The Abyssomicin C family as *in vitro* inhibitors of *Mycobacterium tuberculosis*

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SUMMARY

The antimycobacterial efficacy of the abyssomicin C family of natural products, in addition to a key synthetic intermediate, has been investigated given their reported inhibition of *Bacillus subtilis p*-aminobenzoate biosynthesis. The naturally occurring (−)-abyssomicin C and its atropisomer were found to exhibit low micromolar growth inhibition against the relatively fast-growing and non-virulent *Mycobacterium smegmatis* and the vaccine strain *Mycobacterium bovis* BCG, while their antipodes were slightly less active. (−)-Abyssomicin C and its atropisomer were particularly efficacious against *Mycobacterium tuberculosis* H37Rv, exhibiting MIC values of 3.6 and 7.2 μM, respectively. More specifically, (−)-abyssomicin C was bactericidal. This complex natural product and its analogs, thus, hold promise as chemical tools in the study of *M. tuberculosis* metabolism.

Keywords

Mycobacterium tuberculosis; Abyssomicin; *p*-Aminobenzoate metabolism

Tuberculosis is a global epidemic, claiming nearly 2 million lives and infecting more than 9 million people per annum.[1] The current front-line therapy consists of isoniazid, pyrazinamide, rifampicin, and ethambutol - drugs that arose from discovery efforts more than forty years old. The challenges in tuberculosis drug discovery set by drug resistance, mycobacterial persistence, and latency are significant.[2] The rates at which these hurdles, especially that of drug resistance,[3] have grown have dramatically outpaced the development of new tuberculosis therapies. While drug candidates such as TMC207[4] and PA-824[5] are progressing through clinical trials, the need is evident for new molecules that modulate novel mycobacterial targets.

The natural product (−)-abyssomicin C is a structurally intriguing polyketide-like molecule initially discovered with activity against the *para*-aminobenzoate (*p*ABA) biosynthetic pathway in *Bacillus subtilis*.[6,7] More specifically, (−)-abyssomicin C (compound **1** in Figure

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1) was found to inhibit the biosynthesis of *p*ABA from chorismate. Further investigation by the authors demonstrated the antibacterial activity of (−)-abyssomicin C against a number of Gram-positive bacteria, including clinical isolates of multi drug-resistant *Staphylococcus aureus*. We have previously reported the total synthesis[8] of (−)-abyssomicin C and what was eventually identified by Nicolaou and co-workers as (−)-*atrop*-abyssomicin C.[9] It is interesting to note that (−)-*atrop*-abyssomicin C was later shown to not only be present in the original isolates from *Verrucosispora* strain AB-18-032, but to be the predominant abyssomicin by quantity.[10] We have tested these compounds to determine their efficacy against *Mycobacterium tuberculosis* (*Mtb*). The genes involved in the mycobacterial *p*ABA biosynthesis from chorismate had been located,[11] and data existed to support the essential nature of *p*ABA biosynthesis.[12–14] Importantly, human biosynthesis of *p*ABA is non-existent. Intriguingly, no reported efforts to specifically target mycobacterial *p*ABA biosynthesis had been disclosed in the literature. Thus, the potential for a small molecule that inhibited mycobacterial *p*ABA biosynthesis appeared to hold significant promise in further probing *Mtb* metabolism, with a long-term goal of seeding the discovery of novel antituberculars.

We set out to examine the antimycobacterial efficacy (Table 1) of not only (−)-abyssomicin C, but a larger "abyssomicin family." This family (Figure 1) includes the naturally occurring (−)-abyssomicin C (**1**) and its (+)-antipode (**2**), (−)-*atrop*-abyssomicin C (**3**) and its (+)-antipode **4**, and the so-called Diels–Alder adduct precursors **5** and **6** that were central to our first reported synthesis of the natural product,[8] and have also been reported in the literature by Snider[15] and Couladouros.[16] Initially, these compounds were tested against the non-virulent, faster-growing *Mycobacterium smegmatis* mc[2]155.[17] Experiments with this strain and other mycobacteria were performed with the microplate Alamar Blue assay, as reported by Franzblau.[18] (−)-Abyssomicin C and its atropisomer **3**, in particular, showed modest activities with liquid culture MIC values of 29 and 58 μM, respectively. The respective antipodes **2** and **4** displayed diminished efficacy (MIC = 110 μM). The overall activity trends in *M. smegmatis* held when examining the compound family against *Mycobacterium bovis* BCG. (−)-Abyssomicin C was the most potent compound, with a liquid culture MIC of 7.2 μM and its atropisomer was twofold less potent. The corresponding antipodes of **1** and **3** are 8- and 2-fold less efficacious, respectively, perhaps pointing to some specificity in mode of action. The Diels–Alder adducts **5** and **6** were again much less potent, connoting the importance of structural features other than the presence of the α,β-unsaturated carbonyl systems in driving whole-cell efficacy.

