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Article

Synthesis and Evaluation of Marine Natural Product-Inspired Meroterpenoids with Selective Activity toward Dormant *Mycobacterium tuberculosis*

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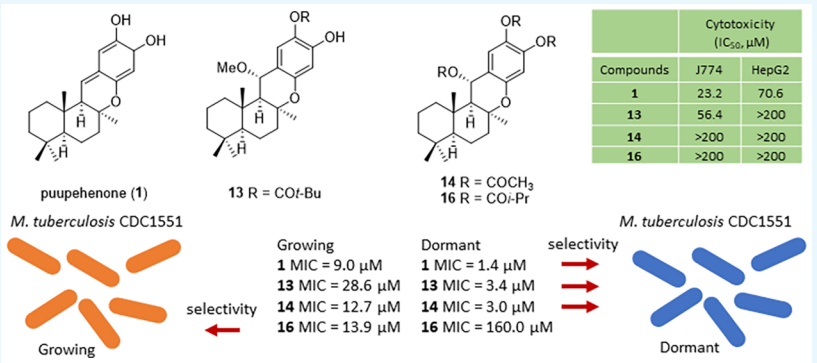

ABSTRACT: Tuberculosis is a disease caused primarily by the organism *Mycobacterium tuberculosis* (*Mtb*), which claims about 1.5 million lives every year. A challenge that impedes the elimination of this pathogen is the ability of *Mtb* to remain dormant after primary infection, thus creating a reservoir for the disease in the population that reactivates under more ideal conditions. A better understanding of the physiology of dormant *Mtb* and therapeutics able to kill these phenotypically tolerant bacilli will be critical for completely eradicating *Mtb*. Our groups are focusing on characterizing the activity of derivatives of the marine natural product (+)-puupehenone (**1**). Recently, the Rohde group reported that puupehedione (**2**) and 15-α-methoxypuupehenol (**3**) exhibit enhanced activity in an in vitro multi-stress dormancy model of *Mtb*. To optimize the antimycobacterial activity of these terpenoids, novel 15-α-methoxy- and 15-α-acetoxy-puupehenol esters were prepared from (+)-puupehenone (**1**) accessed through a (+)-sclareolide-derived β-hydroxyl aldehyde. For added diversity, various congeners related to (**1**) were also prepared from a common borono-sclareolide donor, which resulted in the synthesis of *epi*-puupehenol and the natural products (+)-chromazonarol and (+)-yahazunol. In total, we generated a library of 24 compounds, of which 14 were found to be active against *Mtb*, and the most active compounds retained the enhanced activity against dormant *Mtb* seen in the parent compound. Several of the 15-α-methoxy- and 15-α-acetoxy-puupehenol esters possessed potent activity against actively dividing and dormant *Mtb*. Intriguingly, the closely related triisobutyl derivative **16** showed similar activity to **1** in actively dividing *Mtb* but lost about 178-fold activity against dormant *Mtb*. However, the monopivaloyl compound **13** showed a modest 3- to 4-fold loss in activity in both actively dividing and dormant *Mtb* relative to the activity of **1** revealing the importance of the free OH at C19 supporting the potential role of quinone methide formation as critical for activity in dormant *Mtb*. Elucidating important structure−activity relationships and the mechanism of action of this natural product-inspired chemical series may yield insights into vulnerable drug targets in dormant bacilli and new therapeutics to more effectively target dormant *Mtb*.

INTRODUCTION

Antibiotic resistance is a growing global health crisis. Misuse of antibiotics is endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives. Previously curable infectious diseases are becoming untreatable and new drug development has slowed. Tuberculosis (TB) is often thought of as an archaic disease of the past. However, even with established treatments available, TB remains a global health threat leading to ∼1.5 million deaths annually.[1] Prior to the COVID-19 pandemic, TB topped the list of deaths due to a single infectious disease, beating out HIV/AIDS.[1] The World Health Organization (WHO) estimated that there were approximately 484,000 new cases of drug-resistant TB in

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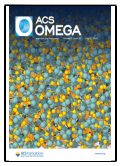

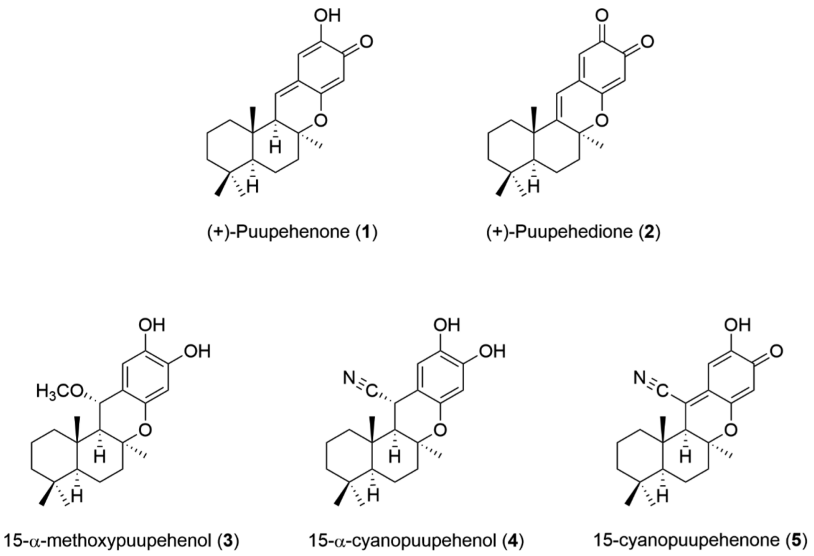

Figure 1. Originally isolated MNPs.

