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Discovery of dipiperidines as new antitubercular agents

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ABSTRACT

As part of our ongoing research effort to develop new therapeutics for treatment of tuberculosis (TB), we synthesized a combinatorial library of 10,358 compounds on solid support using a pool-and-split technique and tested the resulting compounds for activity against *Mycobacterium tuberculosis*. Structure–activity relationship (SAR) evaluation identified new compounds with antitubercular activity, including a novel hit series that is structurally unrelated to any existing antitubercular drugs, dipiperidines. Dipiperidine representatives exhibited MIC values as low as 7.8 μM, the ability to induce promoter Rv0341 activated in response to cell wall biosynthesis inhibition, relatively low nonspecific cellular toxicity in the range of 30–162 μM, and log *P* values less than 4.

Although drugs for treatment of tuberculosis have been available for nearly 50 years, TB remains a global health crisis, killing nearly two million people each year. The increasing prevalence of HIV infection and the growing problem of multi-drug resistant TB (MDR-TB) both emphasize the need for new drugs to more effectively treat the disease in all patients.[1–3]

We previously reported the synthesis of several combinatorial libraries using solid-phase and solution-phase synthetic techniques.[4,5] The first library of asymmetrical ethylenediamines was prepared in an attempt to improve the potency of the existing first line anti-TB drug ethambutol (EMB),[4] which was discovered by Lederle Laboratories from a library of 2000 mostly symmetrical diamines.[6] An evaluation of the structure–activity relationship (SAR) of that original library demonstrated that alterations of the ethylene bridging linker, such as lengthening of the ethylene unit or incorporation of a variety of heteroatoms into the chain, led to a loss of activity. Taking these data into consideration, we generated a library of 63,238 ethylenediamines that was prepared with an emphasis on the introduction of a wide variety of substituents at the two nitrogen atoms of EMB. This led to the identification of the antitubercular candidate SQ109,[7–11] which is currently completing phase 1 human safety studies. After evaluation of the SAR from our ethylenediamine library, we expanded our compound library by investigating alternative diamine linkers. We prepared a second combinatorial library of diamines using amino alcohol pre-loaded resins with amino acids as building blocks. Amino alcohols attached to the resin were convenient starting synthons, as they provided a protected hydroxyl group and an amino group available for modification. Use of amino acids in the synthesis allowed us to introduce diverse elements into the bridging linker between the two amine components, as well as chirality. This synthetic strategy also allowed us to improve hydrophilicity, a characteristic that was lacking in our original ethylenediamine library. The second amino component was diversified by alkylation or acylation after introduction of the amino acids.

In this Letter, we report the solid-phase synthesis of a library of 10,358 diamines based on the general synthetic scheme shown in Figure 1. In vitro screening of the library against *Mycobacterium tuberculosis* and an evaluation of SAR identified a series of compounds structurally unrelated to existing antitubercular drugs.

Library synthesis began with the coupling of the resin-anchored amino alcohols with Fmoc-protected amino acids. This step was performed on a Quest Synthesizer, allowing 20 reactions to occur in parallel. If the starting amino alcohol was protected with Fmoc (for instance, *N*-Fmoc-hydroxylamine or *N*-Fmoc-4-hydroxypiperidine), the protective group was removed using 20% piperidine in DMF prior to coupling (step a, Fig. 1). We chose ten commercially available amino alcohols (Novabiochem; now EMD Biosciences) for the library preparation (Fig. 2 A). Amino acids used as a linker in the synthesis were selected (1) to allow the introduction of various substituents at the bridging ethylenic linker, such as alkyl (Leu, Ile), cycloalkyl (Cha), benzyl (Phe), and heterocycles (His, Trp); (2) to introduce a cyclic moiety between the two nitrogen atoms (Amc), and (3) to incorporate a nitrogen atom into the heterocycle

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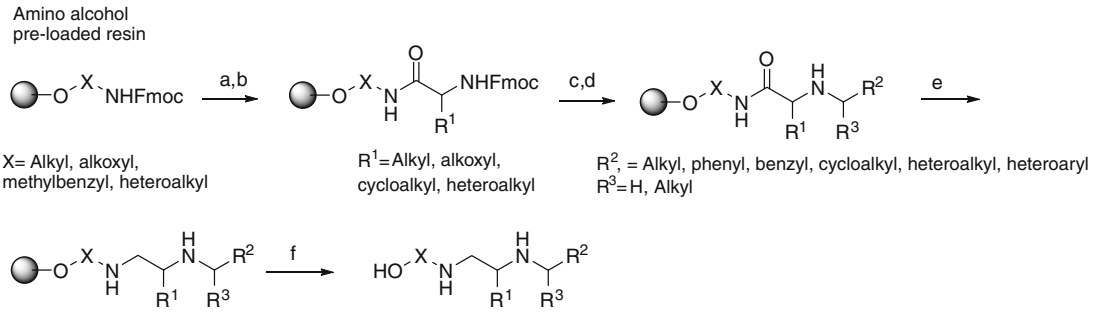


