Article

Antimycobacterial Activity of Laurinterol and Aplysin from *Laurencia johnstonii*

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Abstract: Marine environments represent a great opportunity for the discovery of compounds with a wide spectrum of bioactive properties. Due to the privileged conditions of natural selection, marine natural products are subject to overcome the pressure put on identify novel drugs; not only in the case of newly discovered bioactive metabolites, but also in those previously known. Since drug resistance has caused an increase in infections caused by tuberculous and nontuberculous *Mycobacteria*, the re-evaluation of known bioactive metabolites has been suggested as a means to address this problem. In this sense, this study presents an evaluation of *in vitro* effect of laurinterol (**1**) and aplysin (**2**), two brominated sesquiterpenes isolated from *Laurencia johnstonii* against nine *Mycobacterium tuberculosis* strains and six nontuberculous mycobacteria. Laurinterol (1) exhibited good anti-tuberculous activity, especially against nontuberculous mycobacteria, being remarkable the effect against *M. abscessus* with MIC values lower than the reference drug imipenem. This study provides further evidence for the antimycobacterial activity of some sesquiterpenes from *L. johnstonii*, that can be considered an interesting lead compound for the discovery of novel antimycobacterial molecules to treat NTM infections.

Keywords: Marine natural products; *Laurencia*; brominated sesquiterpenes; antimycobacterial; nontuberculous mycobacteria; tuberculosis

1. Introduction

Antimicrobial resistance is a global health problem that increase difficulties in treating common infectious diseases, as well as a significant increment in health-care costs with concomitant lengthier stays in hospitals and intensive care requirements [1].

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is one of the top 10 causes of death worldwide. In 2018, 1.5 million died from this disease, with over 95% of cases occurring in developing countries [2].

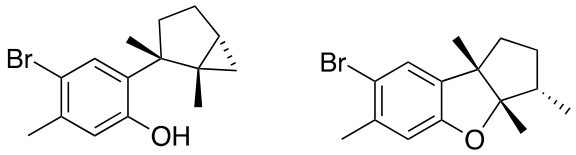
TB can be treated effectively by using the first line drugs (FLDs) isoniazid, rifampin, pyrazinamide, ethambutol and streptomycin. Nevertheless, multidrug-resistant Mtb strains (MDR-TB) is a growingproblem that require the use of second-line drugs with more trouble to procure and furthermore more toxic and expensive than FLDs [3]. WHO estimates that, in 2018, there were about

484,000 new cases with resistance to rifampicin of which 78% had MDR-TB, and only 56% of those cases are currently successfully treated [2]. In addition to Mtb, there are over 170 different species of mycobacterial pathogens defined as nontuberculous mycobacteria (NTM) that cause a wide spectrum of diseases that include TB-like pulmonary, extra-pulmonary disease, and disseminated diseases. Often, NTM do not respond to TB treatments and usually lengthy and complex drug therapies are required to treat them. Furthermore, most NTM have become resistant to many antimicrobials [4].

Natural products are considered privileged molecules because they are fashioned by natural selection to interact with cellular targets with high efficiency and selectivity, and to avoid resistance. Due to these characteristics, natural products and their derivatives represent over one-third of all FDA-approved new molecular entities; with 69% of all antibacterial agents originated from natural products [5]. Nonetheless, the total amount of approved compounds barely represents over 0.1% of the more than 25,000 known antibiotic natural products, mainly due to issues concerning their efficacy, toxicity or stability, among others. Given the efficacy and safety indexes exhibited by the first successfully employed natural drugs, criteria governing novel antibiotic discovery are strict, making it difficult to identify subsequent successful candidates. Due to the pressing need to identify new antibiotic compounds, re-evaluation of known molecules has been suggested [6].

