Discovery of Antimycobacterial Spiro-piperidin-4-ones: An Atom Economic, Stereoselective Synthesis, and Biological Intervention

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An atom economic and stereoselective synthesis of several spiro-piperidin-4-ones through 1,3-dipolar cycloaddition of azomethine ylides generated in situ from isatin and α-amino acids viz. proline, phenylglycine, and sarcosine to a series of 1-methyl-3,5-bis[(*E*)-arylmethylidene]tetrahydro-4(1*H*)-pyridinones is described. These compounds were evaluated for their in vitro and in vivo activity against *Mycobacterium tuberculosis* H37Rv (MTB), multidrug resistant *Mycobacterium tuberculosis* (MDR-TB), and *Mycobacterium smegmatis* (MC^2). Compound 4-(4-fluorophenyl)-5-phenylpyrrolo(spiro[2.3″]oxindole)spiro[3.3′]-1′-methyl-5′-(4-fluorophenylmethylidene)piperidin-4′-one (**4e**) was found to be the most active in vitro with a MIC value of 0.07 μM against MTB and was 5.1 and 67.2 times more potent than isoniazid and ciprofloxacin, respectively. In vivo, compound **4e** decreased the bacterial load in lung and spleen tissues with 1.30 and 3.73−log 10 protections respectively and was considered to be promising in reducing bacterial count in lung and spleen tissues.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* bacteria (MTB)[a] is one of the most prevalent diseases that is responsible for the deaths of about one billion people during the last two centuries.[1]TB remains a serious public health problem in India, accounting for nearly one-third of the global burden, and it has been estimated that 3.5 million of the population are infected with TB.[1,2] Currently, the recommended standard chemotherapeutic regimen for TB treatment is prescribed under DOTS. The chemotherapeutic regimen consists of an initial 2-month phase of treatment with isoniazid, rifampicin, pyrazinamide, and ethambutol followed by a continuation phase of treatment lasting 4 months with isoniazid and rifampicin.[3,4] Poor patient compliance can promote the emergence of drug resistance, and this is particularly true in TB chemotherapy.[3,4] The emergence of strains resistant to either of these drugs causes major concern, as it leaves only drugs that are far less effective, have more toxic side effects, and result in higher death rates, especially among HIV-infected persons. Serial selection of drug resistance, thus, is the predominant mechanism for the development of MDR strains; the patients with MDR strains constitute a pool of chronic infections, which propagate primary MDR resistance. In addition to accumulation of mutations in the individual drug target genes, the permeability barrier imposed by the MTB cell wall can also contribute to the development of low-level drug resistance.[5,6] In the last 50 years, only a few drugs have been approved by the Food and Drug Administration (FDA) to treat TB, which reflects the inherent difficulties in the discovery and clinical testing of new agents and the lack of pharmaceutical industry research in this area.[7] Hence, the discovery of fast-acting effective new drugs to effectively combat TB, including multidrug resistant tuberculosis, is imperative.

1,3-Dipolar cycloaddition of azomethine ylides to exocyclic olefins constitutes a versatile protocol for the construction of poly functionalized spiro-heterocycles viz. pyrrolidines[8] and pyrrolizines,[9] which widely occur in natural products and biologically active compounds. In general, spiro compounds[10] and nitrogen heterocycles such as pyridines,[11] pyrroles,[12] and pyrazolines[13] display good antimycobacterial activities. Recently, we have reported an atom economic synthesis and evaluation of antimycobacterial activities of (i) spiro pyridopyrrolizines and pyrrolidines[14] and (ii) 4*H*-pyrano[3,2-*c*]pyridine derivatives,[15] which inhibited in vitro MTB and MDR-TB. In the course of screening to discover new compounds that could be useful for the chemotherapy of tuberculosis, we identified spiro-piperidin-4-one derivatives **3**−**5**, which inhibited in vitro and in vivo MTB and MDR-TB. We present the preliminary results on the synthesis and the antimycobacterial activities of the first representative series of this family.

