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A novel indigoid anti-tuberculosis agent

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ABSTRACT

The structure of a novel indigoid component was characterized by X-ray crystallography. This compound exhibited excellent anti-tuberculosis activity against *Mycobacterium tuberculosis* H37Rv in whole cell culture showing a submicromolar minimum inhibitory concentration (MIC). A synthesis of this molecule was designed and carried out to produce sufficient material for further testing. The in vitro profile, structure, and first synthesis of this indigoid component is reported.

There exists great demand for new agents capable of combating infections associated with *Mycobacterium tuberculosis* (*M.tb*), the causative bacterium of tuberculosis (TB). This organism afflicts over a third of the world's population with an annual death rate in excess of 2 million.[1] Current first-line therapy involves use of a combination of isoniazid, rifampicin, pyrazinamide, and ethambutol or streptomycin (Fig. 1). These drugs were identified decades ago, and while a number of molecules are currently under study as new agents[2] (e.g., PA-824, OPC-67683), only one (TMC-207)[3] has successfully completed phase III clinical-stage development.

A standard therapeutic course[4] of 6–8 months is required to kill this slow-growing bacteria and to allow for sterilization of the persistent phenotype of *M.tb*. Furthermore, the infected populations are generally found in developing countries where such dosing dynamics and logistics often lead to poor patient compliance. As a result, the non-compliance can exacerbate development of drug resistance; for example, in 2006, 500,000 cases of multi-drug resistant TB (MTR-TB) were estimated with 6.6% of these cases carrying the extensively-drug resistant TB (XTR-TB) strains.[5] Considering all infectious diseases, TB is the #1 cause of death of HIV-infected populations, and the epidemic has now become an urgent global health problem.

High-throughput screening (HTS) of compound libraries in search of new anti-TB actives is fraught with problems related to the unique lipophilic cell wall[6] of *M.tb* serving as a barrier to some structural types, along with the relative abundance of cytochrome P450 enzymes[7] (20 isoforms) which inactivate functionalized molecules.

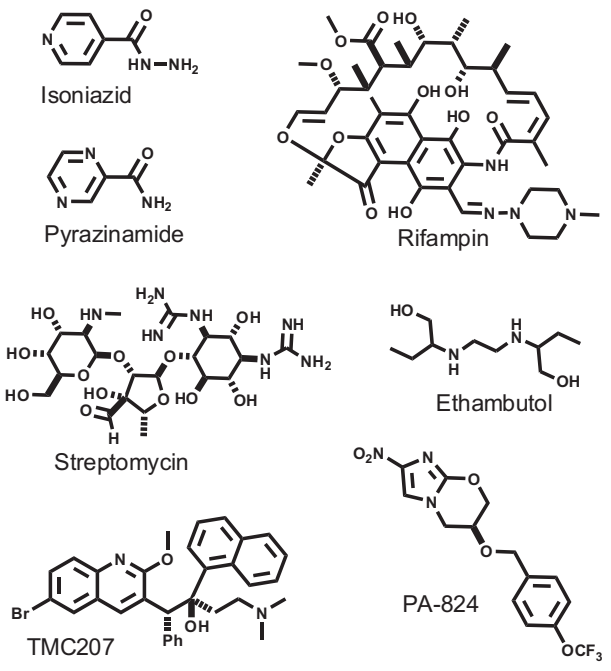

Figure 1. Anti-TB agents.

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Table 1
In vitro profile of compound **1** (μM)

Assay[a]		(**1**)	Isoniazid	Rifampin
1	MIC *H37Rv*	0.57	0.22	0.038
2	Cytotoxicity *Vero cell*	>32	>100	>182
3	LORA MIC	5.3	>100	0.49
4	Protein shift MIC (4% BSA)	2.1	0.45	0.089
5	Protein shift MIC (10% FBS)	0.57	0.24	0.21
6	MIC *M. smegmatis*	>50	18.9	16.7
7	MIC *C. albicans*	>50	ND	ND
8	MIC *S. aureus*	>50	ND	ND
9	MIC *E. coli*	>50	ND	ND

[a] Description of these assays are listed in Supplementary data section.