Figure 1. General synthetic approach for the library preparation. Reagents and conditions: (a) piperidine/DMF (20%, v/v), 20 min, rt; (b) Fmoc-protected amino acid (2.5 equiv), HATU (2 equiv), DIEA (10 equiv), DMF/CH_2Cl_2 (1/1, v/v), rt, 18 h; (c) piperidine/DMF (20%, v/v), 20 min, rt; (d) carboxylic acid (2.5 equiv), PyBrop (2 equiv), DIEA (10 equiv), DMF/CH_2Cl_2 (1/1), rt, 18 h or an aldehyde or ketone (5 equiv), 1 M solution of $NaBH_3CN$ in THF, THF, rt, 18 h; (e) Red-Al (65+ wt % in toluene)/THF anhydrous (1/6, v/v), rt, 6 h; (f) 10%TFA/CH_2Cl_2, rt, 20 min.

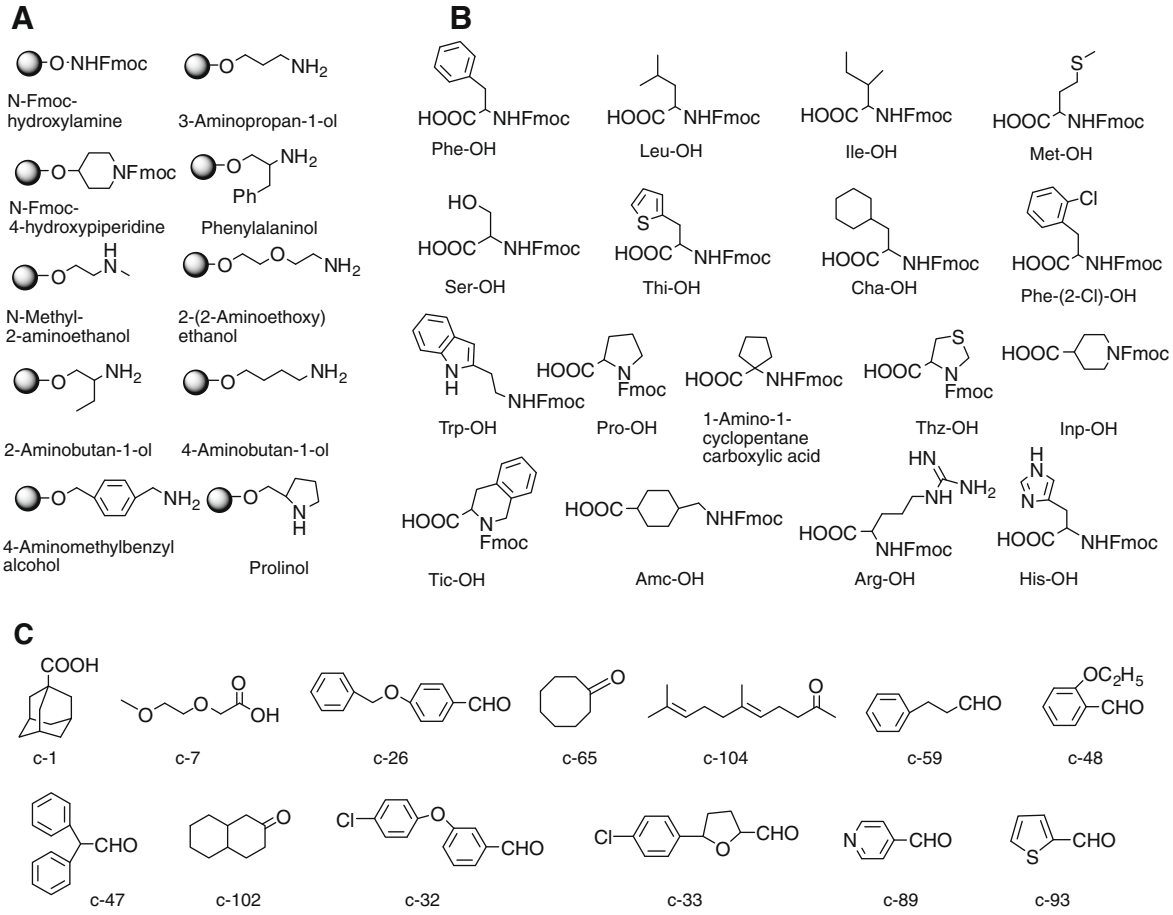


Figure 2. Reagents used in the library preparation: A—amino alcohol pre-loaded resins; B—amino acids (amino acids used were of the L configuration (if applicable)); C—representatives of the acylating/alkylating reagents.

(Inp). Chemical structures of the amino acids that were used in the compounds synthesis are shown in Figure 2 B. Amino acid names and their standard abbreviations are available as Supplementary data.

After completion of the coupling step (step b in Fig. 1), we confirmed the formation of intermediate amide for every amino alcohol used. This was necessary because some of the pre-loaded amino alcohols were isomers (3-aminopropan-1-ol and *N*-methyl-2-aminoethanol, *N*-Fmoc-4-hydroxypiperidine and prolinol, and 2-aminobutanol and 4-aminobutanol) that could not be distinguished by mass spectrometry (MS) after pooling of the intermediates into the mixtures for further modification and screening. To confirm the synthesis, we cleaved a small amount of each intermediate amide from the resin and analyzed it by positive ion electrospray MS. With the exception of amino acid Arg, all the amino alcohols successfully produced intermediate amides with amino acids.

The synthesized intermediates were pooled into groups of 10, leaving ~50 mg of each resin available for deconvolution. Deconvolution was performed if a group was found to have antibacterial activity. These pools of 10 compounds were evenly distributed into 96-well plates. After Fmoc removal (step c, Fig. 1), the second amino group was derivatized by acylation with a carboxylic acid, or by

reductive alkylation with aldehydes or ketones in the presence of cyanoborohydride (step d, Fig. 1). The selection of alkylating/acylating reagents (12 carboxylic acids, 20 ketones, and 64 aldehydes) was made both to guarantee structural diversity and ensure that the final diamine products would carry the same or similar types of substituents that had been observed in the hit compounds identified from previous diamine libraries.[4,5] The carbonyls were organized in a 96-well master plate as 1 M solutions of individual compounds per well and were added to each individual pool of 10 resins, one carbonyl compound per well. Representatives of carbonyls are shown in Figure 2 C; a complete list of the carbonyl compounds used is available as Supplementary data.