Chemistry

The 1,3-dipolar cycloaddition of azomethine ylides generated in situ from the reaction of isatin with (i) proline, (ii) phenylglycine, and (iii) sarcosine, to **1a**−**m** afforded spiro-[2.3″]oxindole-spiro-[3.3′]-5′-arylmethylidene-1′-methyltetrahydro-4′(1*H*)-pyridinone-4-aryl-hexahydro-1*H*-pyrrolizines (**3a**−**m**), 4-aryl-5-phenylpyrrolo(spiro[2.3″]-oxindole)-spiro[3.3′]-5′-arylmethylidene-1′-methylpiperidin-4′-ones (**4a**−**m**), and 1-methyl-4-arylpyrrolo-(spiro[2.3″]-oxindole)-spiro[3.3′]-5′-arylmethylidene-1′-methylpiperidin-4′-ones (**5a**−**m**) in excellent yields, respectively (Scheme 1). All the reactions proceed (i) chemoselectively, as the cycloaddition occurs on only one C═C of **1** furnishing exclusively the mono spiro-cycloadducts **3**−**5**, ascribable to the steric hindrance exerted by these cycloadducts for the second cycloaddition, (ii) regioselectively, as the electron-rich carbon of the dipole adds to the β carbon of **1**, and (iii) stereoselectively,

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[a] Abbreviations: MTB, *Mycobacterium tuberculosis*; MDR-TB, multidrug resistant tuberculosis; MC^2, *Mycobacterium smegmatis*; MIC, minimum inhibitory concentration, DOTS, directly observed treatment short-course.

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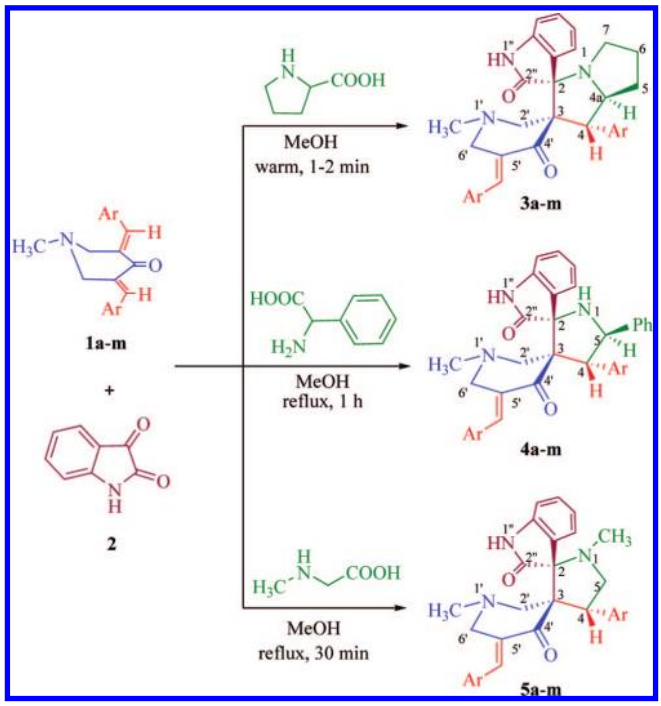
Scheme 1. Synthesis of Spiro-piperidin-4-ones **3**−**5**



as only one diastereomer is obtained exclusively in quantitative yields, although more than one stereocenter is present in these cycloadducts **3**−**5**.

Thirty-nine spiro-piperidin-4-ones have been synthesized in this study by employing cycloadditions. These reactions were effected by refluxing an equimolar ratio of the corresponding reactants in methanol over a water bath. After completion of the reactions (TLC), the reaction mixtures were poured into water to get pure **3**−**5** as yellow solids. It is interesting to note that the reaction of **1** with azomethine ylide generated from isatin and proline is completed within 1−2 min, resulting in quantitative yields of **3**, whereas the reaction with phenylglycine or sarcosine is completed respectively in 1 h and 30 min. The only byproduct of these cycloadditions are water and carbon dioxide, and hence the atom economy is very high (89−90%). The structure of spirocycloadducts **3**−**5** was elucidated with the help of ^{1}H, ^{13}C and 2D NMR spectroscopic data and single crystal X-ray crystallographic studies (Figures 1 and 2).[16] The X-ray structure determination of **3a**, which we have noted after the acceptance of this manuscript, has also been reported by Kumar et al.[16b] and the data agree with the data reported by us except with slight variations. All the spiro compounds obtained in this work are in racemic form, although two of the amino acid precursors (proline and phenylglycine) employed for the generation of the azomethine ylides are chiral. This is ascribable to the fact that the intermediates involved in the cycloaddition, viz. the azomethine ylides generated from the amino acids are achiral.