The structure–activity relationships (SAR) against *M. smegmatis* and *M. bovis* BCG are indicative of definite structural requirements for efficacy. Clearly, abyssomicins **1** and **2** and their respective atropisomers **3** and **4** are significantly more active than the Diels–Alder adducts **5** and **6**. This points toward the importance of the oxabicyclic ring, which may serve as a conformational constraint, and the secondary hydroxyl. We observe twofold differences in MIC values between **1** and atropisomer **3**, which are small but may be attributed to the positioning of the potentially reactive α,β-unsaturated enone in the 11-membered macrocyclic ring. Nicolaou and co-workers reported **3** to be a better Michael acceptor (under 1,4-reduction conditions with hydride reagent) than **1**, which they proposed to be responsible for a small (15 μM vs. 20 μM) MIC differential against methicillin-resistant *S. aureus*.[19] Finally, it is noteworthy that the naturally occurring enantiomer of (−)-abyssomicin **1** is 4–8 times more active than its antipode **2**, supporting inhibition through a specific binding event.

The *Mtb* H37Rv strain was utilized in liquid cultures to assess the MIC values of the two most potent compounds **1** and **3**. The MIC of (−)-abyssomicin C was 3.6 μM and the MIC of its atropisomer **3** was 7.2 μM. Cultured *Mtb* H37Rv (OD$_{600}$ ~ 0.5) were exposed to ca. 4×,

40×, and 80× multiples of the MIC of **1**, incubated for 18 h, and then plated and serially diluted in duplicate. Incubation with the compound resulted in bacterial death (data not shown). Bacterial outgrowth after ca. 6 weeks was only observed with the first dilution of the lowest concentration of **1** (4×; 14 μM), and at a visibly lower number of colony-forming units as compared to the control. At higher concentrations of **1**, no visible bacterial outgrowth at any of the dilutions was noted, in stark contrast to the control. While there was not enough compound available to test the MBC formally, we did observe bactericidal activity.

While these abyssomicins are ca. 10–20 fold less active against *Mtb* H37Rv than the front-line drug isoniazid on a molar basis, they compare favorably to second-line drugs such as ethionamide (MIC = 18 μM).[20] With very limited amounts remaining of these abyssomicins, we were unable to determine if this promising efficacy is extended to other members of the *Mycobacterium* genus. It should be noted that Bister and colleagues reported a lack of efficacy of **1** against *Mycobacterium phlei* DSM 750 on complex medium.[7]

In liquid culture, we investigated whether antimycobacterial activity is rescued by added *p*ABA. We began by examining whether *p*ABA added to liquid cultures of *M. bovis* BCG could reverse growth inhibition of the abyssomicins. It has been reported that the efficacy of (−)-abyssomicin C versus *B. subtilis* was reversed by a 5 mM solution of *p*ABA.[7] At concentrations ranging from 2.5 to 20 mM, *p*ABA was observed, through the Alamar Blue assay, to not be toxic to the mycobacteria. As a control experiment, *p*ABA (at ca.1.5 times the concentration of drug by weight) was found to reverse the inhibition of *M. bovis* BCG by *p*-aminosalicylate (PAS),[21] in sync with the original experiments with *Mtb* by Youmans and colleagues.[22] In contrast, upon adding *p*ABA (at seven concentrations from 27 μM to 5 mM) to *M. bovis* BCG exposed to (−)-abyssomicin C (at 7.2, 14, or 29 μM), we failed to observe reversal of mycobacterial growth inhibition.

The antimycobacterial activity of the abyssomicin C family has been established through liquid culture MIC determinations. In particular, bactericidal (−)-abyssomicin C and its atropisomer have demonstrated single-digit micromolar MICs versus *M. tuberculosis* H37Rv. The biological data encourage studies to explore the SAR around this family of molecules while further probing the mechanism of action of these natural products in order to better understand *M. tuberculosis* metabolism.

1. Materials and methods

1.1. Synthesis of abyssomicin C family members

The syntheses of the family members **1**–**6** have been described previously.[8,23]

1.1.1. Determination of MIC values for abyssomicin C family members versus M. smegmatis mc²155, M. bovis BCG, and M. tuberculosis H37Rv—A drug susceptibility assay in 96-well plate format by Alamar Blue was modified from Franzblau and co-workers.[18] The respective bacteria were grown to mid-log phase (OD_{600} = 0.5) and diluted to OD_{600} = 0.001 or 0.003 for *M. smegmatis* or *M. bovis* BCG/*M. tuberculosis*, respectively. 200 μL of sterile deionized water was added to all outer-perimeter wells of sterile 96-well plates to minimize evaporation of the medium in the test wells during incubation. Compounds were dissolved in DMSO and subsequent serial dilutions were performed in 7H9 complete medium.100 μL of serial diluted drug solutions were added to the wells in columns 3–10, and 100 μL 7H9 medium were added in columns 2 and 11. 100 μL of mycobacterial inoculum was added to the wells in rows B to G in columns 2–9 by using a multichannel pipette (yielding a final volume of 200 μL per well). Thus, the wells in column 2 served as drug-free (inoculum-only) controls, the wells in column 10 served as