2018.[1] One of the major problems in treating *Mtb* infection is that subpopulations of bacteria can enter a dormant state during treatment of active TB.[2,3] As a result, phenotypically drug-tolerant bacilli are able to persist in the presence of antitubercular drugs, which lays the foundation for the development of resistance. These persistent bacilli are the reason that treatment periods for *Mtb* are 6−9 months or longer. Finding better drugs effective against dormant and drug-resistant *Mtb* is a critical step in reducing the global burden of TB. Highlighting the prolonged lack of investment in the development of new antibiotics, bedaquiline (BDQ), approved in 2012, was the first new drug to be approved for the treatment of multidrug-resistant TB in 40 years.[4] It is notable that BDQ has a novel mechanism of action and inhibits the bacterial adenosine triphosphate (ATP) synthase of mycobacteria.[5] This mechanism of action is effective against both drug-resistant *Mtb* as well as against dormant *Mtb*.[6] Thus, the targeting of dormant *Mtb* which exhibit phenotypic resistance to antibiotics is now considered an important goal for improving treatment times and reducing rates of drug resistance.[2,7]

There are several diverse classes of compounds that have recently been reported to inhibit the growth of *Mtb* including quinazolines, benzothiazoles, benzoimidazoles, oxadiazoles, nitroimidazoles, diarylquinoline, and many five- and six-membered heterocycles.[8] Many of these compounds were discovered through fragment screening on actively replicating bacteria. In the current study, we have made systematic modifications to a previously identified marine natural product (MNP) exhibiting selective activity against dormant *Mtb* with the goal of identifying more potent compounds that may contribute to shorter, more effective treatment regimens for TB.

To exploit the rich chemical diversity of MNPs, we previously conducted the first large-scale screen of MNPs against replicating and dormant *Mtb*.[9] This yielded two meroterpenoid compounds that intriguingly exhibited enhanced potency against non-replicating dormant *Mtb* versus replicating *Mtb*. Our labs are currently focusing on the meroterpenoid (+)-puupehenone (**1**), originally isolated in 1979 from an encrusting yellow sea sponge in Hawaii but subsequently also isolated from sea sponges of the orders *Verongida*, *Dictyoceratida*, *Dendroceratida*, and *Haplosclerida*.[10] (+)-Puupehenone's activity as an antitubercular agent was first reported by El Sayed et al. in 1999. At a concentration of 38.1 μM, (+)-puupehenone (**1**), 15-α-cyanopuupehenol (**4**), and 15-cyanopuupehenone (**5**) (Figure 1), exhibited 99, 96, and 90% inhibition of *Mtb*, respectively.[11] Recently, we made the exciting discovery that puupehedione (**2**) and 15-α-methoxypuupehenol (**3**), metabolic derivatives of puupehenone shown in Figure 1, have minimum inhibitory concentrations (MIC) of 268.4 and 31.3 μM, respectively, against active *Mtb* and MICs of 47.2 and 1.4 μM, respectively, in an in vitro multistress dormancy model of *Mtb*.[9] These compounds are particularly noteworthy because they demonstrate an unusual selectivity for dormant *Mtb* over replicating *Mtb*, pointing to a novel mechanism of action.

The synthesis of these compounds has been carried out by a few groups resulting in unique chemical approaches. Previous routes to make these meroterpenoids used methods including: a regioselective Friedel−Crafts alkylation,[12] an aldol addition-based approach,[13] a Michael-type Friedel−Crafts alkylation on a α,β-enone,[14] and a Diels−Alder approach.[15] Many of these methods form the C-8 epimer of (+)-puupehenone, which is the sterically favored epimer. The C-8 epimer has not been evaluated against *Mtb*.

Intrigued by the activity seen in non-replicating *Mtb*, we sought to define structure activity relationships (SARs) and possibly identify new compounds or prodrugs that may improve potency in both replicating and dormant *Mtb*. One way to improve drug specificity and efficacy is to implement a prodrug strategy. For example, pyrazinamide (PZA) and isoniazid are both prodrugs that are metabolized by *Mtb* to activate them inside the mycobacteria. This illustrates one advantage of prodrugs whereby activation of the drug within the pathogen minimizes potentially deleterious off-target effects on the host. Ester prodrugs of 15-α-methoxypuupehenol were envisioned to add more specificity and hopefully maintain their potent activity. This concept has been demonstrated by the observation that protection of PZA using an ester prodrug strategy restored the activity in strains of *Mtb* that had become resistant to PZA.[16] *Mtb* contains a

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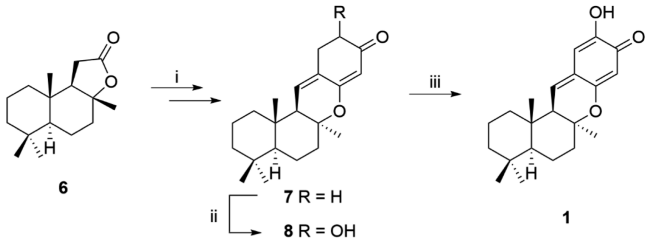
significantly higher number of serine hydrolases by proteome percentage than other common bacteria or even humans, which could cleave the ester group.[17] We employed a previously reported stereospecific route to prepare naturally occurring (+)-puupehenone (**1**). Compound **1** was then modified to make the 15-α-methoxy ester derivatives.

We also employed a separate method to synthesize a library of open-ring compounds with various substitution patterns on the aromatic ring. These compounds were prepared to explore the SAR related to the aromatic motif found in the parent compound. As a point of diversification, we also thought to prepare an additional library of open-ring puupehenol-like compounds using a Suzuki–Miyaura reaction by taking dvantage of a borono-sclareolide terpenoid donor first synthesized by Dixon et al.[18] Alternatively, the boronic acid was coupled with novel quinones allowing for the generation of new tetracyclic compounds that we hoped would mimic the activity of 15-α-methoxy puupehenol.