Biological Results and Discussion

Antimycobacterial Activity. The spiro-piperidin-4-ones **3**−**5** were screened for their in vitro antimycobacterial activity against MTB, MDR-TB, and MC^2 by agar dilution method for the determination of MIC in duplicate at pH value of 7.40. The pH value employed points to the fact that the amino groups in the compounds cannot be significantly protonated. This, in turn, suggests that the species with free amine functionality is

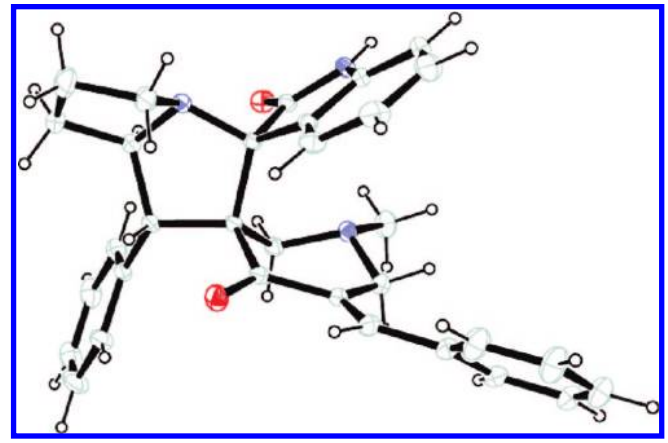


Figure 1. ORTEP diagram of **3a**.

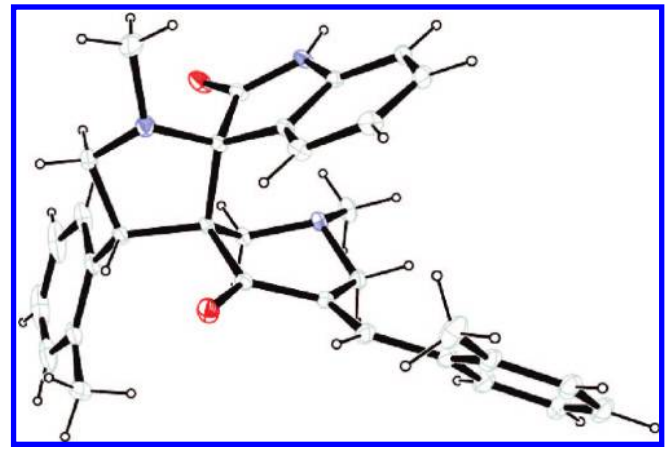


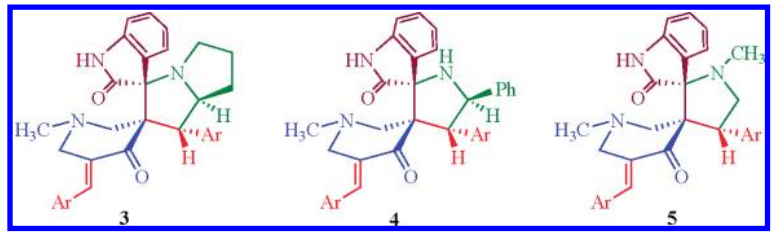
Figure 2. ORTEP diagram of **5g**.[18]

responsible for the activity. The MDR-TB clinical isolate was resistant to isoniazid, rifampicin, ethambutol, and ofloxacin. The MIC is defined as the minimum concentration of compound required to completely inhibit the bacterial growth. The MIC's of the synthesized compounds along with the standard drugs for comparison are reported in Table 1.

In the first phase of screening against MTB, all the compounds showed excellent in vitro activity against MTB with MIC ranging from 0.07−53.30 μM. Eleven compounds (**3f**, **3h**, **3k**, **4a**, **4c**−**e**, **5d**, and **5f**−**h**) inhibited MTB with MIC of less than 1 μM, and 23 compounds were more potent than standard fluoroquinolone ciprofloxacin (MIC: 4.71 μM). When compared to isoniazid (MIC: 0.36 μM), three compounds (**3f**, **4e**, and **5f**) were found to be more active against MTB, and one compound **5h** was equipotent as isoniazid. Compound **4e** was found to be the most active in vitro with MIC of 0.07 μM against MTB and was 5.1 and 67.2 times more potent than isoniazid and ciprofloxacin, respectively.