Recently, scientists from The Shaw Group, Inc. have successfully employed genetic engineering to modify the active site of the oxidative enzyme, toluene-4-monooxygenase(T4MO).[8] The wild type T4MO enzyme is able to hydroxylate a wide range of aromatic and aliphatic chemicals, and through their efforts, the substrate range has been expanded by creating new isoforms with different substrate specificities and product distributions. This approach, termed 'combinatorial biocatalysis',[9] proceeds by incubating the organism (*Escherichia coli*) expressing the cloned T4MO isoform, with the substrate(s), and the resultant microbial processing of these substrates is followed by HPLC analysis. Indole-based substrates were selected for this experiment due to their status as a privileged core in medicinal chemistry, their propensity for oxidation and subsequent dimerization under these culture conditions. Following the microbial processing of this modified oxidative system, novel secondary metabolites were produced and isolated.[22] Upon screening these indigoid libraries from the aforementioned bacterial culture for antimicrobial bioactivities, several structural sub-sets typified by compound **1** were found to exhibit potent anti-TB activity.[10] Herein, we report the anti-TB profile, structural characterization of **1**,[12] and the first chemical synthesis of this compound.[11]

The in vitro profile of **1** is listed in Table 1 along with that of the anti-TB agents, isoniazid and rifampin. The biocatalytic reaction described above utilized indole and anthranil as the lone substrates (Fig. 2) and produced multiple components from which **1** was isolated. Compound **1** exhibits good potency and is not greatly affected by protein-shift assays 4 and 5 (Table 1) showing a 1- and 3.7-fold increase in the MIC, respectively. This compound has excellent selectivity in the breadth of spectrum assays (6–9) with

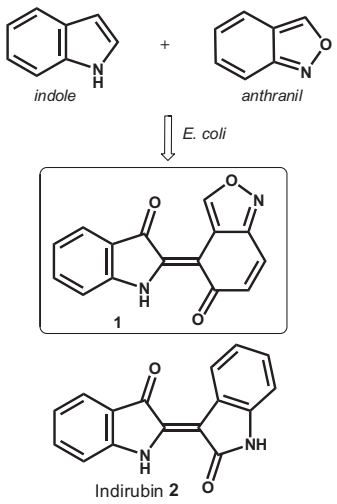


Figure 2. Biocatalytic formation of **1** in *E. coli* and structure of indirubin (**2**).

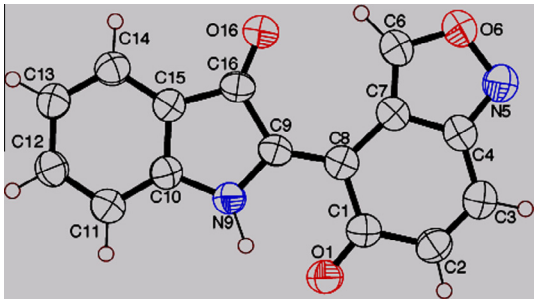
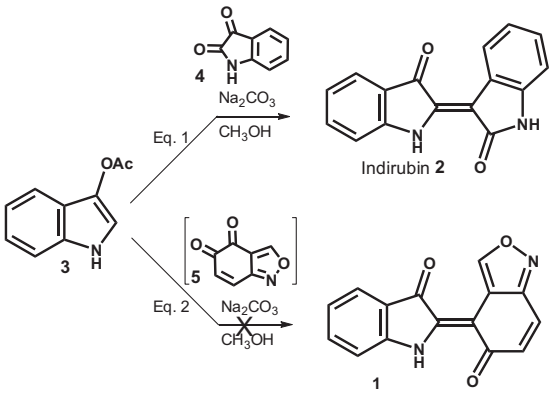
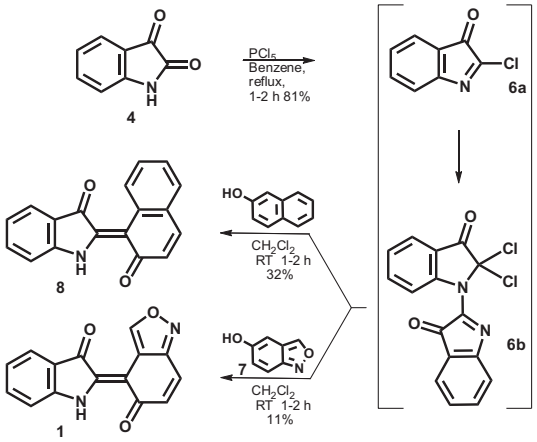


Figure 3. X-ray structure of indigoid **1** (CCDC 919159).



Scheme 1. Indoxyl route to indigoids.



Scheme 2. Chemical synthesis of compound **1**.

all MICs >50 μM. The Low Oxygen Recovery Assay (LORA), designed to test those mycobacteria in the non-replicating phenotype, shows approximately a 10-fold effect.

Single crystal X-ray analysis,[13] of **1** confirmed its novel structure (Fig. 3). Compound **1** is structurally related to indirubin (**2**), a dye-like indole dimer, such that both share a 3-indolone core; however, as indirubin lacked anti-TB activity,[14] both the structural and biological novelty of **1** was established.[15]

In order to determine the viability of compound **1** as an anti-TB agent, we required quantities much greater than the scale-limited cellular system could provide. Initially, synthetic routes were based upon the classical synthesis[16] of indirubin utilizing 3-acetoxylindole (**3**) as the nucleophilic partner and isatin (**4**) as the electrophile (Scheme 1; Eq. 1). In a similar manner, coupling of **3** with the putative quinone **5** (attempted prep of **5** via oxidation[17]

of 5-hydroxybenzisoxazole; Scheme 1; Eq. 2) led to a complex mixture of reaction products none of which was the desired product.

