concentration reducing the mean cell viability by 50%. The mean TD_{50} values were calculated from at least three independent experiments.

***Ex Vivo* Activity.** *Fibroblast Assay.* The ability of the drugs to protect fibroblast cells (MRC-5) against the MTB Erdman strain was tested as described previously.[38,39] Briefly, MRC-5 cells ($0.8-1.2 \times 10^6$ cells/flask) were cultured in minimum essential media + GlutaMAX (MEM-Gibco) supplemented with 1 mM sodium pyruvate, 1× MEM NEAA (stock concentration 100×), and 10× heat-inactivated fetal bovine

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serum (FBS). Cells were grown for 10−14 days at 37 °C in 5% CO_2. (The medium was changed twice a week.) On the day of the experiment, the fibroblasts were trypsinated (trypsin-EDTA) and then washed once with 1× phosphate-buffered saline (PBS). Fibroblasts were then counted using tryptan blue and adjusted to a concentration of 7.35×10^4 cells/mL in the MEM medium. Fibroblasts (40 μL) were added to a 384 black well plates (Corning number 3683), and the compounds (1 μL) were added to the fibroblasts. The drugs were then 2-fold serially diluted. Fibroblasts were incubated for 2 to 3 h at 37 °C in 5% CO_2 before the bacteria were added. The MTB Erdman strain was cultured in the 7H9 complete to mid-logarithmic phase (OD 0.5) and concentrated to an $OD_{600\ nm}$ of 1 (3×10^8 cells/mL) in 7H9 complete. Bacteria were diluted to a concentration of 4×10^4 bacteria/10 μL by adding 133 μL of Erdman culture $OD_{600\ nm}$ = 1 to 10 mL MEM. Bacteria (10 μL) were added to the 384 fibroblast-containing plates. The plates were incubated for 3 days at 37 °C in 5% CO_2. Plates were read using a TECAN infinite M200 microplate reader (by excitation at 560 nm and emission at 590 nm) after 1 h of incubation with 5 μL of PrestoBlue cell viability reagent (Invitrogen). Wells containing fibroblasts alone, fibroblasts with DMSO, and medium alone were used as controls. The experiment was performed in duplicate.

Macrophage Assay. THP-1 cells were grown in RPMI-1640 medium containing 10% fetal bovine serum and incubated at 37 °C in a 5% CO_2 environment. THP-1 cells (100 000) per well were differentiated into macrophages by incubating the cells with 100 nM phorbol myristate acetate (PMA) for 12 h. Cell differentiation was checked by microscopy, and PMA was removed and replaced with fresh RPMI. *M. tuberculosis* Erdman bacteria were prepared at an optical density of 1 (3×10^8 cells/mL) in Middlebrook 7H9 media. Cells were infected at a multiplicity of infection (MOI) of 5 per macrophage and incubated for 4 h at 37 °C with 5% CO_2. The medium containing bacteria was removed, and cells were washed twice with PBS. Test compounds (pyranoindolones and RIF) dissolved in RPMI were added to the 96-well plates and incubated for 3 days at 37 °C with 5% CO_2. On day 3, macrophage survival was tested by exposing the cells to PrestoBlue cell viability solution (Life Technology) for 1 h. Fluorescence was recorded using a TECAN Infinite M200 microplate reader.

Microsomal Stability. Compound **10a** (10 mg/mL) or control compounds nifedipine (Sigma N7634) and carbamazepine (Sigma C4024) (10 mg/mL) in DMSO were diluted in ddH_2O to a final concentration of 0.2 mg/mL. An aliquot of this solution was used for a further dilution in potassium phosphate pH 7.4 buffer to a final concentration of 4 μg/mL followed by incubation at 37 °C with mouse CD-1 liver microsomes (2 mg/mL) (Invitrogen MSMC-PL) and human liver (Invitrogen HMMC-PL) for 10 min (50 μL). The reaction was initiated by adding an NADPH regeneration system (Promega V9510) (50 μL) and incubating at 37 °C with gentle agitation. At 0, 5, 15, 30, and 60 min, the reaction was stopped by the addition of 100 μL of ice-cold acetonitrile. Samples were then centrifuged at 16 100*g* (13 200 rpm) at 4 °C for 10 min, and the supernatants were analyzed using HPLC-UV. Samples without NADPH cofactor (0 and 60 min) were used as controls to monitor the nonenzymatic degradation of the compounds.

Isolation and Characterization of Resistant Mutants 10a-R1−10a-R6. *M. bovis* BCG cells cultured in 10 mL of 7H9 liquid medium containing 0.2% glycerol, 0.05% Tween 80, and 10% ADC were plated on 7H11 agar containing 10% OADC and **10a** at final concentrations of 0, 10, and 20 μg/mL. Compound **10a** was dissolved in DMSO to give a final concentration of 2% in the medium. Culture (200 or 1000 μL) with OD_{600} = 1 was used for plating. In the latter case, the culture was centrifuged (10 min, 3500*g*, 23 °C), and the resulting pellet was suspended in 200 μL of 7H9 medium and then spread on the plate. The plates were incubated for 22 days at 37 °C. Out of about 1000 obtained colonies, approximately 75% had a small diameter (about 0.5 mm) and the rest were larger (about 2 mm) and the calculated frequency of resistance was 10^{-7}. Twelve colonies from each phenotype were inoculated into 5 mL of 7H9 liquid medium with 0.2% glycerol, 10% ADC, and ±0.05% Tween 80 containing 10 or 20 μg/mL compound **10a**. Cultures were incubated for about 16 days at 37 °C with shaking at 130 rpm. The resistant phenotype was confirmed for six strains, labeled 10a-R1−10a-R6. Mutant strains 10a-R1 and 10a-R2 originated from the small colonies, and the rest originated from the larger colonies.

