Antitubercular prodrugs

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Abyssomicins from a South China Sea deep-sea sediment *Verrucosispora* sp.: Natural thioether Michael addition adducts as antitubercular prodrugs

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Tuberculosis (TB) is a leading cause of death in the world today, exacerbated by the prevalence of multi (MDR-TB), extensively (XDR-TB) and totally (TDR-TB) drug resistant strains. Despite the threat to human health, existing front-line TB therapeutics remain constrained to a handful of vintage antibiotics prescribed in a combinatorial format to achieve efficacy. The current shortfall in antitubercular drugs demands urgent attention, to develop new antibiotics effective against all strains of tuberculosis.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

In responding to this challenge, we screened a library of marine-derived bacteria (4024) and fungi (533) for growth inhibitory activity against Bacille Calmette Guerin (BCG), an attenuated strain of the bovine tuberculosis bacillus *Mycobacterium bovis*.[1] BCG serves as a non-pathogenic but nevertheless valuable screening surrogate for the far more hazardous and pathogenic *M. tuberculosis*. Our screening detected 27 (0.6%) extracts with anti-BCG activity, including a South China Sea deep-sea (–2733 m) sediment-derived actinomycete, *Verrucosispora* sp. (MS100128). Bioassay-directed fractionation of a large scale (21 L) culture of MS100128 yielded three new members of the rare class of abyssomicin polyketide, J (**1**), K (**2**) and L (**3**), and four known[2] abyssomicins, B (**4**), C (**5**), D (**6**) and H (**10**) (Figure 1). All structures were assigned by detailed spectroscopic analysis, with the known abyssomicins **4**–**6** and **10** documented in the Supporting Information, and the new abyssomicins **1**–**3** discussed below.

The abyssomicins B–D (**4**–**6**) were first reported in 2004[2a,2b] from a deep-sea (abyssal) *Verrucosispora* sp. (AB-18-032), since proposed as the new taxon *Verrucosispora maris* sp. nov.[3] A subsequent 2007 reinvestigation[2c] of AB-18-032 described three additional co-metabolites in the form of abyssomicins G (**9**) and H (**10**), and *atrop*-abyssomicin C (**8**). The deep-sea status of the abyssomicin chemotype was challenged by a 2007 report[4] of abyssomicin E (**7**) from a Senegalese soil *Streptomyces* sp. (HK10381), a 2010 report[5] of abyssomicin I (**11**) from a Mexican soil *Streptomyces* sp. (CHI39), and a 2011 report[6] of *ent*-homoabyssomicins A and B from a German soil *Streptomyces* sp. (Ank 210). Ongoing interest in the synthesis, biosynthesis and pharmacology of the abyssomicins has been fuelled by the observation that C (**5**), an inhibitor of *p*-aminobenzoic acid (*p*-ABA) biosynthesis (a putative molecular target for next-generation antibiotics),[6] exhibits promising anti-MRSA[2b] and antitubercular[7] activities.

HRESI(+)MS measurements on J (**1**) revealed an adduct ion ($[M+Na]^+$) consistent with a molecular formula of $C_{38}H_{46}O_{12}S$ (Δmmu +0.4). Examination of the ^{13}C NMR ($CDCl_3$) data (Supporting Information, Table S1) revealed only 19 carbon resonances, requiring a level of symmetry. Further analysis of the NMR data revealed a high degree of similarity with those previously reported for D (**6**),[8] with the most significant difference being the replacement of methylene C-9 (δ_H 2.00/1.54, δ_C 26.1) in **6** with a thiomethine (δ_H 3.83, δ_C 41.1) in **1**. Detailed analysis of 2D COSY, HMBC and ROESY NMR correlations (Supporting Information, Figure S1e) confirmed a common pentacyclic core between **1** and **6**, with an HMBC correlation from H-9 (δ_H 3.83) to C-9 (δ_C 41.1) suggesting dimerization through a C-9 to C-9′ thioether bridge. A C-9 β thioether configuration was assigned by comparing experimental data for H-9 ($J_{8,9}$ 10.8 Hz; $J_{9,10}$ 3.6 Hz) with calculated values for energy minimized (MM2) *in silico* models of α ($J_{8,9}$ 6–7 Hz; $J_{9,10}$ <1 Hz) and β ($J_{8,9}$ 7–8 Hz; $J_{9,10}$ 3–4 Hz) thioethers,[4] and with the