Subsequently, compounds having MIC less than 3 μM were evaluated against MDR-TB, and the compounds inhibited MDR-TB with MIC ranging from 0.08−5.31 μM. All of the 21 compounds screened were found to be more active than isoniazid (MIC: 45.57 μM) and ciprofloxacin (MIC: 37.73 μM). Eight compounds (**3f**, **3h**, **3k**, **4d**, **4e**, **5d**, **5f**, and **5g**) inhibited MDR-TB with MIC of less than 1 μM. Compound **5f** was found to be the most active in vitro with MIC of 0.08 μM against MDR-TB and was 569.6 and 471.6 times more potent than isoniazid and ciprofloxacin, respectively. The compounds were also evaluated against MC^2 in which all the compounds inhibited

Table 1. Minimum Inhibitory Concentrations (μM) of Spiro-piperidin-4-ones **3**−**5** against Mycobacterial Species[a]



			MIC (μg/mL)					MIC (μg/mL)					MIC (μg/mL)		
aryl	compd	IC_{50}(μM)	MTB	MDRTB	MC^2	compd	IC_{50} (μM)	MTB	MDRTB	MC^2	compd	$IC_{50}$$\mu$M)	MTB	MDRTB	MC^2
phenyl	**3a**	NT	25.56	NT	102.25	**4a**	119.05	0.74	1.49	2.97	**5a**	NT	13.49	NT	53.99
4-chlorophenyl	**3b**	NT	22.73	NT	90.91	**4b**	NT	5.27	NT	42.09	**5b**	117.49	2.93	NT	5.88
4-methylphenyl	**3c**	NT	12.09	NT	24.18	**4c**	113.02	0.71	1.41	2.82	**5c**	NT	6.37	NT	50.92
4-methoxyphenyl	**3d**	NT	5.70	NT	45.54	**4d**	106.84	0.67	0.67	1.33	**5d**	119.50	0.75	0.36	2.98
4-fluorophenyl	**3e**	119.05	2.97	2.97	23.81	**4e**	111.41	0.07	0.16	0.69	**5e**	NT	6.27	NT	50.10
2-chlorophenyl	**3f**	113.64	0.35	0.71	5.69	**4f**	105.22	2.63	5.27	10.52	**5f**	117.48	0.17	0.08	1.47
2-methylphenyl	**3g**	NT	12.09	NT	24.18	**4g**	113.02	2.82	2.82	11.30	**5g**	127.29	0.39	0.39	1.59
2-methoxyphenyl	**3h**	113.84	0.71	0.71	2.84	**4h**	106.84	1.33	2.67	1.33	**5h**	119.50	0.36	1.49	2.98
3-fluorophenyl	**3i**	NT	5.96	NT	23.81	**4i**	111.41	2.78	NT	5.58	**5i**	125.25	1.56	1.56	6.27
2,4-dichlorophenyl	**3j**	NT	19.94	NT	79.74	**4j**	94.27	2.35	2.35	9.43	**5j**	103.99	1.29	2.59	5.21
2-thienyl	**3k**	124.75	0.38	0.38	3.11	**4k**	NT	11.64	NT	11.64	**5k**	131.58	1.64	3.28	6.59
1-naphthyl	**3l**	106.11	2.65	5.31	10.61	**4l**	NT	5.01	NT	20.00	**5l**	NT	11.10	NT	22.20
2-furyl	**3m**	NT	53.30	NT	106.61	**4m**	NT	24.75	NT	49.51	**5m**	141.08	1.76	3.52	0.88
isoniazid			0.36	45.57	45.57			0.36	45.57	45.57			0.36	45.57	45.57
ciprofloxacin			4.71	37.73	2.35			4.71	37.73	2.35			4.71	37.73	2.35

[a] MTB: *Mycobacterium tuberculosis*; MDR-TB: Multidrug resistant *Mycobacterium tuberculosis*; MC^2: *Mycobacterium smegmatis*; NT: not tested.