Alternatively, through the use of the polarity inversion concept, the 5-hydroxybenzisoxazole (**7**) was utilized as the nucleophilic partner with the indole-related fragment serving as the electrophile via its corresponding 2-chloroindolen-3-one (**6a**/**b**) (Scheme 2). As previously reported, treatment of isatin (**4**) with phosphorus pentachloride actually leads to dimeric structure **6b**.[18] Although this dimer is isolable via rapid chromatographic purification techniques, its instability to moisture encouraged direct use of the crude material in subsequent reactions. The addition of **6** to other aromatic systems has precedent; for example, reaction of **6** with 2-naphthol afforded adduct **8**[19] which did not exhibit any anti-TB activity. Direct combination of **6** with 5-hydroxybenzisoxazole (**7**) at 25 °C produced **1**.[20] Generally, these products were obtained in a pure state by filtration from the dichloromethane (DCM) reaction mixtures. Further purification, if necessary, was accomplished via silica gel chromatography using methanolic-DCM as eluting solvents.

These compounds exhibit poor water solubility and are unstable in the presence of secondary amines. The possibility of this chemical instability being related to the poor metabolic stability[21] is presently under investigation. Efforts to modify the solubility and stability characteristics and expand the structure–activity relationship is presently underway using new chemical approaches for functionalization. The excellent potency, ease of access, and low molecular weight of this novel anti-TB hit provides the encouragement for these efforts.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.11.024.

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11. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 6.76 (d, 1H, J = 9.85 Hz), 7.11 (dt, 1H, J = 7.38, 7.59 Hz), 7.49 (d, 1H, J = 7.79 Hz), 7.62 (dt, 1H, J = 7.38, 7.59 Hz), 7.70 (d, 1H, J = 7.38 Hz), 7.90 (d, 1H, J = 9.85), 10.06 (s, 1H), 12.40 (br s, 1H). ^{13}C NMR (400 MHz, DMSO-d_6) δ ppm 100.59, 112.16, 114.08, 119.00, 122.82, 124.82, 128.14, 134.10, 137.27, 140.74, 151.59, 153.60, 156.66, 187.87, 188.89. HRMS [ESI] calculated for $C_{15}H_8O_3N_2$ (M+H$^+$) 265.0608; found 265.0603.
12. Several structures were proposed as possible matches for the 1-D and 2-D NMR data for the isolate, which included the correct structure; G. Pauli, D. Lankin; Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago 833 S. Wood Street, Chicago, IL 60612.
13. For X-ray data, see Supplementary data. The author has deposited atomic coordinates for 1 with the Cambridge Crystallographic Data Centre and allocated the deposition number: CCDC 919159. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 lEZ, U.K.
14. Indirubin, indigo, and isoindigo were tested under standard conditions and found to show MICs of >32 μM, >32 μM, and 27 μM, respectively, against *M.tb*.
15. SciFinder (Chemical Abstracts Service) and Reaxys (Elsevier Properties) were analyzed via substructure, term, and patent searches.
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17. Attempted oxidation of 5-hydroxybenzoisoxazole afforded 76% of the putative quinone. ^{1}H NMR ($CDCl_3$) δ ppm 9.06 (s, 1H), 7.73 (d, 1H, J = 8.0 Hz), 6.74 (d, 1H, J = 8.0 Hz). Mass spectral analysis showed only a dimer signal [M+H] = 299 rather than the parent [M+H] = 150.
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20. A sample (25 mg) was purified for an analytical sample via silica gel chromatography using DCM as eluent to give 9 mg (11%) of the desired product, **1** whose ^{1}H, ^{13}C NMR data, HRMS and HPLC data agreed in total with material obtained from the incubation.[11]
21. Unpublished results, L.L. Klein, V. Petukhova; Treatment of **1** with secondary amines such as diethylamine, piperidine at room temperature led to slow loss of starting material and formation of blue-green products which are, as yet, unidentified.
22. (a) Baeyer, A. *Chem. Ber.* **1881**, *14*, 1741; (b) McClay, K.: Wan, B.; Wang, Y.; Cho, S.; Yu, J.; Santarsiero, B.; Mehboob, S.; Johnson, M.; Franzblau, S.; Steffan, R. US patent US2011/0082180 A1, 2013; *Appl. Microbiol. Biotechnol.* **2013**, *97*, 7151.