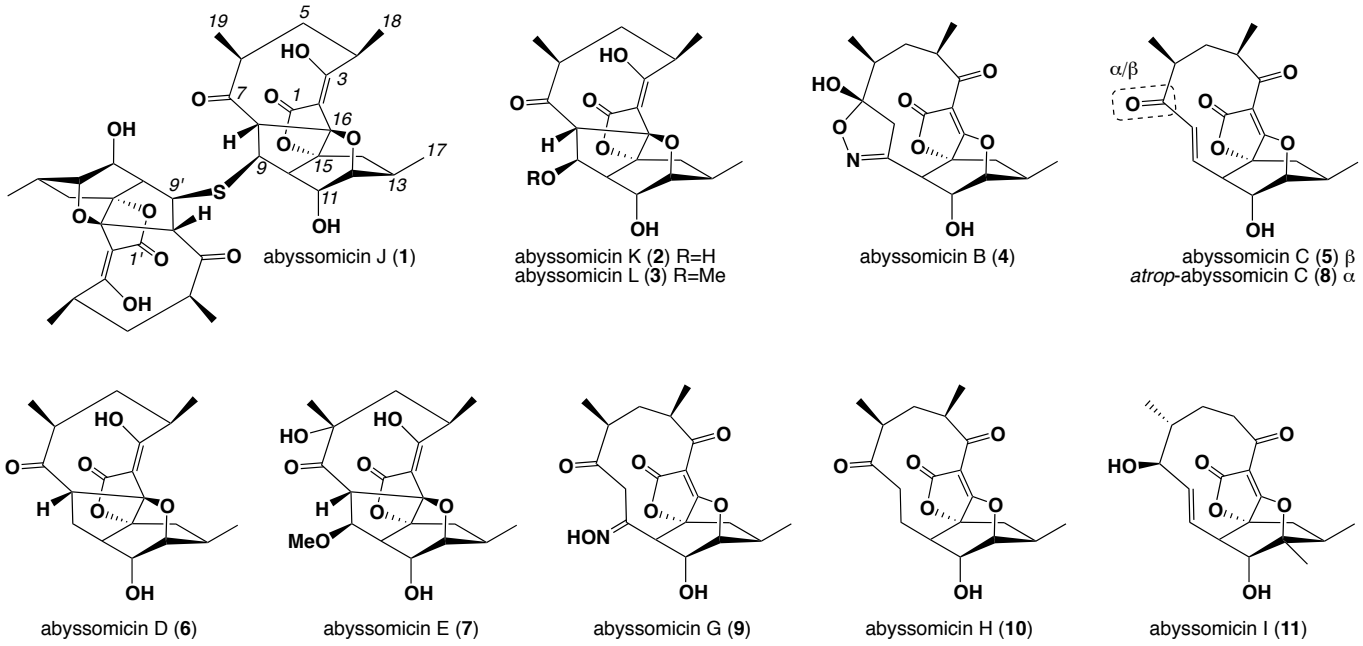

Figure 1. Structure of abyssomicins **1–11**

literature data for E (**7**) ($J_{8,9}$ 8 Hz; $J_{9,10}$ 4 Hz). Thus the complete relative stereostructure for J (**1**) could be assigned as shown in Figure 1.