Table 2. In Vivo Activity Data of **4e** and Isoniazid against *Mycobacterium tuberculosis* ATCC 35801 in Mice

compd	lungs (log CFU ± SEM)	spleen (log CFU ± SEM)
control	7.31 ± 0.11	8.93 ± 0.20
isoniazid (25 mg/kg)	5.61 ± 0.13	4.92 ±0.15
4e (25 mg/kg)	6.01 ± 0.19	5.20 ± 0.13

MC^2 with MIC ranging from 0.69−106.61 μM, and 31 compounds were found to be more active than isoniazid (MIC: 45.57 μM).

With respect to structure−MTB activity relationship, the results demonstrated that the antimycobacterial activity was in the order: **5** > **4** > **3**. This is evident from the fact that eight compounds in series **5** (**5d**, **5f**−**k**, and **5m**), five in series **4** (**4a**, **4c**−**e**, and **4h**), and three in series **3** (**3f**, **3h**, and **3k**) were more active against MTB. Among the compounds from the series **3**−**5**, compounds **3k** (MIC: 0.38 μM), **4e** (MIC: 0.07 μM), and **5f** (MIC: 0.17 μM) was found to be more active. The influence of substituents at *N* as well as at carbon α to the *N* of the pyrrolidine ring and substituents at different positions of aryl rings was examined for SAR. Bridging between *N* and the adjacent carbon leads to loss of antimycobacterial activity as seen with series **3**. *Ortho* substitution in the aryl ring enhances activity in both series **3** and **5**, whereas *para* substitution at the aryl ring facilitates activity in series **4**. Electronegative and monosubstitution favor the antimycobacterial activity in all series. When the aryl ring is a sulfur heterocycle, activity is favored as seen in **3k**, whereas the furan ring in **3m** and **4m** renders these compounds inactive.

Compound **4e**, being the most active in vitro against MTB, was tested for efficacy against MTB at a dose of 25 mg/kg (Table 2) in CD-1 mice. The mice were infected intravenously with *Mycobacterium tuberculosis* ATCC 35801. Drug treatment by intraperitoneal route began after 10 days of inoculation of the animal with microorganism and continued for 10 days. After 35 days postinfection, the spleens and right lungs were aseptically removed, the number of viable organisms was determined and compared with the counts from negative (vehicle treated) controls (Mean culture forming units (CFU) in lung: 7.31 ± 0.11 and in spleen: 8.93 ± 0.20). Compound **4e** decreased the bacterial load in lung and spleen tissues with 1.30 and 3.73−log 10 protections respectively and was considered to be promising in reducing bacterial count in lung and spleen tissues. When compared to isoniazid at the same dose level, **4e** was found to be less active. This could be ascribed to various pharmacokinetic reasons.

The compounds with MIC less than 3 μM were further examined for toxicity (IC_{50}) in a mammalian Vero cell line up to 62.5 μg/mL concentration by serial dilution method. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 nonradioactive cell proliferation assay[19] and the results are reported in Table 1. Twenty-three compounds when tested showed IC_{50} values ranging from 94.27−131.58 μM. The compound **4e** was found to be nontoxic up to 62.5 μg/mL (111.41 μM) and showed selectivity index (IC_{50}/MIC) of 1634.

Conclusions

The 1,3-dipolar cycloaddition of azomethine ylide generated in situ from isatin and α-amino acids to 1-methyl-3,5-bis[(*E*)-arylmethylidene]tetrahydro-4(1*H*)-pyridinones afforded spiropiperidin-4-ones **3**−**5** in quantitative yields. These spiroheterocycles displayed good in vitro antimycobacterial activity against MTB, MDR-TB and MC^2. Further studies on the synthesis and examination of structure−activity relationships of a wide range of heterocyclic compounds with various substituents at phenyl ring and *N* atom of the oxindole moiety and varying ring size of 1-methyl-4-piperidone are in progress in our research groups.

Experimental Section

General. The melting points were measured in open capillary tubes and are uncorrected. The ^{1}H, ^{13}C, and the 2D NMR spectra were recorded on a Bruker (Avance) 300 MHz NMR instrument

using TMS as internal standard and $CDCl_3$ as solvent. Standard Bruker software was used throughout. Chemical shifts are given in parts per million (δ-scale), and the coupling constants are given in hertz. Silica gel-G plates (Merck) were used for TLC analysis with a mixture of petroleum ether (60−80 °C) and ethyl acetate as eluent. Elemental analyses were performed on a Perkin-Elmer 2400 Series II elemental CHNS analyzer.