RESULTS/DISCUSSION

We synthesized five 1,6-conjugated derivatives of (+)-puupehenone with the use of commercially available and inexpensive (+)-sclareolide (**6**) as the key intermediate which is useful for an "atom- and step-economical" synthesis of (+)-puupehenone using chemistry reported by Wu (Scheme 1) with minor

Scheme 1[a]

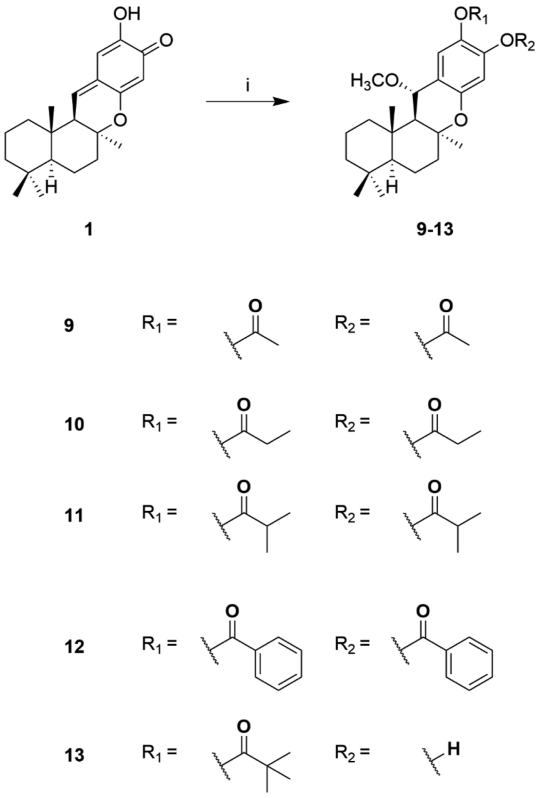


[a]Reagents and conditions: synthesis of (+)-puupehenone: (i) six steps as previously reported, 10% overall; (ii) Davis oxaziridine (2 equiv), KHMDS (1.5 equiv), THF, −78 °C, 4 h, 55%. (iii) As previously reported 60%.

revisions as noted.[13] We treated compound **7** with KHMDS at −78 °C and subsequently reacted it with O_2 and $P(OMe)_3$ to furnish the products **8** and **1** with only 5 and 10% yield, respectively.[13] To improve the yield at that step, we attempted other oxidation methods such as *t*-BuOK/18-crown-6/O_2,[19] *t*-BuOK/*t*-BuOH/O_2,[20] I_2/DMSO,[21] PIDA/KOH/MeOH,[22] NHMDS/camphorsulfonyloxaziridine,[23] and Davis' oxaziridine[24] mediated reaction conditions, based on literature procedures. However, only the Davis' oxaziridine oxidation went well in our hands to afford compound **8** in moderate yield.[25] Treatment of compound **7** with 3-phenyl-2-(phenylsulfonyl)oxaziridine in the presence of KHMDS at −78 °C in THF introduced an α-hydroxyl group and furnished the product **8** in a 55% yield. Due to instability of product **8** observed during isolation by flash column chromatography, the crude product was subjected to further reaction without purification. For the regioselective dehydrogenation of compound **8** to obtain (+)-puupehenone (**1**), we tried several conditions including using Dess–Martin periodinane[26] and bismuth(III)oxide-mediated oxidation[27] conditions. However, none of these methods improved the yield for the desired product. Finally, the α-hydroxylated product **8** was treated with *t*-BuOK in *tert*-butyl alcohol at room temperature to obtain (+)-puupehenone (**1**) in a 60% yield.[13]

After obtaining (+)-puupehenone, we proceeded with the synthesis of a library of 1,6-addition derivatives of (+)-puupehenone (Scheme 2). It was previously reported

Scheme 2[a]



[a]Reagents and conditions: (i) $Mg(OMe)_2$/MeOH, alkyl/aryl anhydride, py, 0 °C to rt, **9** (15%), **10** (17%), **11** (30%), **12** (30%), **13** (25%).

that the addition of 1.0 equivalent of $Mg(OMe)_2$ in methanol furnished stereospecific 1,6-conjugate addition to quinone-methide system of (+)-puupehenone to give 15-α-methoxypuupehenol as the only product over the β-isomer, which was later converted to more stable diacetate (**9**) using acetic anhydride-pyridine (4:1 v/v) in a one-pot reaction system.[28] Therefore, (+)-puupehenone was allowed to react with $Mg(OMe)_2$ in methanol, followed by a series of alkyl/aryl anhydrides in the presence of pyridine to give 15-α-methoxypuupehenol derivatives **9**–**13** in a moderate yield (16–30%). Among them the derivative made using pivalic anhydride furnished 15-α-methoxypuupehenol monopivalate (**13**) instead of dipivalate derivative. In that case, the di-acylation is prevented due to the bulky nature of the pivalic group. For compound **13**, the aromatic carbons C-18 and C-19 (106 and 147 ppm, respectively) are shifted more downfield compared to other C-18 and C-19 shifts (111 and 142 ppm, respectively) found in disubstituted compounds **9**–**12**. This clearly indicates that only the C-20 hydroxyl group was esterified in compound **13**. It is noteworthy that the formation of other isomers was not observed under these reaction conditions.

To modify the hydroxyl group of (+)-puupehenone by acetylation, we treated **1** with acetic anhydride and pyridine

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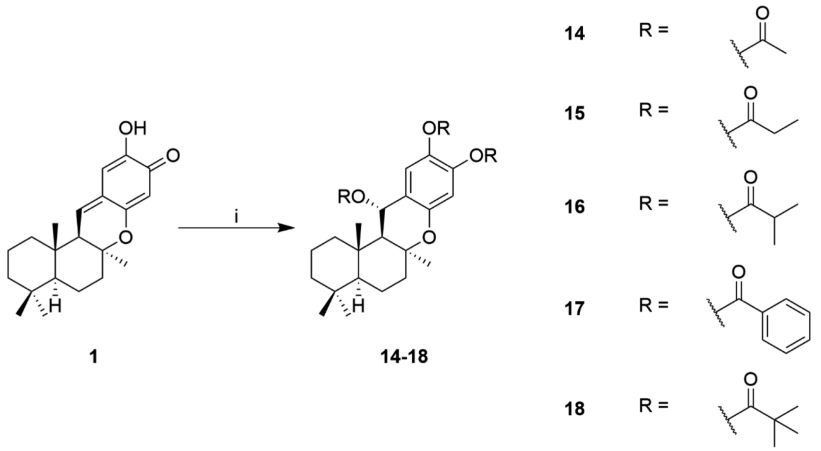
Scheme 3[a]



[a]Reagents and conditions: (i) alkyl/aryl anhydride, py, rt, 2 h, **14** (34%), **15** (25%), **16** (34%), **17** (16%), **18** (30%).