Marine organisms have become an important source of several thousands of bioactive compounds [7]. Eight of these compounds have gained clinical approval and around thirty are candidates in different clinical phases of drug development. Nevertheless, none of them have been exploited as antibacterial [8]; however other approved compounds are under study on diseases such as severe pain, hypertriglyceridemia, schizophrenia, cognitive disorder, and antiviral effect [8,9].

Among marine organisms, algae of genus *Laurencia* are considered one of the richest sources of novel compounds showing a wide range of bioactive properties, such as the brominated sesquiterpenes laurinterol (**1**) and aplysin (**2**) (Fig. 1) [10]. Laurinterol has been evaluated for its antibacterial [11-13], cytotoxic [13-15], antifouling [16], anti-*Acanthamoeba* [17], Na/K ATPase inhibition [18], insecticidal and repellent properties [19]. Meanwhile, aplysin has shown moderate cytotoxic activity, however it has been evaluated for its mechanisms of action on different biological models [20-23].



Laurinterol (**1**) Aplysin (**2**)

Figure 1. Structures of natural sesquiterpenes laurinterol (**1**) and aplysin (**2**)

Recently, membrane proteins, such as P-type ATPases and ATP synthases, have been suggested as promising targets to treat mycobacterial infections, becoming possible to overcome the limitations that suppose the lipid-rich cell of mycobacteria [24,25]. In this sense, laurinterol (**1**) has been reported as a potent Na/K ATPase inhibitor [18], which encouraged us to explore its antimycobacterial properties and to proceed to evaluate aplysin (**2**), a related structural compound, due to its abundance and low toxicity, as well as the possibility to obtain it from **1** [17].

2. Results and Discussion

The emergence of drug resistance is one of the challenges to control TB and other mycobacterial infections, resulting in the evident need to develop new drugs. Several new drugs and compounds are currently undergoing clinical evaluation as treatment options against mycobacterial infections [26-29]. Taking into consideration the important role of natural products and their semi-synthetic derivatives as drug leads, it is not surprising that some antimicrobials such as rifampicin, streptomycin, amikacin, viomycin, kanamycin, capreomycins and cycloserine are currently used in combination with other antitubercular agents in the treatment of mycobacterial infections [30].

More than 170 compounds isolated from marine organisms, mostly from microorganisms and invertebrates, have been reported to possess anti-TB properties and they are represented by several biosynthetic classes such as alkaloids, peptides, terpenoids, sterols, and others. Despite that, only ten out of those compounds are considered as potential for further development, with MIC values below 64 μg/mL and a selectivity index (SI, CC_{50}/MIC) higher than 10 [31].

Table 1 shows the activity of **1** and **2** against nine *M. tuberculosis* strains. Compound **1** inhibited eight out of the nine strains of Mtb with MICs ≤ 100 μg/mL. *M. tuberculosis* CIPTIR-F296 was the most susceptible strain for both compounds; laurinterol was more active, showing MIC value of 25 μg/mL, even lower than rifampicin (32 μg/mL), the most used drug in TB treatment [32].

Table 1. MIC values of laurinterol (**1**) and aplysin (**2**) for *Mycobacterium tuberculosis* strains

Strain	Laurinterol (**1**) [μg/mL]	Aplysin (**2**) [μg/mL]	*Rifampicin [μg/mL]
M. tuberculosis H37Rv	100	Nt	0.1
M. tuberculosis CDC 1551	50	Nt	1
M. tuberculosis LIID-28-99	50	Nt	1
M. tuberculosis LIID-582-15	25	>100	1
M. tuberculosis LIID-619-15	25	>100	1
M. tuberculosis LIID-853-15	100	>100	1
M. tuberculosis CIPTIR-F296	25	50	32
M. tuberculosis CIPTIR -*D152*	50	>100	1
M. tuberculosis CIPTIR -C131	>100	>100	0.1

