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

Anshupriya Si, Alexander D. Landgraf, Sandra Geden, Steven J. Sucheck,* and Kyle H. Rohde*

ABSTRACT: Tuberculosis is a disease caused primarily by the organism Mycobacterium tuberculosis (Mtob), which claims about 1.5 million lives every year. A challenge that impedes the elimination of this pathogen is the ability of Mtob 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 Mtob and therapeutics able to kill these phenotypically tolerant bacilli will be critical for completely eradicating Mtob. 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 Mtob. 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 Mtob, and the most active compounds retained the enhanced activity against dormant Mtob 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 Mtob. Intriguingly, the closely related triisobutyl derivative 16 showed similar activity to 1 in actively dividing Mtob but lost about 178-fold activity against dormant Mtob. However, the monopivaloyl compound 13 showed a modest 3- to 4-fold loss in activity in both actively dividing and dormant Mtob 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 Mtob. 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 Mtob.

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. Prior to the COVID-19 pandemic, TB topped the list of deaths due to a single infectious disease, beating out HIV/AIDS. The World Health Organization (WHO) estimated that there were approximately 484,000 new cases of drug-resistant TB in 2020, more than doubling the number of cases from 2015. Developing new antibiotics and alternative approaches to treat drug-resistant TB is crucial to prevent the emergence of multidrug-resistant TB (MDR-TB) and the threat of extensively drug-resistant TB (XDR-TB), which is near impossible to treat. The search for new TB therapies has been driven by the urgent need for novel treatments that are capable of overcoming resistance and acting on the unique physiology of the dormant or drug-resistant bacterial form. The understanding of the dormant state of TB has evolved, and recent advances indicate that this state is characterized by a slow-growing, nutrient-starved, and metabolically quiescent phase.

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One of the major problems in treating Mycobacterium tuberculosis (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 antimicrobial 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 noteworthy because they demonstrate an unusual selectivity for dormant 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.\(^5\,7\)

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 \(\mu\text{M}\), (+)-puupehenone (1), 15-\(\alpha\)-cyanopuupehenol (4), and 15-cyanopuuphehene (5) (Figure 1), exhibited 99, 96, and 90% inhibition of Mtb, respectively.\(^11\) Recently, we made the exciting discovery that puupehedione (2) and 15-\(\alpha\)-methoxy-puuphehene (3), metabolic derivatives of puupehenone shown in Figure 1, have minimum inhibitory concentrations (MIC) of 268.4 and 31.3 \(\mu\text{M}\), respectively, against active Mtb and MICs of 47.2 and 1.4 \(\mu\text{M}\), respectively, in an in vitro multitress 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 \(\alpha,\beta\)-ene,\(^14\) 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-\(\alpha\)-methoxy-puuphene-nol 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
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 advantage of a borono-sclareolide terpenoid donor first synthesized by Dixon et al.¹⁸ Alternatively, the boronic acid compounds using a Suzuki–Miyaura reaction were also employed as a point of diversification. For the regioselective dehydrogenation of (+)-puupehenone (C in THF introduced an α-hydroxyl group and furnished (+)-puupehenone (1) 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 modifications as noted.¹³ We treated compound 7 with KHMDMS at −78 °C and subsequently reacted it with O₂ 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/O₂,¹⁹ t-BuOK/t-BuOH/O₂,²⁰ I₂/DMSO,²¹ PIDA/KOH/MeOH,²² NHMDMS/camphorsulfonyloxaziridine,²³ and Davis’ oxaziridine 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.²⁴ Treatment of compound 7 with 3-phenyl-2-(phenylsulfonyl)oxaziridine in the presence of KHMDMS 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 and bismuth(III) oxide-mediated oxidation²⁵ 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 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 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) found in disubstituted compounds 9–12 are shifted more downfield compared to other C-18 and C-19 shifts (111 and 142 ppm, respectively) found in unsubstituted 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
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. 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-puupehenol 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) 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-
dibenzoxyl-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 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(methoxyethoxy)-2-bromobenzene 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, 9–28, 30–29, 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 CDC155132 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, 16, 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...
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-pupeiisol, 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 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 have activity because of its C8 conformation as well.
inhibition. It is interesting to compare compound 24 in replicating M. smegmatis to its benzyl protection of the phenyl alcohol benzyl-protected precursor 23, respectively, in replicating Mtb-lux. This observation for compound 24 shows any activity seen, N.A. = not applicable. M. smegmatis mc2155 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.

Table 1. MIC against M. tuberculosis and M. smegmatis

| compounds | Mtb H37Ra | Mtb-lux | R/D | M. smegmatis |
|-----------|-----------|---------|-----|-------------|
| 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    | 2.0 | 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 | 0.09        | - |
| 20        | 100       | 62.0    | 6.0 | 1.0         | - |
| 21        | 100       | 79.6    | 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       | - |

Table 2. Cytotoxicity Data for Compounds 1, 9, 10, and 13–16

| compounds | cytotoxicity (IC50 μM) | SI/6/SI10 | 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 |

*SI was calculated as IC50/MIC. SI is the SI for replicating Mtb-Lux. SI10 is the SI for dormant Mtb-Lux.

The IC50 was calculated for each compound against J774/A.1 and HepG2 cell lines. The selectivity index (SI) for both replicating and dormant conditions was then calculated by taking the IC50 and dividing it by the corresponding MIC (SI = IC50/MIC). 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 tricatex-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. tuberculosis 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 following well-known reported chemistry. Among these 24 compounds, 14 compounds have superior activity against dormant Mtb-lux versus the parent natural product.

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.

**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.
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* (mc²15S) 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 OD₆₀₀ 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 derived strain containing the autoluminescent reporter plasmid pMV306bhp + LuxG13 (a gift from Brian Robertson and Siouxsie Wiles – Addgene plasmid #26161; http://n2t.net/record/bio planta:26161; RRID: Addgene_26161) was used in this study as previously described. *Mtbb−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% CO₂. Kanamycin at 103 μM (KAN) was added for maintenance of the reporter plasmid.

**MIC for Replicating Mtb-Lux (MICₚ).** 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 OD₆₀₀ 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 (MICₚ).** To assess the activity of compounds against non-replicating dormant Mtb, a multi-Stress dormancy (MSD) model was used as previously described. 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% O₂, 10% CO₂) for 9 days prior to addition of compounds. Dormant cultures at OD₆₀₀ = 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 MICₚ and MICₚ 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.

**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. IC₅₀ values were determined using nonlinear regression fitting of the database GraphPad Prism. The SI was calculated as IC₅₀/MIC.

**ASSOCIATED CONTENT**

**Supporting Information**

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

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

**AUTHOR INFORMATION**

**Corresponding Authors**

Steven J. Suchecz − Department of Chemistry and Biochemistry, University of Toledo, Toledo, Ohio 43606,
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The authors declare no competing financial interest.

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