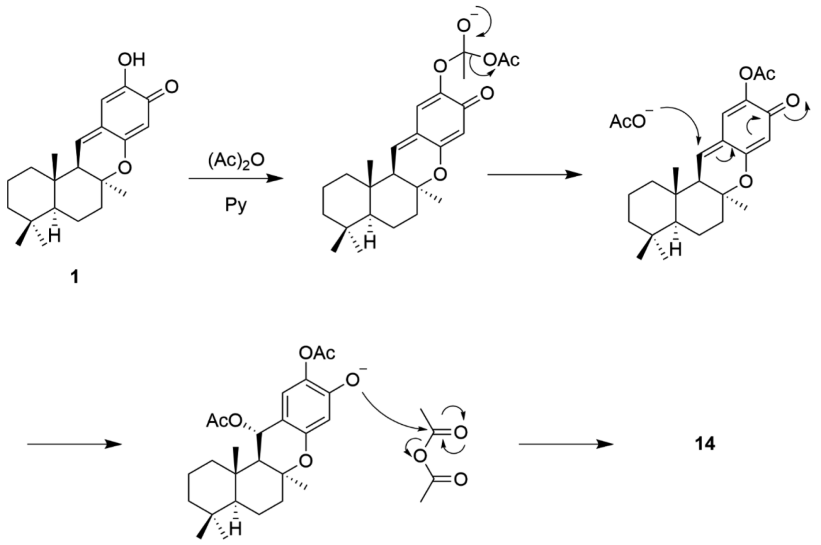


Figure 2. Hypothesized abbreviated mechanism for tri-substituted derivative formation.

(Scheme 3). Interestingly, we produced the 15-α-acetoxypuupehenol triacetate (**14**) in a 30% yield. Only a single isomer was observed in that case which was characterized carefully by spectral analysis. Then, we attempted similar reactions using different alkyl/aryl anhydrides and afforded the tri-substituted derivatives **15-18** which were produced in moderate yield (16−34%). The hypothesized mechanism behind the formation of those derivatives is shown in Figure 2. First, the alcohol present in (+)-puupehenone would be alkylated/arylated by the anhydride. This would in situ generate a carboxylate anion which would perform a stereospecific 1,6-conjugate addition into the quinone−methide system of (+)-puupehenone to produce a new phenolic position. This position would eventually be esterified by the excess amount of reagent in the reaction medium. All newly prepared compounds were characterized using spectral analysis (see Supporting Information). This strategy also represents a way to quickly prepare ester libraries as potential prodrugs.

We also worked on developing structural derivatives of the (+)-puupehenone skeleton. This utilized the borono-sclareolide intermediate developed by Dixon et al.[18] Following this route, (+)-sclareolide (**6**) was transformed to boronic acid (**19**) and then to (+)-chromazonarol (**21**) (Scheme 4). We then tried to selectively oxidize chromazonarol to generate *epi*-puupehenone using 2-iodoxybenzoic acid (IBX); however, we were unable to separate the resulting mixture of isomers. Using a different method for a one-pot oxidation/reduction and protection, we were able to generate acetate-protected *epi*-puupehenol (**22**)[29] with a 90% yield. To avoid the oxidation of **21**, direct alkylation was attempted on 2-methoxybenzoquinone which gave 8-*epi*-19-methoxypuupehenol (**20**) with a 20% yield. Electron donation from the methoxy group decreases the overall yield for this reaction due to differences in site reactivities leading to the formation of various side products.

We then shifted focus to using intermediate **19** in several Suzuki couplings using 10% palladium diacetate, 15% SPhos, and cesium fluoride to generate new ring-open derivatives (Scheme 5a). The phenol compound **23** was prepared first by coupling the boronic acid to 1-benzyloxy-4-bromobenzene in a 90% yield. The benzyl group was easily removed by hydrogenation with 5% palladium on carbon to afford compound **24** in a 73% yield. This same method was used to prepare the resorcinol derivative **25** by coupling to 1,3-

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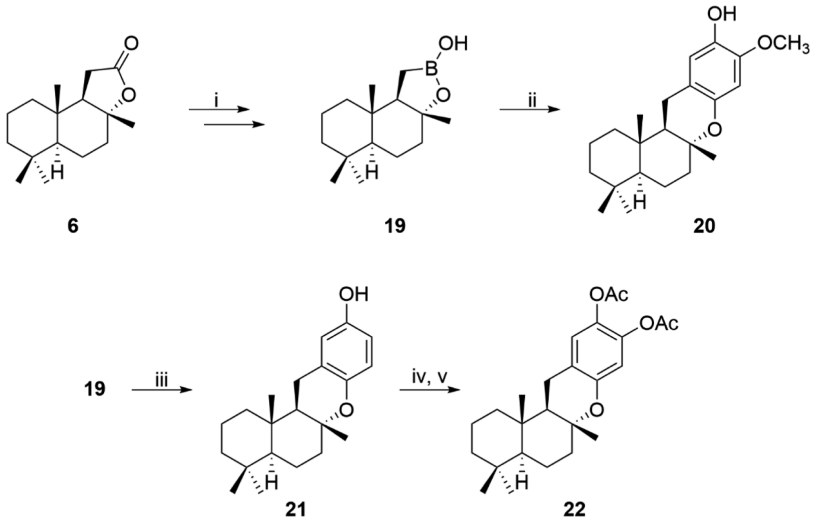
Scheme 4[a]