Synthesis of 1-Methyl-3,5-bis[(*E*)-arylmethylidene]tetrahydro-4(1*H*)-pyridinones (1). General Procedure. A mixture of 1-methyl-4-piperidone (0.113 g, 1 mmol), aromatic aldehyde (2 mmol), and NaOH (1 mL, 30%) in alcohol (25 mL) was stirred for 15 min. The separated solid was filtered and washed with water (100 mL) to obtain pure **1** as yellow solid.

Synthesis of Spiro-[2.3″]-oxindole-spiro[3.3′]-1′-methyl-5′-(arylidene)tetrahydro-4′-(1*H*)-pyridinone-4-arylhexahydro-1*H*-pyrrolizine (3). General Procedure. A mixture of **1** (1 mmol), isatin **2** (0.147 g, 1 mmol), and proline (0.115 g, 1 mmol) was dissolved in methanol (10 mL) and warmed on a water bath for 1−2 min. After completion of the reaction as evident from TLC, the mixture was poured into water (50 mL). The precipitated solid was filtered and washed with water to obtain pure **3**.

Synthesis of 4-Aryl-5-phenylpyrrolo(spiro[2.3″]-oxindole)-spiro-[3.3′]-1′-methyl-5′-(arylidene)piperidin-4′-ones (4). General Procedure. A mixture of **1** (1 mmol), isatin **2** (0.147 g, 1 mmol), and phenylglycine (0.151 g, 1 mmol) in methanol (10 mL) was refluxed for 1 h. After completion of the reaction as evident from TLC, the mixture was poured into water (50 mL). The precipitated solid was filtered and washed with water to obtain pure **4**. The yield, melting point and NMR spectroscopic data of **4a**−**f** and **4k**−**m** agree well with those reported by our group earlier.[17]

Synthesis of 1-Methyl-4-arylpyrrolo-(spiro[2.3″]oxindole)-spiro-[3.3′]-1′-methyl-5′-(arylidene)piperidin-4′-ones (5). General Procedure. A mixture of **1** (1 mmol), isatin **2** (0.147 g, 1 mmol), and sarcosine (0.089 g, 1 mmol) were dissolved in methanol (10 mL) and refluxed for 30 min. After completion of the reaction as evident from TLC, the mixture was poured into water (50 mL). The precipitated solid was filtered and washed with water to obtain pure **5**. The physical and spectroscopic data of **5a**−**d** agree well with those reported in the literature.[20]

MIC Determination. All compounds were screened for their in vitro antimycobacterial activity against MTB, MDR-TB, and MC^2 in Middlebrook 7H11 agar medium supplemented with OADC by agar dilution method similar to that recommended by the National Committee for Clinical Laboratory Standards for the determination of MIC in duplicate.[19] The MDR-TB clinical isolate was obtained from Tuberculosis Research Center, Chennai, India, and was resistant to isoniazid, rifampicin, ethambutol, and ofloxacin. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound required to give complete inhibition of bacterial growth.

Cytotoxicity. All the compounds were further examined for toxicity (IC_{50}) in a mammalian Vero cell line up to concentration of 62.5 μg/mL[21] by serial dilution method. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 nonradioactive cell proliferation assay.

In Vivo Studies. One compound was tested for efficacy against MTB at a dose of 25 mg/kg in six-week-old female CD-1 mice six per group. In this model, the mice were infected intravenously through caudal vein approximately 10^7 viable *Mycobacterium tuberculosis* ATCC 35801. Drug treatment by intraperitoneal route began after 10 days of inoculation of the animal with microorganism and continued for 10 days. After 35 days postinfection, the spleens and right lungs were aseptically removed and ground in a tissue homogenizer, the number of viable organisms was determined by serial 10-fold dilutions and subsequent inoculation onto 7H10 agar plates. Cultures were incubated at 37 °C in ambient air for 4 weeks prior to counting. Bacterial counts were measured and compared with the counts from negative controls (vehicle treated) in lung and in spleen (Table 2).

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Supporting Information Available: Experimental methods for compounds **3**, **3a**,**b**, **3e**−**m**, **4**, **4g**−**j**, **5**, **5e**−**l**, MIC determination, cytotoxicity, in vivo studies, This material is available free of charge via the Internet at http://pubs.acs.org.

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