HRESIMS measurements on K (**2**) and L (**3**) revealed adduct ions consistent with molecular formulae (**2**, $C_{19}H_{24}O_7$, Δmmu +0.8; **3**, $C_{20}H_{26}O_7$, Δmmu +0.5) attributed to H_2O and MeOH Michael addition adducts of C (**5**), respectively. In support of this hypothesis, the NMR ($CDCl_3$) data for **2** and **3** (Supporting Information, Tables S2 and S3) proved to be very similar to those of **1**, with significant differences being limited to replacement of the thiomethine in **1** (δ_H 3.83; δ_C 41.1) with a hydroxymethine in **2** (δ_H 4.81 and δ_C 67.7), and a methoxymethine in **3** (δ_H 4.43 and δ_C 76.7; OMe δ_H 3.30 and δ_C 58.2). The 2D NMR data for **2** and **3** (Supporting Information, Figures S2e and S3e) also revealed diagnostic correlations supportive of the proposed structures.

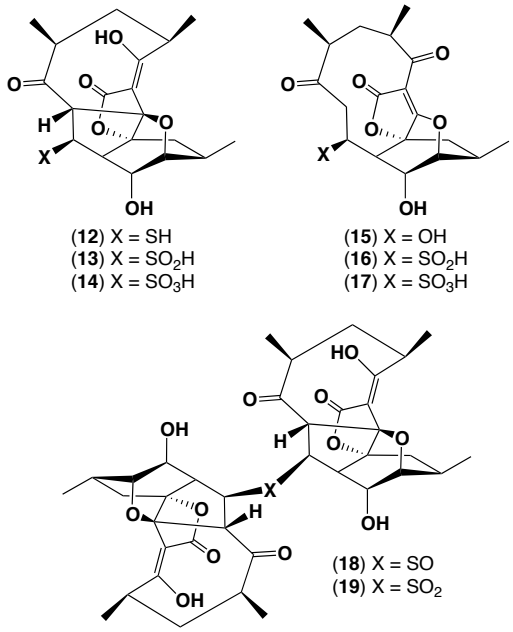

Figure 2. Abyssomicin semi-synthetic analogues **12–19**

Absolute configurations were assigned to **1**–**3** on biogenetic grounds, given that they are co-metabolites of **4**–**6** and **8**, all of which have been assigned to a common antipodal series.[2] Also supportive of this biosynthetic relationship, we demonstrated that **1**–**3** could be formed as Michael addition adducts of **5**. For example, a sample of **5** exposed to 0.1 M Na_2S resulted in near quantitative conversion to three products. The major product was identified as J (**1**), while the minor products were identified as the intermediate thiol **12** and its oxidation product, the sulfonic acid **14** (Figure 2). By contrast, exposure of **5** to 0.05 M NaOH returned only a single product identified as K (**2**), while exposure to 0.5 M TFA lead to a mixture of K (**2**) and a new isomer **15** (Figures 2 and 3). The structure for **15** was assigned by detailed spectroscopic analysis (Supporting Information, Figure S10c), and its formation rationalized as an acid-mediated H_2O Michael addition adduct of **5**, lacking the cascading second intramolecular Michael addition needed to form the caged-carbon skeleton of **2**. Exposure of **15** to 0.05 M NaOH resulted in quantitative conversion to K (**2**), while exposure of **5** to 0.5 M TFA in MeOH resulted in facile conversion to a single product identified as L (**3**) (Supporting Information, Figures S17 and S18). Significantly, this latter transformation proceeded (albeit at a far slower rate) without exposure to acid, during handling/storage of C (**5**) in MeOH. The observations listed above confirm that **1**–**3** are biosynthetically related to and are likely derived from **5**, and reveal for the first time an acid-mediated strategy capable of accessing a new abyssomicin scaffold (i.e. **15**).