[a]Reagents and conditions: (i) five steps as previously reported, 69%; (ii) 2-methoxy-1,4-benzoquinone, $K_2S_2O_8$, $AgNO_3$, $PhCF_3/H_2O$ (1:1), 60 °C, 2.5 h, 20%; (iii) one step as previously reported 60%; (iv) IBX, DMF, 0.5 h, rt; (v) 10% Pd/C, K_2CO_3, Ac_2O, DMF, 1 atm H_2, rt, 24 h, 90% from **21**. IBX = 2-iodoxybenzoic acid.

dibenzyloxy-4-bromobenzene in a 32% yield, followed by deprotection to afford compound **26** in an 89% yield. The catechol derivative surprisingly did not survive the benzyl deprotection conditions, and so, a *para*-methoxy benzyl group was used. The same coupling conditions allowed for the addition of the 4-bromo-1,2-bis[(4-methoxyphenyl)methoxy]-benzene giving compound **27** in a 65% yield. Deprotection of the *para*-methoxybenzyl group was achieved by hydrogenation using 5% palladium on carbon to afford compound **28** in a 70% yield. The triol derivative **29**, however, did not survive the standard deprotection conditions of the benzyl group yielding a complex mixture of byproducts that were not isolated. To circumvent this problem, we investigated the possibility of making a trimethoxy-protected triol, first reported as an intermediate by Wang et al. Using the same Suzuki coupling techniques, we were able to couple 2,4,5-trimethoxy-1-bromobenzene to the boronic acid **19** in a 68% yield. Treatment of compound **32** with cerium ammonium nitrate should have yielded a *para*-quinone and allowed for cyclization of the structure to the *ortho*-quinone; however, the quinone methide **33** was formed instead (Scheme 5b). Finally, we made (+)-yahazunol (**31**), another MNP originally isolated from the brown seaweed *Dictyopteris undulata* in 1979.[30] Compound **31** is a structural isomer of compounds **26** and **28**. This was accomplished by coupling 1,4-bis(methoxymethoxy)-2-bromo-benzene to boronic acid **19** to afford compound **30** in a 25% yield. Compound **30** was then deprotected using 1,2-ethanedithiol to yield compound **31** in a 67% yield. All synthesized compounds were characterized using spectral analysis.

Biological Results. Compounds **1**, **9**−**18**, **20**−**29**, and **31**−**33** were screened against *Mycobacterium smegmatis* (*M. smegmatis*) and *Mycobacterium tuberculosis* H37Ra (*Mtb* H37Ra) using the resazurin microtiter assay (REMA) plate method (Table 1).[31]

These same compounds where also screened against a *M. tuberculosis* CDC1551[32] derived strain containing the autoluminescent reporter plasmid pMV306hsp + LuxG13 (*Mtb-lux*) in a replicating and multistress dormancy model of non-replicating *Mtb*; these results are also shown in Table 1. The only compound with activity against both replicating *Mtb* H37Ra and *Mtb-lux* (MICs of 6.25 and 20.1 μM, respectively) and dormant *Mtb-lux* (MIC of 3.8 μM) and *M. smegmatis* (MIC of 50 μM) was compound **9**. Compounds **1**, **9**−**10**, **13**−**15**, **21**, **24**, and **28** all show enhanced activity against dormant *Mtb-lux* than against replicating *Mtb-lux*. However, the parent compound **1** still exhibited the most potent activity against dormant *Mtb*-lux with an MIC of 1.4 μM. Compounds **1**, **10**, **23**, **28**, **14**, **15**, **20**, and **21** are also more active in replicating strain *Mtb-lux* than in strain *Mtb* H37Ra. The latter is an attenuated biosafety level 2 (BSL-2) strain derived from *Mtb* H37 parent strain in 1935.[33] This observed increase in activity toward *Mtb-lux* could be partially due to differences in the methods used for quantification of the MIC in the respective strains or batch variations in this work and previously reported literature.

The addition of the prodrug ester groups improved the activity of the compounds in *Mtb* H37Ra for compounds **14** and **15** (MIC of 25 μM each) and replicating *Mtb-lux* for compound **15** (MIC of 5.5 μM); however, the activity dropped as esters became bulkier. Increasing the bulk of the ester resulted in little to no activity such as compound **12** in *Mtb*. The monopivalate-substituted compound **13** showed reduced activity in *Mtb* H37Ra (100 μM) but surprisingly maintains activity in *Mtb-lux* in both replicating and dormant states (MICs of 28.6 and 3.4 μM, respectively). This activity could be explained by the compound's ability to be converted more readily to a reactive quinone methide intermediate due to the presence of the free hydroxyl group. This conversion would happen quicker since it would not have to undergo the removal of the pivalate group by enzyme modification. Once converted to the quinone methide intermediate, the compound would be able to react with nucleophilic amino acid side chains in target proteins. Compound **11** begins to lose activity in dormant *Mtb*-lux (20.1 μM) similar to the trend seen for related compound **16** (160.0 μM) indicating the increased steric bulk of the isobutyl group affects the activity. Compound **12**, with the larger pivaloyl group, still retains some activity in dormant