In step d, Figure 1, we observed that partial double alkylation occurred on amino acids bearing a primary amino groups (leucine [Leu], isoleucine [Ile], β-cyclohexylalanine [Cha], or 4-aminomethylcyclohexane carboxylic acid [Amc]) when aldehydes were used as alkylating reagents. The ratio of mono to double alkylated compounds did not exceed 5:1. For the amino acids that have a secondary amino group (isonipecotic acid [Inp], proline [Pro], and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid [Tic]), overalkylation did not occur. When ketones or carboxylic acids were used in this step, only mono derivatives were formed.

In the next step (step e, Fig. 1), the intermediate amides were reduced using Red-Al solution in THF. This step proceeded to completion with tertiary amides, while with secondary amides the ratio of reduced to unreduced products varied between 1:1 and 1:10.

Finally, the resulting compounds were cleaved from the resin with 10% of TFA in DCM (step f, Fig. 1). Formation of the products was monitored by positive electrospray mass spectrometry using two randomly selected rows, which allowed us to analyze 25% of the entire library. From this analysis, we calculated a library yield of 63%, suggesting that 10,358 of the targeted 16,320 compounds were formed.

Compound mixtures containing 10+ compounds per well were moved into primary screening without additional purification. After identification, active mixtures were deconvoluted and single compounds from the active group were screened for activity against *M. tuberculosis* using a whole cell assay (see below). The components of the active wells, including side products such as unreduced and double alkylated diamines, were separated by chromatography and screened again to identify the active compound(s).

The 10,358 compound library was tested in vitro against the laboratory strain of *M. tuberculosis* (H37Rv) in two screens: (1) direct determination of minimal inhibitory concentration (MIC) using viable organisms in a broth microdilution assay, and (2) a whole cell high-throughput bioluminescence based assay (Luc) with a recombinant *M. tuberculosis* strain that produces light in response to the inhibition of cell wall biosynthesis.[4,5] Compounds active in both tests were of particular interest to us, since this suggests that inhibition of bacterial growth is likely due to disruption of cell wall biosynthesis, rather than a more generalized toxicity.