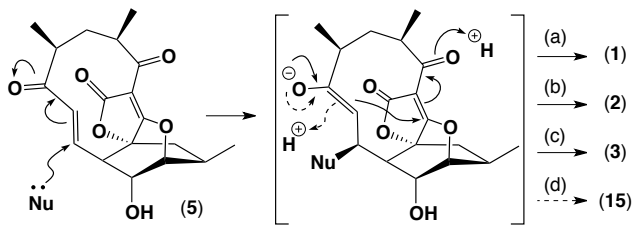

Figure 3. Michael addition on **5** to yield the adducts **1**–**3** and **15**. (a) Nu = Na_2S; (b) Nu = NaOH; (c) Nu = MeOH; (d) Nu = H_2O/H^+

To address the possibility that one or more of **1**–**3** were handling artifacts, a fresh EtOAc extract of a small-scale culture of *Verrucosispora* sp. (MS100128) was prepared and subjected to HPLC-DAD-MS analysis, avoiding exposure to acid, base and hydroxylic solvents. This analysis detected all of **1**–**3**, as well as the metabolites **4**–**6** and **10**, confirming their natural product status (Supporting Information, Figure S13).

Among the known abyssomicins, only the atropisomers **5** and **8** have been attributed anti-TB properties – against the fast growing non-pathogenic *M. smegmatis*, the TB surrogate BCG, and *M. tuberculosis* (H37Rv)[7] – emphasizing the critical structure activity importance of the Michael acceptor enone moiety.[2a] Given this history, we were initially surprised to discover that, along with **5**, the thioether **1** was the principle anti-TB agent in *Verrucosispora* sp. (MS100128). Indeed, the anti-BCG activities for **1** (MIC 3.125 μg/mL) compared favorably with those of **5** (MIC 6.25 μg/mL) (Supporting Information, Table S11). To explain this apparent departure from the established Michael acceptor pharmacophore paradigm, we hypothesized that **1** was a natural prodrug, undergoing *in situ* reverse Michael addition to deliver an abyssomicin anti-TB antibiotic (presumably **5** and/or **8**). As **1** was stable during isolation and handling, we speculated that the reverse Michael addition process required activation by *in situ* enzymatic oxidation (i.e. P450). This view was based in part on a review of the literature, which confirmed that P450 enzymes can transform thioethers through sulfoxides to sulfones, and that sulfones can undergo a reverse Michael addition. For example, the synthetic vasodilator thioether flosequinan sulfide is transformed by rat and human liver P450 enzymes to its sulfoxide and sulfone,[9] while cancer cell enzymatic oxidation of synthetic thioether prodrugs of brefeldin yield sulfones, which in turn undergo rapid reverse Michael addition to deliver brefeldin.[10] In yet another example of sulfone mediated reverse Michael addition, the semi-synthetic sulfone antibiotic dalfopristin undergoes metabolism in human plasma to the natural product Michael acceptor pristinamycin IIA.[11] These examples notwithstanding, based on our hypothesis **1** would represent the first example of a natural thioether adduct (dimer or otherwise) that serves as a prodrug for its associated Michael acceptor.

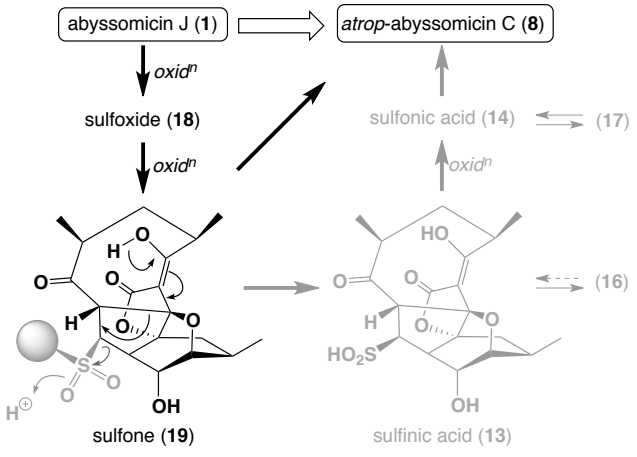

Figure 4. Oxidative activation of the prodrug abyssomicin J (**1**), leading to *atrop*-abyssomicin C (**8**)