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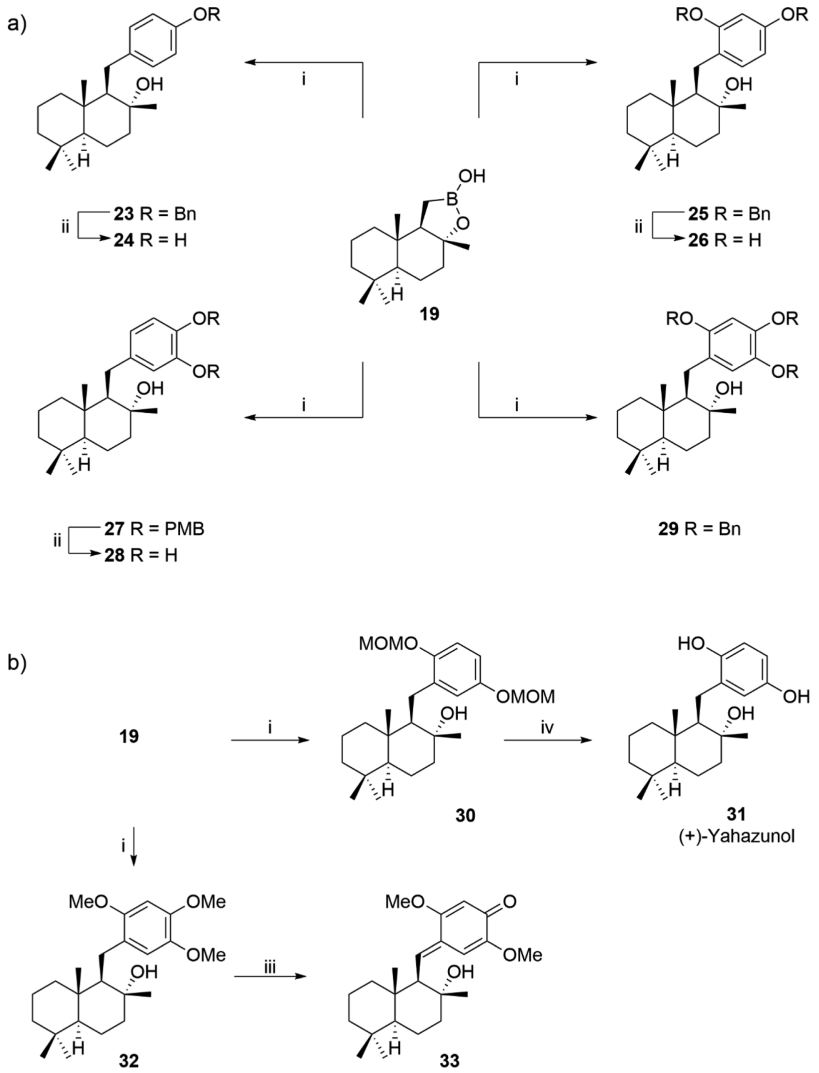
Scheme 5[a]



[a]Reagents and conditions: (i) 10% $Pd(OAc)_2$, 15% SPhos, CsF, 1-4 dioxanes, ArBr. 50 °C, 12 h, (24−90%); (ii) 5% Pd/C MeOH 1 atm H_2 rt 12 h, (70−89%); (iii) CAN MeCN/H_2O (1:1) −5 °C to rt 0.5 h, 78%; (iv) $MgBr_2$ *n*-butanthiol EtO_2 rt 24 h, 67%. CAN = cerium(IV) ammonium nitrate.

Mtb-lux (84.9 μM); however, it is greatly reduced compared to **11** and **12**. These effects are likely due to differences in protein expression or cellular metabolism between replicating and dormant *Mtb*. This change in cellular activity leads to the MIC of these 15-α-methoxy derivatives (**9**, **10** and **13**) being 4−5 times lower in dormant *Mtb*-lux than that of replicating. Such compounds that display an unusually enhanced antimicrobial activity against dormant versus replicating *Mtb* represent valuable chemical biology tools to identify vulnerable targets or pathways in drug-tolerant bacilli.

Changing the substitution at the C15 position from an α-methoxy group in compound **9** to an α ester in compound **14** increased the activity slightly in replicating *Mtb-lux* (20.1 to 12.7 μM, respectively) and **10** compared to **15** (18.8 to 5.5 μM, respectively). Compound **15** has the lowest MIC in replicating *Mtb-lux* (5.5 μM) and the second lowest in dormant *Mtb-lux* (2.0 μM) out of the tested compounds. Compound **16** has an MIC of 13.8 μM against replicating *Mtb-lux* but an MIC of 160.0 μM against dormant *Mtb-lux*. Interestingly, compound **16** is the only compound that loses activity in dormant *Mtb-lux* but still retains activity in replicating *Mtb-lux*. We speculate that this could be due to downregulation of key esterases that would allow removal of the bulky tripivaloyl group in the dormant *Mtb-lux*. This downregulation of esterases would not affect compound **13** since removal of the 15-α-methoxy group may generate the quinone methide from the free hydroxyl resulting in an active compound.

Chromazonarol (**21**), which is epimeric at C-8, shows significantly reduced activity against the strains of *Mtb*. The addition of a methoxy group in compound **20** seems to improve the activity of the compound core against *Mtb-lux* (62.0 μM for **20** vs 179.6 μM for **21**). The relatively lower activity of these compounds could also be a result of the inverted conformation at the C8 position or may be due to the loss of functionality required for putative quinone methide formation. We speculate that the core has activity independent of any putative quinone methide formation based on this data. Compound **22**, acetate-protected *epi*-puupehenol, might not have activity because of its C8 conformation as well. It is noted that compounds with activity lower than 200 μM were not listed in Table 1. The open-ring derivatives also did not

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Table 1. MIC against *M. tuberculosis* and *M. smegmatis*[a]

		Mtb-lux			
compounds	*Mtb* H37Ra	MIC_R	MIC_D	*R/D*	*M. smegmatis*
MIC (μM) for puupehenone derivatives against mycobacteria					
1	100	9.0	1.4	6.4	-
9	6.25	20.1	3.8	5.3	50
10	50	18.8	3.6	5.2	-
11	50	17.2	20.1	0.9	-
12	-	>200	84.9	N.A.	-
13	100	28.6	3.4	8.4	-
14	25	12.7	3.0	4.2	-
15	25	5.5	2.0	2.8	-
16	-	13.9	160.0	0.09	-
20	100	62.0	60.0	1.0	-
21	100	179.6	101.9	1.8	-
23	100	141.3	99.4	1.4	-
24	-	361.2	97.6	3.7	-
28	-	174.4	126.3	1.4	-