Nt: No tested; * Reference drug

In comparison with TB treatments, the anti-NTM therapies have been remarkably neglected. Hence, it has been suggested to screen known anti-Mtb compounds against strains of NTM [33]. For this reason, we evaluated **1** and **2** against six clinical isolates of NTM (Table 2); three strains of *M. abscessus*, one strain of *M. fortuitum* and two strains of *M. intracellulare*. Both compounds were active against NTM; whereas compound **2** inhibited *M. intracellulare* LIID-01 at MIC of 50 μg/mL, compound **1** exhibited good activity against all the strains tested, particularly against *M. abscessus* strains (MIC 6.2 μg/mL). It is important to emphasize that *M. avium* complex (*M. avium* and *M. intracellulare*) and *M. abscessus* represent prevalent sources of infections and, in addition to the lengthy and complex treatment (18-24 months of at least three combinate drugs), they are resistant to many common antibiotics [28]. In this study imipenem and linezolid were selected as drug control because they are part of the recommended treatment for NTM infections [34-37].

Table 2. MIC values of laurinterol (**1**) and aplysin (**2**) for nontuberculous mycobacteria clinical strains

Strain	Laurinterol (**1**) [μg/mL]	Aplysin (**2**) [μg/mL]	*Linezolid [μg/mL]	*Imipenem [μg/mL]
M. abscessus LIID- 01	6.2	100	1	32
M. abscessus LIID- 02	6.2	100	1	32
M. abscessus LIID-03	6.2	>100	1	8
M. fortuitum LIID- 01	12.5	>100	8	4
M. intracellulare LIID-01	12.5	50	2	1
M. intracellulare LIID-02	25.0	100	8	>64

* Reference drug

Previously, laurinterol (**1**) and aplysin (**2**) were evaluated for their toxicity against murine macrophages and they exhibited half-maximal cytotoxic concentration (CC_{50}) of 23.7 and 323.7 μg/mL, respectively [17]. In our study, given the antimycobacterial activity of the sesquiterpenes, **1** exhibit a selectivity index (SI) of 3.8 for *M. abscessus* meanwhile **2** showed SI between 3.2 and 6.5 for two strains

of *M. intracellulare* and *M. abscessus* and one strain of *M. tuberculosis*. The SI is used to estimate the therapeutic window of a drug and to identify drug candidates for further studies, a SI value less than 2 indicates general toxicity of the pure compound, therefore both compounds can be good candidates for subsequent analysis.

To date, algae metabolites represent over 2% of the marine anti-TB compounds reported, and among the metabolites isolated from *Laurencia* that have been tested against Mtb (Fig. 2), obtusol (**3**), elatol (**4**) and deschloroelatol (**5**) exhibited MICs of 32 μg/mL; debromolaurinterol (**6**) and isolaurenisol (**7**) showed MICs of 64 and 38 μg/mL, respectively and, allolaurinterol (**8**) and allolaurinterol acetate (**9**) of 16 and 9 μg/mL, respectively [38-40]. Axisonitrile-3, a sesquiterpene isonitrile isolated from the sponge *Acanthella klethra*, is one of the most active marine compounds against Mtb, which exhibited MIC of 2 μg/mL [38].

Although the urge of new anti-NTM treatments, marine natural products have been weakly evaluated; Biá Ventura *et al*. investigated the antimycobacterial activity of two extracts of *L. dendroidea* and three of its halogenated sesquiterpenes against *M. bovis*, noting obtusol (**3**) with a half-maximal inhibitory concentration (IC_{50}) of 31.4 μg/mL [39].