drug-only controls and the wells in column 11 served as medium-only controls. The plates were sealed with breathable film (VWR) and were incubated at 37 °C for 24 h for *M. smegmatis* or 4 days for *M. bovis* BCG/*M. tuberculosis*. Alamar Blue (Biosource) reagent was added to every well. The plates were reincubated at 37 °C for 6 h for *M. smegmatis* or 24 h for *M. bovis* BCG/*M. tuberculosis*, and the colors of all wells were recorded. A blue color in the well was interpreted as no growth, and a pink color was scored as growth. A few wells appeared violet after 24 h of incubation, but they invariably changed to pink after another day of incubation and thus were scored as growth (while the adjacent blue wells remained blue). The MIC was defined as the lowest drug concentration that prevented a color change from blue to pink.

1.1.2. Determination of bactericidal activity of (−)-abyssomicin C versus M. tuberculosis H37Rv—*M. tuberculosis* H37Rv was grown at 37 °C to log phase in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol, 0.05% Tween-80, 0.5% bovine serum albumin, 0.2% dextrose, and 0.085% sodium chloride. A 1 mL aliquot was removed and incubated in the presence of (−)-abyssomicin C, at concentrations of 5, 50, and 100 μg/mL, or in the absence of compound as a control, for 18 h. Post incubation, the cultures were serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, and plated in duplicate. The plates were 7H10 agar (Difco) supplemented with oleic acid, albumin, dextrose and catalase. The plates were examined for the outgrowth of bacterial colonies after 21 days and then again after a total of ca. 6 weeks.

1.1.3. Evaluation of pABA effect on PAS treated M. bovis BCG—The Alamar blue assays were conducted as described above. *p*ABA solutions, at concentrations ranging from 0.1 to 6 μg/mL, in ethanol/media and PAS (MIC ~ 0.03 μg/mL; concentrations ranging from 0.045 to 4 μg/mL) were added to *M. bovis* BCG. Bacteria were cultured for 4 days and followed by addition of Alamar blue to evaluate their growth.

1.1.4. Evaluation of pABA effect on (−)-abyssomicin C treated M. bovis BCG— A similar procedure was utilized as described for the rescue of PAS treated *M. bovis* BCG by *p*ABA. At concentrations ranging from 2.5 to 20 mM, *p*ABA solutions in ethanol/media were added to cultured *M. bovis* BCG and evaluated after 4 days by the addition of Alamar blue to check for *p*ABA toxicity. Similarly, *p*ABA solutions, at concentrations ranging from 27 μM to 5 mM, in ethanol/media and (−)-abyssomicin C, at the concentration of 7.2, 14, or 29 μM, were added to *M. bovis* BCG culture. Bacteria were cultured for 4 days and followed by addition of Alamar blue to evaluate their growth.

Acknowledgments

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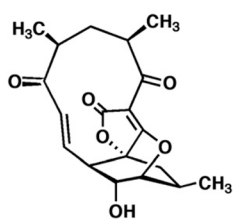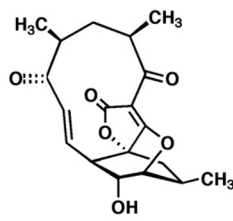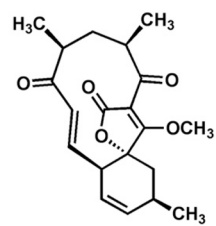
Figure 1.
Chemical structures of the studied abyssomicin C family.

Table 1

Minimum inhibitory concentration (MIC) data for (−)-abyssomicin C family and drug controls.

Compound	MIC* in *M. smegmatis* mc²155 in μg/ml (μM)	MIC* in *M. bovis* BCG in μg/ml (μM)	MIC* in *M. tuberculosis* in μg/ml (μM)
rifampicin	1.2 (1.5)	0.00064 (0.00078)	ND
isoniazid	2.5 (18)	0.16 (1.2)	0.040 (0.29)
(−)-abyssomicin C	10 (29)	2.5 (7.2)	1.2 (3.6)
(+)-abyssomicin C	40 (110)	20 (58)	ND
(−)-*atrop*-abyssomicin C	20 (58)	5.0 (14)	2.5 (7.2)
(+)-*atrop*-abyssomicin C	40 (110)	10 (29)	ND
(−)-Diels-Alder adduct	>80 (>230)	40 (120)	ND
(+)-Diels-Alder adduct	>80 (>230)	40 (120)	ND

*The values reported represent the mean of three or more independent determinations.