[a]MICs for *Mtb* H37Ra and *M. smegmatis* were determined visually by REMA assay and do not represent results for >99% killing of TB. MIC values for *Mtb-lux* were determined by Luciferase reporter assay and do represent results for 99% killing of TB cells. (-) = no activity seen, N.A. = not applicable. *M. smegmatis* mc^2155 ATCC 700084, *M. tuberculosis* H37Ra ATCC 25177, and *M. tuberculosis* CDC1551.

perform very well with only **23**, **24**, and **28** showing any inhibition. It is interesting to compare compound **24** to its benzyl-protected precursor **23** (361.2 and 141.3 μM, respectively, in replicating *Mtb-lux*) which shows an increase in the MIC due to the benzyl protection of the phenyl alcohol in replicating *Mtb-lux*. This observation for compound **24** and **23** is then reversed in dormant *Mtb-lux* (97.6 and 99.4 μM, respectively). These SARs are visually summarized in Figure 3

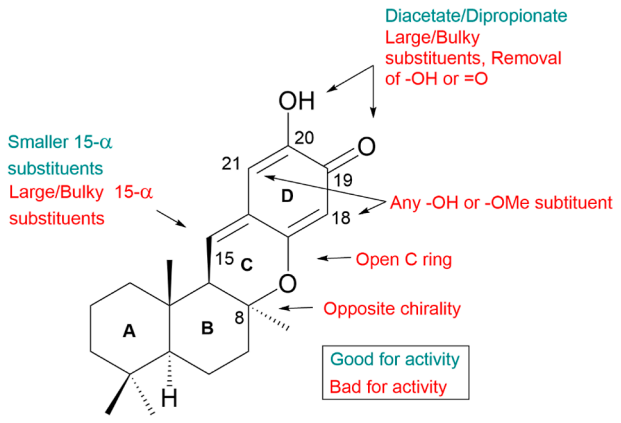

Figure 3. Summary of SARs against *M. tuberculosis*.

on the core structure of (+)-puupehenone. In general, smaller substituents were better at the 15-α position, while larger substituents negatively affected the activity. Similarly, the smaller acetate and propionates were well tolerated at positions 19 and 20 on the D ring, while large acyl groups or removal of the 19-carbonyl or 20-hydroxyl were not well tolerated. Addition of OH or OMe were unhelpful at positions 18 and 21 as was the opening of the C ring or epimerization of the C-8 position.

To evaluate the selectivity of the top six compounds from the *Mtb-lux* activity screen (Table 2), cytotoxicity for two different mammalian cell lines was determined.

Table 2. Cytotoxicity Data for Compounds 1, 9, 10, and 13−16[a]

	cytotoxicity (IC_{50}, μM)		SI_R/SI_D	
compounds	J774	HepG2	J774	HepG2
1	23.2	70.6	7.7/46.4	23.5/141.2
9	59.1	~107	6.6/34.8	12.0/62.9
10	60.6	>200	6.8/35.7	>22.5/>117.6
13	56.4	>200	4.4/37.6	>15.7/>133.3
14	>200	>200	>33.3/>142.3	>33.3/>142.3
15	26.4	64.2	9.4/26.4	22.9/64.2
16	>200	>200	82.4/1.01	>26.3/>2.25

[a]SI was calculated as IC_{50}/MIC. SI_R is the SI for replicating *Mtb*-Lux. SI_D is the SI for dormant *Mtb*-Lux.

The IC_{50} was calculated for each compound against J774A.1 and HepG2 cell lines. The selectivity index (SI) for both replicating and dormant conditions was then calculated by taking the IC_{50} and dividing it by the corresponding MIC ($SI_R = IC_{50}/MIC_R$, $SI_D = IC_{50}/MIC_D$).[34] Consistent with our experience, the J774 macrophage cell line was more sensitive to all compounds tested. For compounds **9**, **10**, and **13**, reductions in cytotoxicity were offset by lower potency, yielding minimal changes in selectivity. However, compound **14** retained potent activity toward both dormant and active *Mtb-lux*, while causing no cytotoxicity even at the highest tested concentrations. The significantly improved SI relative to the parent natural product affords a promising therapeutic window for elimination of both replicating and dormant bacilli. Based on these observations, this triacetate-protected puupehenone represents a promising candidate for further optimization.

CONCLUSIONS

The discovery of novel antimycobacterials able to eradicate phenotypically drug-tolerant dormant *Mtb* is critical for developing effective regimens to shorten the treatment course for TB. Our recent discovery of a meroterpenoid MNP with enhanced activity against dormant versus replicating *Mtb* presented an opportunity to address this problem. To elucidate SARs and optimize the selective antimycobacterial activity of this scaffold, a series of meroterpenoid derivatives were synthesized and evaluated for selective antimycobacterial activity against *M. smegmatis* (*M. smegmatis*), *M. tuberculosis* H37Ra (*Mtb* H37Ra), and *M. tuberculosis* CDC1551 (*Mtb-lux*) in both the replicating and nonreplicating states. A library of ester derivatives was prepared from commercially available (+)-sclareolide through the formation of (+)-puupehenone as an intermediate using a previously reported protecting group-free stereospecific synthetic route with a minor modification at the last step. Another library related to structural derivatives of (+)-puupehenone as well as ring-open derivatives was also synthesized following well-known reported chemistry. Among these 24 compounds, 14 compounds have high to moderate activity against *Mtb*. Only compound **9** has activity against both replicating *Mtb* H37Ra and *Mtb-lux* and dormant *Mtb-lux* and M. smegmatis. Seven compounds (**1**, **9**−**11**, and **13**−**15**) have superior activity against dormant *Mtb-lux* versus

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replicating *Mtb*. Structural modifications that yielded compound **14** eliminated all detectable in vitro cytotoxicity, a key step toward future in vivo studies. Overall, the summarized SAR of the active compounds against *Mtb* will inform subsequent medicinal chemistry optimization of this scaffold. In addition, this novel chemical series provides chemical biology tools for the discovery of novel targets or pathways vulnerable to inhibition in drug-tolerant dormant *Mtb*.