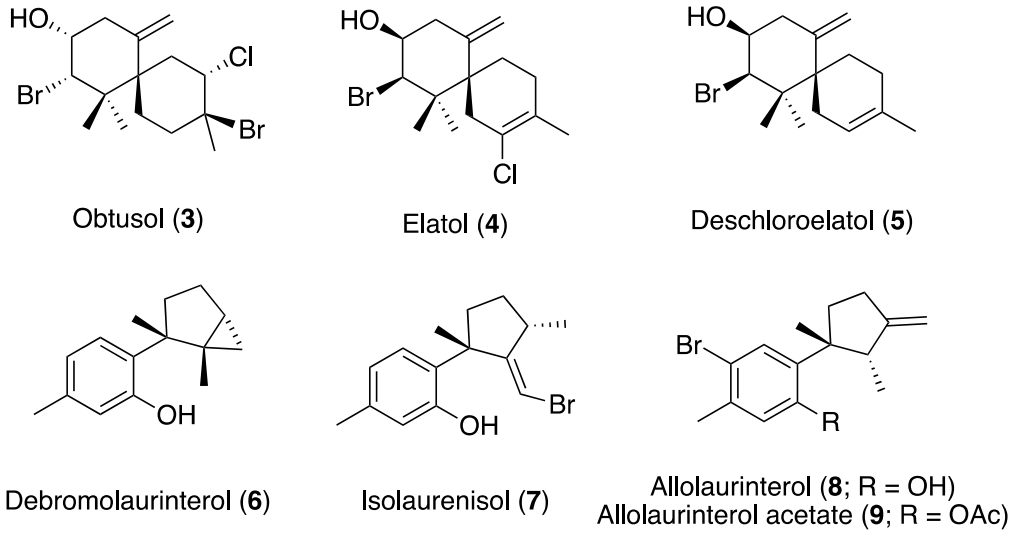


Figure 2. *Laurencia*-derived sesquiterpenes evaluated for its antimycobacterial activity

Laurencia-derived sesquiterpenes with chamigrane (compounds **3**-**5**), cyclolaurane (compound **6**), and laurane (compounds **7**-**9**) skeletons have showed antitubercular properties. The presence of lipophilic groups such as an exocyclic double bond, an aromatic ring, or halogenated substitutions, particularly bromine atoms, as well as the presence of a hydroxyl group, are common structural features that all these small active molecules share. These structural characteristics are also found in laurinterol (**1**) and aplysin (**2**), with cyclolaurane and laurane frameworks, respectively. Nonetheless, despite the bromide atom, the formation of an ether bond between the aromatic ring and the cyclopentane fragment of **2** decreases the antimycobacterial activity, revealing the relevance of the hydroxyl group for the activity.

In this work, we observed that the brominated cyclolaurane, laurinterol (**1**), can be considered an interesting lead compound for the discovery of novel antimycobacterial molecules to treat NTM infections. Furthermore, one of the major challenges in the biodiscovery process is the supply problem, which would be overcome if we consider that laurinterol (**1**) is the major metabolite found in *L. johnstonii*, with an estimated abundance of 70% in the total crude extract. These results are of remarkable importance since NTM infections, especially those due to *M. avium* and *M. abscessus* complexes, have increased worldwide. It means an important concern for healthcare due to the intrinsic resistance to most conventionally utilized antimicrobials, with *M. abscessus* being one of the most drug-resistant mycobacteria [41]. Additionally, it has been reported the person-to-person transmission of *M. abscessus* among cystic fibrosis patients [42], overturning the general belief that

NTM infections are acquired through environmental sources. This poses a major challenge for drug development, where hit rates in primary screens for *M. abscessus* can be lower than 0.1%. Thus, generation of attractive lead compound represent a bottleneck [43].

In the search for potential compounds with anti-TB properties, those identified as active have MIC of 50-64 µg/mL. Nonetheless, less active or inactive derivatives are also recommended to be revisited if they highlight structure-activity relationships, in order to increase the opportunities to identify new antimycobacterial treatments [44,45]. Sesquiterpenes from *L. johnstonii* can be considered interesting lead compounds for the discovery of novel antimycobacterial molecules to treat NTM infections reinforcing the fact for that anti-NTM compounds may not be necessarily active against Mtb strains. Reinvestigation of known natural compounds and their synthetic derivatives will open the opportunities to access to undiscovered scaffolds as drug leads. In this context, the vast chemical diversity of marine natural products represents an immense opportunity to be exploited as new antimycobacterial drugs and although the progress, it is still necessary a systematic study that provides novel scaffolds for further optimization.