To test this hypothesis *in vitro*, a MeCN/H_2O solution of **1** was treated with the oxidizing reagent Oxone (as a chemical P450 surrogate[12]) to yield four products identified by spectroscopic analysis as the sulfoxide (**18**), sulfone (**19**), sulfonic acid (**14**) and *atrop*-abyssomicin C (**8**). The sulfoxide (**18**) identified by HPLC-DAD-HRESI(+)MS, proved unstable to handling, undergoing rapid air oxidation to the sulfone (**19**). Likewise, while the sulfone **19** was sufficiently stable for ^{1}H NMR analysis, when handled in MeCN at 40 °C (1 h) it underwent a reverse Michael addition to yield **8**, together with four minor intermediates. The latter products were identified by HPLC-DAD-ESI(+)MS as the sulfinic acids (**13** and **16**), and sulfonic acids (**14** and **17**). Although a sample of **14** could be purified and characterized by ^{1}H NMR, even short (10 min) exposure to MeCN at r.t. led to equilibration to a **14**:**17** mixture, while heating to 70 °C (12 h) transformed this mixture to **8**. Of note, following oxidative activation by Oxone to form the sulfoxide **18**, all subsequent transformations leading to **8** could be accommodated by air oxidation and inherent chemical reactivity. Based on these observations, a plausible mechanism for the transformation of **1** into **8**, inclusive of the intermediates **13**–**14** and **16**–**19**, is illustrated in Figure 4 (Supporting Information Figures S19–21). In this mechanism, the formation of a single atropisomer **8** (i.e. no trace of **5**) was particularly interesting and prompted closer examination.

To better understand the chemical and biological significance of atropisomer selectivity in the reverse Michael addition transformation of **1** to **8**, we carried out analytical studies on the Michael acceptors **5** and **8**. Earlier workers have demonstrated,[13] and we have independently confirmed (Supporting Information Figures S12a and S12b), that **5** and **8** equilibrate under anhydrous acid-mediated conditions (e.g. $CDCl_3$). Importantly, as this equilibration was not evident under non-anhydrous *in vivo* conditions, we reasoned that **5** and **8** acted independently as anti-TB agents, with an antibiotic potency correlated to their respective strengths as Michael acceptors. Building on this hypothesis, and having established **8** as the sole atropisomer arising from reverse Michael addition, we reasoned that **8** was optimally configured as a superior Michael acceptor (compared to its atropisomer **5**). To test this hypothesis, separate MeCN/H_2O solutions of **5** and **8** were exposed to 0.1 M TFA to initiate an acid-mediated Michael addition leading to **15**. A time course (18 h) analysis clearly established **8** as a far more potent Michael acceptor (Figure 5), consistent with its prior history as a superior antibacterial agent.[2c,13] The high Michael acceptor potency of **8** also suggested a low *in vivo* half-life. Consistent with all of the above, we detected **8** in BCG cells exposed to **1**.

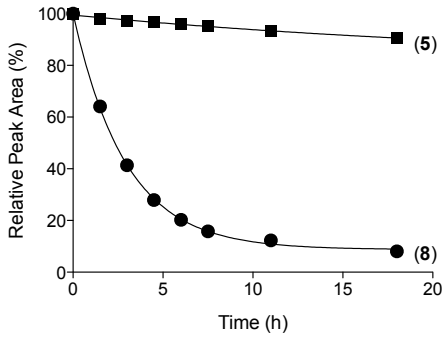

Figure 5. HPLC (254 nm) analysis of abyssomicin C (**5**) and *atrop*-abyssomicin C (**8**) exposed to 0.1 M TFA in 90% H_2O/MeCN at 40 °C.