Compounds were screened in pools, as prepared, in a dose response, with concentrations ranging from 50 μM to 0.39 μM. In this initial screen, we assumed that compounds were synthesized with 100% yield. EMB was used as a positive control in the Luc assay, and both izoniazid (INH) and EMB were used as positive controls in the broth microdilution assay. Wells were considered active if (1) the MIC was equal to or less than 12.5 μM, or (2) if the MIC was equal to or less than 50 μM and the response in the Luc assay was at least 1.5× over the background luminescence of the untreated control. Based on these criteria, 198 mixtures were found to be active. Active mixtures were then deconvoluted in a 96-well plate format by re-synthesis of the individual diamine components of each active mixture, followed by the screening of the deconvoluted plates in the same assays. Screening results revealed 118 individual compounds with an MIC equal to or lower than 50 μM, out of which 109 compounds had an MIC equal to or less than 12.5 μM.

Evaluation of SAR showed that 80 of the 118 hits were derived from two amino alcohols—prolinol (45 hits) and its structural isomer 4-hydroxypiperidine (35 hits). Five amino acids produced the vast majority of the hits (Fig. 3); these were Amc, Ile, phenylalanine [Phe], thienylalanine [Thi], and Inp. The carbonyl compounds that contributed most to activity of the final products were 3-(4-chlorophenoxy)-benzaldehyde (c-32, 23 hits), 5-(4-chlorophenyl)furfural (c-33, 12 hits), geranylacetone (c-104, 10 hits), benzyloxyacetaldehyde (c-27, 10 hits), and 4-(benzyloxy)benzaldehyde (c-26, 10 hits). These data correlate well with our previous findings[4,5], suggesting that these fragments might be important features for antitubercular activity.

Only 38 of the 118 hits showed a positive response in the Luc assay, suggesting that the target of the majority of compounds was independent of the cell wall synthetic pathway. Certain amino acids were more likely to produce compounds with a positive Luc response, as shown in Figure 3. Interestingly, 9 hits in the Inp series were active in both assays, exhibiting MIC between 3.13 and 25 μM. The luminescence level in the Luc assay of these hits was between 59% and 125% of the response of the assay for the EMB, a cell wall active drug, implying that the compounds are likely interfering with cell wall biosynthesis. In contrast, the activity of Amc hits in the Luc assay was only about 6% of the EMB control response, while the MIC for this series fell in the range of 3.13–12.5 μm. Compounds from other series that were considered active from the microdilution assay also showed poor activity in the Luc assay. Therefore, we chose to perform further evaluations on hits from the Inp series because of the apparent cell wall activity.

The nine compounds from the Inp series were prepared on a 150–200 mg scale with at least 95% purity to confirm the MIC and for more in-depth in vitro screening. Purity evaluation was performed by GC and/or HPLC. The structures of synthesized compounds were characterized by mass spectroscopy and ^{1}H NMR. MS and ^{1}H NMR spectra are available as Supplementary data.

The MIC of seven of the nine purified compounds were between 7.8 and 31.25 μM, as determined by the microdilution method described previously (Table 1); the remaining two compounds did not have activity. The MIC of the most active compounds (SQ609, SQ614, and SQ615) were confirmed using growth in BACTEC medium. The hits were then tested for activity against mammalian cells (HepG2) using an assay that measures in vitro metabolism of tetrazonium salts (MTS) to determine IC_{50} (concentration of compound at which 50% of cells remain viable). The selectivity index (SI) is calculated as the ratio of IC_{50} to the MIC, and is designed