METHODS

Bacterial Growth Conditions. *M. smegmatis* (mc^2155) and *M. tuberculosis* (H37Ra) were obtained from American Type Culture Collection (ATCC). A frozen stock of *M. smegmatis* and *Mtb* was grown in Middlebrook 7H9 media (10% OADC-0.05% Tween 80) to a log phase growth OD_{600} of 0.6 taken using a Laxco MicroSpek DSM micro cell density meter.

MIC *M. smegmatis*. The MIC of compounds **1**, **9**−**18**, **20**−**29**, **31**−**33**, and ampicillin was determined by broth-dilution assays and staining with resazurin. Freshly grown cultures of *M. smegmatis* were used as inoculum at a dilution of 1:1000 in Middlebrook 7H9 media (10% OADC−0.05% Tween 80). Plates were sealed with a breathable membrane and incubated for 24 h at 37 °C. After a 24 h incubation in the presence of twofold serial dilutions of compounds, the plates were stained with resazurin (30 μL 0.02% w/v), incubated for 4−5 h plates, and observed for color change from blue to pink. The MIC was scored at the lowest concentration that retained its blue color. The assay was repeated in triplicate.

MIC *M. tuberculosis* H37Ra. The MIC of compounds **1**, **9**−**18**, **20**−**29**, **31**−**33**, and rifampicin was determined by broth-dilution assays and staining with resazurin. A freshly grown culture of *Mtb* H37Ra was used as inoculum at a dilution of 1:1000 in Middlebrook 7H9 media (10% OADC−0.05% Tween 80). Plates were sealed with a breathable membrane and incubated for 14 days at 37 °C in a resealable plastic bag along with a plate of ultrapure water to increase the humidity and prevent evaporation. After the 14-day incubation period in the presence of twofold serial dilutions of the antibiotic, the plates were stained with resazurin (30 μL 0.02% w/v) and incubated for 24 h before the plates were observed for color change from blue to pink. The MIC was scored at the lowest concentration that retained its blue color. Each assay was repeated in triplicate.

MIC *M. tuberculosis* CDC1551. *Preparation of Compound Stock Solutions.* A rifampicin (RIF) stock solution was prepared at 60 mM in 100% dimethyl sulfoxide (DMSO), followed by the preparation of a 60 μM working stock in deionized water. An isoniazid (INH) stock solution was prepared at 10 mM in deionized water, followed by the preparation of a 2.5 mM working stock. Puupehenone analogues were prepared from powder at 20 mM in 100% DMSO. Working stocks of each compound were prepared in deionized water at a concentration of 1 mM (5% DMSO final). All stocks were stored at −80 °C.

Bacterial Strains and Culture Conditions. A *M. tuberculosis* CDC1551[32] derived strain containing the autoluminescent reporter plasmid pMV306hsp + LuxG13 (a gift from Brian Robertson and Siouxsie Wiles − Addgene plasmid #26161; http://n2t.net/addgene:26161; RRID: Addgene_26161) was used in this study as previously described.[9,35] *Mtb-lux* was cultured in Middlebrook 7H9 supplemented with 0.05% Tween 80 and 10% oleic acid/albumin/dextrose/catalase (OADC) and incubated stationary at 37 °C and 5% CO_2. Kanamycin at 103 μM (KAN) was added for maintenance of the reporter plasmid.

MIC for Replicating Mtb-Lux (MIC_R). To determine the MIC against replicating *Mtb-lux*, 10-point dose−response curves were carried out using twofold serial dilutions of compounds. Compounds were added to solid bottom white 384-well plates (Corning) by an Integra AssistPlus automated liquid handler. *Mtb-lux* cultured to mid-log phase was diluted to an OD_{600} of 0.02 and added to each well in a total volume of 30 μL. Following incubation for 4 days, luminescent signal in each well was determined using a Synergy H4 plate reader (Biotek). Each 384-well plate contained positive (12 μM RIF) and negative (1% DMSO) controls. To accurately determine the MIC of more potent compounds, subsequent 10-point dose−response curves were carried out using a lower range of concentrations.

MIC for Dormant Mtb (MIC_D). To assess the activity of compounds against non-replicating dormant *Mtb*, a multi-Stress dormancy (MSD) model was used as previously described.[9] Briefly, *Mtb-lux* cultures were grown to log phase in Complete Dubos media, pelleted and resuspended in MSD media (10% Complete Dubos at pH 5.0 with 0.018% tyloxapol, no glycerol), and incubated in a hypoxia chamber (37 °C, 5% O_2, 10% CO_2) for 9 days prior to addition of compounds. Dormant cultures at OD_{600} = 0.4 were treated with serial dilutions of compounds in white 384-well plates (30 μL total volume per well) as described above. The luminescent signal was read after 2 days of treatment using a Synergy H4 plate reader (BioTeK). To ensure the phenotypic drug tolerance of dormant *Mtb*, 16-point dose−response curves for RIF and INH (starting at 12 and 500 μM, respectively) were conducted against dormant bacteria and replicating *Mtb*-Lux in complete Dubos media.

The MIC_R and MIC_D values reported represent the average of four data sets (two independent replicates with two technical replicates on each plate). Data were normalized in such a way that the highest and lowest output values in the curve were set to be 100 and 0% growth, respectively. Dose−response curves were analyzed using Graphpad Prism with curves fitted using a modified Gompertz model to determine MIC values representing 99% killing.[9]

Cytotoxicity Assay. Cytotoxicity was assessed using J774A.1 (murine macrophage-like) and HepG2 (human liver carcinoma) cell lines using twofold dilution of the compounds as described previously.[9,36] IC_{50} values were determined using nonlinear regression fitting of the databy GraphPad Prism. The SI was calculated as IC_{50}/MIC.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c01887.

^{1}H NMR spectra and ^{13}C NMR spectra of compounds and experimental details for the preparation of compounds **7**−**19**, **20**−**33** (PDF)

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Notes

The authors declare no competing financial interest.

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