In summary, our investigations into the anti-TB properties of the South China Sea deep-sea *Verrucosispora* sp. (MS100128) lead to the isolation, identification and anti-TB evaluation of new (**1**–**3**) and

known (**4**–**6, 10**) abyssomicins. Structures were assigned to **1**–**3** on the basis of detailed spectroscopic analysis, biosynthetic considerations, mechanistic studies, and semi-synthesis from the co-metabolite **5**. Detailed analytical studies into abyssomicin Michael addition chemistry informed our understanding of the chemical reactivity, stability and anti-TB properties of this rare structure class. We established **8** as a far more potent Michael acceptor than **5**, and used this to rationalize its superior antibacterial properties. We transformed **5** into the Michael adduct **1**, and used both *in vitro* and *in vivo* analytical studies to demonstrate that **1** can act as a prodrug, responding to oxidative activation to selectively deliver the anti-TB antibiotic **8**.

Our studies make a contribution beyond the specifics of the abyssomicin pharmacophore by drawing attention to the possible utility of thioether Michael addition adducts as a means to stabilize highly reactive Michael acceptors, thereby enhancing bioavailability and improving therapeutic potential. The thioether Michael adduct prodrug concept, inspired by abyssomicins from deep below the South China Sea, offers a promising new approach to "chemically package" bioactive Michael acceptors, improving their chances of being developed into clinically useful drugs.

Experimental Section

Full details of microbial cultivation and extraction, isolation, purification and structure elucidation of compounds, bioassays and analytical and mechanistic studies are provided in the Supporting Information.

Microbial Isolation: Strain MS100128 was isolated using oatmeal agar from a sediment sample collected in April 2010 from the South China Sea (20° 9.795′ N, 118° 18.124′ E) at 2733 m below sea level and was identified as a *Verrucosispora* sp. using 16S rRNA gene sequence analysis (GenBank accession no. JQ724543). The strain has been preserved at the China General Microbiological Culture Collection Center (accession no. 5847).

Abyssomicin J (1): white powder; $[\alpha]^{24}_{D}$ +188 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 296 (3.83), 267 (4.13), 202 (4.01) nm; NMR data, see Supporting Information Table S1; HRESIMS *m/z* 749.2606 $[M+Na]^+$ (calcd. for $C_{38}H_{46}O_{12}SNa$, 749.2602).

Abyssomicin K (2): white powder; $[\alpha]^{24}_{D}$ +48 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 296 (3.32), 264 (3.71), 202 (3.60) nm; NMR data, see Supporting Information Table S2; HRESIMS *m/z* 365.1603 $[M+H]^+$ (calcd. for $C_{19}H_{25}O_7$, 365.1595).

Abyssomicin L (3): white powder; $[\alpha]^{24}_{D}$ +101 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 296 (3.40), 265 (3.74), 204 (3.54) nm; NMR data, see Supporting Information Table S3; HRESIMS *m/z* 377.1611 $[M-H]^-$ (calcd. for $C_{20}H_{25}O_7$, 377.1606).

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Entry for the Table of Contents (Please choose one layout)

Layout 1:

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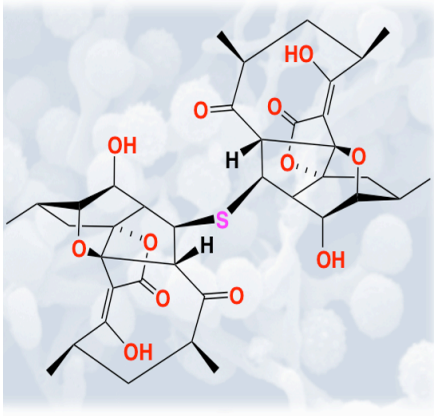

A Chinese deep-sea *Verrucosispora* sp. yielded three new abyssomicins J–L. The dimeric thioether J represents a unique example of a masked Michael acceptor, with its anti-TB properties attributed to *in situ* oxidative activation of a twin reverse Michael addition cascade to *atrop*-abyssomicin C. We present analytical studies that provide mechanistic insights into the biosynthesis, biomimetic synthesis, stability and biological mechanism of action of abyssomicins, and that inform our understanding of, and prospects for the developing, Michael acceptor based drugs.