3. Materials and Methods

2.1 Extraction and isolation

Ethanolic extract of *Laurencia johnstonii* collected in Baja California Sur, Mexico was chromatographed in Sephadex LH-20 in order to obtain five fractions. Fraction 3 was rechromatographed by Flash Silicagel using a stepwise gradient from *n*-hexane to ethyl acetate. Fraction 3.2 (95% *n*-hexane) was partitioned in a Silicagel open column using a stepwise gradient from *n*-hexane to ethyl acetate to yield pure compounds **1** and **2** as previously described [17].

2.2 Mycobacterial strains and culture conditions

Six clinical isolates belonging to NTM, including three strains of *M. abscessus*, two of *M. intracellulare* and one of *M. fortuitum*, as well as nine strains of *M. tuberculosis* were obtained from the Laboratorio Interdisciplinario y de Investigación Dermatológica (LIID) belonging to the Hospital Universitario José E. González (Monterrey, Nuevo León, México). The strains were activated from the frozen stocks in Lowenstein-Jensen Medium and Blood Agar incubating at 37°C for 7-14 days.

2.3 Determination of the Minimum Inhibitory Concentration (MIC)

MIC, defined as the minimal concentration of compound which prevents visible growth of the bacteria, was determinated by measurement of turbidity in the wells using the broth microdilution method [46]. Susceptibility of Mtb to compounds **1** and **2** was evaluated using Alamar Blue [47] and *M. tuberculosis* H37Rv was used as a susceptible-strain control, meanwhile the antibiotic rifampicin was also tested for activity comparison.

Subject to NTM strains, cation-adjusted Mueller-Hinton broth (CA-MHB) was used for rapidly growing mycobacteria (*M. abscessus* and *M. fortuitum*) and Middlebrook 7H9 broth supplemented with oleic albumin dextrose catalase (OADC) for slowly growing mycobacteria (*M. intracellulare*) [46]. The MIC was determinated after 72 h at 37 °C for *M. abscessus* and *M. fortuitum* strains, and after 7 to 10 days for *M. intracellulare* strains. *Staphylococcus aureus* ATCC 29213 was used as external control and the antibiotics linezolid and imipenem at concentrations established by the CLSI (maximum 64 µg/mL) were used for activity comparison. Compounds **1** and **2** were evaluated from 3.12 to 100 µg/mL. The experiments were performed in duplicates.

4. Conclusions

With the emerging threat of drug resistance, particularly infections caused by NTM, our results highlight the relevance to revisit marine-sourced compounds as potential lead molecules in the development of new therapies. Laurinterol (**1**) showed a moderated activity (MIC 25-50 µg/mL) against six out of the nine *Mycobacterium tuberculosis* strains tested. However, it showed good activity

(MIC 6.25-25 μg/mL) against all NTM strains tested. In the case of *M. abscessus* LIID-01, LIID-02 and LIID-03, as well as *M. intracellulare* LIID-02 and M. *tuberculosis* CIPTIR -F296, the MIC values either improve or are similar to those showed by the reference drugs. Attending the abundance of laurinterol (**1**) in the whole extract of *L. johnstonii* and its anti-mycobacterial activity, this study opens the path for **1** to be considered a potential lead compound as well as to continue with structure-activity relationship studies and to evaluate its mode of action.

Author Contributions: E.V.V. and C.A.M.T. conceived and designed the experiments; S.G.D. performed the extraction and isolation experiments and wrote the paper; A.R.D.M. and J.J.F. analyzed the chemical data; K.L.L., C.A.M.T. and L.V.C. performed the antimycobacterial assays and analyzed the activity data; P.C.R. and E.V.V. contributed to the activity analysis data. All the authors reviewed and commented the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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