To monitor the susceptibility of the selected resistant mutants to different drugs, an REMA method was used.[21] The cultures were inoculated to the starting OD_{600} value of 0.02 in the total volume of 200 μL of 7H9 medium with 0.2% glycerol, 10% ADC, and 0.05% Tween 80 and incubated for 7 days in the presence of various concentrations of selected inhibitors in a 96-well microtiter plate at 37 °C. Subsequently, 60 μL of 0.01% (w/v) resazurin was added to the cultures, and the plates were incubated for 48 h at 37 °C. The MICs of the inhibitors were determined on the basis of the visual evaluation of the color changes from blue to pink in the individual wells.

The susceptibility of selected mutants toward **10a** (from 0.1 to 20 μg/mL) and pretomanid (from 0.01 to 20 μg/mL) was also established on 7H11 agar plates containing 10% OADC. The drugs were dissolved in DMSO, and its final concentration in the medium was 2%. The cultures were diluted to OD_{600} = 0.5, and 4 μL portions of these cultures diluted by factors of 10^0, 10^{-1}, and 10^{-2} were applied to the plate. Subsequently, the plates were incubated at 37 °C for about 1 month.

DNA was extracted from the wild-type *M. bovis* BCG and selected resistant mutants 10a-R1−10a-R6 as described in the Supporting Information. The *fgd1*, *fbiA*, *fbiB*, and *fbiC* genes were PCR amplified as described in the Supporting Information and subjected to sequencing (Microsynth). Whole-genome sequencing (WGS, Illumina HiSeq) of the parent *M. bovis* BCG and mutant strain 10a-3 was performed and analyzed as described in the Supporting Information.

HPLC-FLD. The samples for HPLC-FLD analyses were obtained as described,[33] with minor modifications. The cells were incubated in the buffer containing 27.5 mM sodium acetate (pH 4.7) and 2% (v/v) acetonitrile at 95 °C for 20 min. The mixtures were centrifuged twice for 10 min at 15 000*g* and 23 °C. The supernatants from the second centrifugation were used directly for quantification of the F_{420} forms using a Dionex Ultimate 3000 HPLC system (Thermo Scientific) equipped with an autosampler and a fluorescence detector (FLD). Samples were kept at 15 °C in the autosampler. Analyses were performed according to Choi et al.[33] with some modifications. Briefly, coenzyme F_{420} species were separated on a reverse-phase Eclipse XDB-C18 column (4.6 mm i.d. × 150 mm, 5 μm) (Agilent) maintained at 28 °C, and the injection volume was 10 μL. The mobile phase consisted of 27.5 mM sodium acetate (pH 4.7) containing 2%

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acetonitrile (buffer A) and 100% acetonitrile (buffer B) with gradient elution at a flow rate of 1 mL min^{-1} and a run time of 31 min. The applied gradient was 0−2 min 0% B, 2−6 min from 0 to 2% B, 6−15 min from 2 to 9% B, 15−22 min from 9 to 28% B, 22−25 min from 28 to 50% B, 25−26 min from 50 to 0% B, and 26−31 min at 0% B. For fluorescence detection, an excitation wavelength was set at 400 nm, emission was set at 470 nm, and the flow cell temperature was set at 42 °C.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00622.

Characterization of the novel pyrano[3,2-*b*]indolone-based compounds; optimized synthesis of pretomanid; identification of mutations in resistant strains 10a-R1−10a-R6 by PCR and WGS; construction and susceptibility testing of the complementants; bactericidal activity of lead compound **10a** against replication and non-culturable *M. tuberculosis* cells in the Salina model of dormant *M. tuberculosis*; susceptibility testing of *M. bovis* BCG and mutant strains 10a-R1−10a-R6 by the REMA method; identification of mutations in isolated resistant *M. bovis* BCG strains 10a-R1−10a-R6 by PCR and Illumina sequencing; testing of susceptibility of *M. bovis* BCG and mutant strains 10a-R1−10a-R6 complemented with the functional copy of *fgd1* provided on pVV2-$fgd1_{BCG}$ plasmid against pretomanid and **10a**; and investigation of the effects of **10a** and **11126066** by macromolecular synthesis assays with *M. tuberculosis* H37Rv in Sauton's medium (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EMA, European Medicines Agency; FDA, Food and Drug Administration; HIV, human immunodeficiency virus; MIC_{99}, minimal inhibitory concentration; OADC, oleic acid−albumin−dextrose−catalase; REMA, resazurin reduction microplate assay; TD_{50}, 50% toxic dose; WHO, World Health Organization; WGS, whole genome sequencing

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