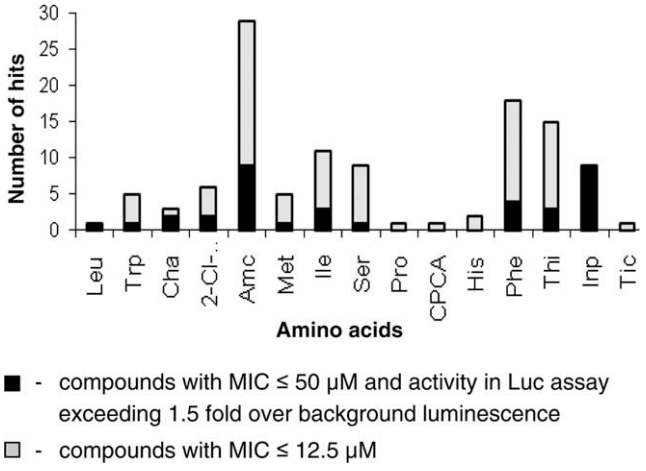
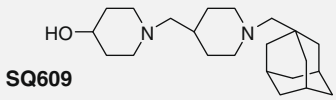
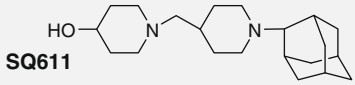
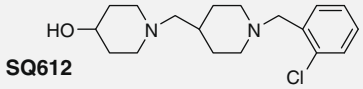
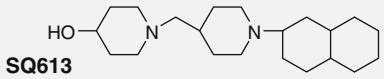
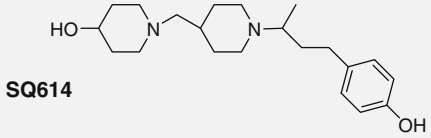
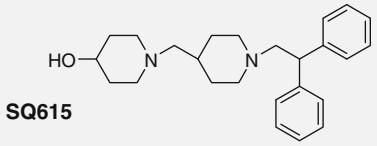
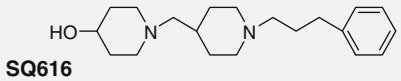


Figure 3. Occurrence of amino acids in library hits. ■—Compounds with MIC ⩽50 μM and activity in Luc assay exceeding 1.5-fold over background luminescence. □—Compounds with MIC ⩽12.5 μM.

Table 1
In vitro screening data for Inp series

Compound	MIC[a], μM microdilution	MIC[b], μM BACTEC	Luc, % to EMB[c]	IC_{50}[d], μM	SI[e]	Log *P*[f]
 **SQ609**	6.25	15.6	59	56	8.96	3.76
 **SQ611**	31.25	—	102	25	0.80	3.24
 **SQ612**	31.25	—	107	91	2.91	2.37
 **SQ613**	31.25	—	108	41	1.31	3.95
 **SQ614**	7.8	15.65	90	162	20.77	1.91
 **SQ615**	7.8	16	125	30	3.85	3.16
 **SQ616**	15.63	—	70	39	2.50	2.85

[a] MIC of the controls in the broth microdilution assay: INH 0.063 μg/ml (0.46 μM); Rif 0.03 μg/ml (0.03 μM).
[b] MIC of the controls in the BACTEC assay: INH 0.05 μg/ml (0.36 μM); EMB 1.95 μg/ml (7.04 μM).
[c] Percent of luminescence level of tested compounds as compared to the level of EMB (control).
[D] Determined with HepG2 cells (human hepatocellular liver carcinoma cell line) in MTS assay.
[e] Selectivity index SI = IC_{50}/MIC.
[f] Log *P*, octanol–water partitioning coefficient, was calculated using ACD software.

to estimate a range of drug concentration for in vivo studies. Based on the SI, SQ609, SQ614, and SQ615 were the most potent hits for further evaluation. Log *P* values, also known as the octanol–water partition coefficient, were calculated using ACD software. Log *P* is used as an indicator of compound lipophilicity and solubility to predict transport properties across cell membranes. For all compounds of the series, log *P* values were less than 4, suggesting good absorption. In vitro screening data obtained for the Inp hit series is presented in Table 1.

In conclusion, a diverse library of diamine compounds was prepared on solid support and screened in vitro against *M. tuberculosis* in two assays. Several compound series were identified. The dipiperidine series was the most promising, since hits of the series were active in both assays, exhibited low mammalian cell toxicity, and had favorable log *P* values. Although compounds of this series demonstrated similar MIC to EMB as well as activity in bioluminescence assay, they are structurally different than the ethylenediamine class of compounds, and may have improved properties. The more rigid structure of dipiperidines is likely to reduce the propensity of the compounds to form stable chelates with divalent metal ions, a quality which is associated with the ocular toxicity of ethambutol. In addition, they may have a different mechanism of action from ethambutol, as well as modified pharmacokinetics. This new class of compounds is now being tested in more extensive in vitro studies and in mouse models to determine efficacy against *M. tuberculosis* in vivo. These data will allow us to more fully explore their potential as antitubercular drugs.

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Supplementary data

Complete list of amino acid and their abbreviation, list of carbonyl compounds used in the library synthesis, ^{1}H NMR and MS spectra for hit compounds, detailed experimental procedures. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.135.

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