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ANTIMYCOBACTERIAL EVALUATION OF GERMACRANOLIDES

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

NIKOLAUS H. FISCHER,*† TIANSHENG LU,† CHARLES L. CANTRELL,† JOSÉ CASTAÑEDA-ACOSTA,† LEOVIGILDO QUIJANO‡ and SCOTT G. FRANZBLAU§

†Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803-1804, U.S.A.; ‡Instituto de Quimica, Universidad Autónoma de Mexico, Mexico, D.F., Mexico; §GWL Hansen's Disease Center, Laboratory Research Branch, P. O. Box 25072, Baton Rouge, Louisiana 70894, U.S.A.

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Key Word Index—Sesquiterpene lactones; germacranolides; antimycobacterial activity; *Mycobacterium tuberculosis*; *M. avium*

Abstract—The minimum inhibitory concentrations (MIC) against *Mycobacterium tuberculosis* and *M. avium* of parthenolide, costunolide, 1 (10)-epoxycostunolide and other germacranolide-type sesquiterpene lactones and derivatives were determined by use of a radiorespirometric bioassay. Structure-activity relationship studies with natural and semisynthetic sesquiterpene lactones suggested that the α-methylene-γ-lactone moiety is an essential, but not sufficient, structural requirement for significant *in vitro* activity against *M. tuberculosis* and *M. avium*.

INTRODUCTION

Tuberculosis (TB) is a bacterial disease caused mainly by *Mycobacterium tuberculosis* and *M. bovis*. It is estimated that one-third of the world's population is infected with TB [1, 2]. Therefore, it remains a major world health problem, in particular, since the incidence of multidrug-resistant TB has increased in many countries [1–3]. Furthermore, *M. avium* represents an opportunistic invader in immune-deficient individuals, in particular, AIDS patients [4]. These acute problems have led to a renewed initiative to search for new structural types of drugs effective against this infectious disease. Our interests are directed toward a bioassay-guided search for structurally new and novel antituberculosis natural products from extracts of higher plants [5]. In this effort, evaluation of a series of extracts of plants from the southeastern United States, using a radiorespiratory bioassay [5, 6], resulted in a number of extracts with significant antimycobacterial activity. For instance, bioassay-guided chromatographic separation of active extracts of the sea daisy (*Borrichia frutescens*) provided cycloartane-type triterpenes which exhibited significant *in vitro* activity against *M. tuberculosis* with minimum inhibitory concentrations (MICs) of $< 10\,\mu g/ml^{-1}$ [7].

In recent years, the germacranolide-type sesquiterpene lactone (SL) parthenolide (**3**) has received considerable attention due to its wide spectrum of biological activities, including cytotoxic, antitumor, antibacterial, and antifungal properties [8, 9]. Reports on its antiinflammatory activity [10] and its action as the major active principle in European feverfew commonly used for the prophylactic treatment of migraine [11, 12] have stimulated renewed interest in this germacranolide. We recently found that parthenolide and other SLs inhibit the expression of inducible cyclooxygenase (COX-2) and proinflammatory cytokines in macrophages, activities which correlated with the inhibition of mitogen-activated protein kinases (MAPKs) [13].

Considerable antimycobacterial activity was exhibited by a number of extracts of plants from the southeastern United States [5], including *Magnolia grandiflora* [14, 15] and *M. virginiana* [16]. Therefore, their pure constituents, costunolide (**1**) and parthenolide (**3**), as well as a series of related germacranolides and derivatives [14], were tested against *M. tuberculosis* and *M. avium*, using a respirometric bioassay [5–7].

The biological activity of SLs is generally attributed to the alkylating property of the α-methylene-γ-lactone moiety, and the presence of other alkylating sites (e.g. epoxides and conjugated carbonyl groups) may enhance their biological activities [8]. Germacradienolides, as well as their monoepoxide derivatives, possess configurational and conformational

*Author to whom the correspondence should be addressed. Phone: (504)-388-2695; Fax: (504)-388-2695; E-mail: fischer@chemgate.chem.lsu.edu.

Table 1. Antimycobacterial activity of costunolide (**1**), parthenolide (**3**) and derivatives against *Mycobacterium tuberculosis* and *M. avium*

Compound (Structure Number)	MIC ($\mu g\,ml^{-1}$) *M. tuberculosis*	*M. avium*
Costunolide (**1**)	32	128
11βH, 13-Dihydrocostunolide (**2**)	128	128
Parthenolide (**3**)	16	64
11βH, 13-Dihydro-parthenolide (**4**)	128	128
1,10-Epoxycostunolide (**5**)	64	128
1,10-Epoxy-11βH, 13-dihydrocostunolide (**6**)	128	128
1,10-Epoxyparthenolide (**7**)	128	128
1,10-Epoxy-11βH,13-dihydroparthenolide (**8**)	128	128
Santamarine (**9**)	64	>128
11βH, 13-Dihydrosantamarine (**10**)	>128	>128
Reynosin (**11**)	64	>128
11βH, 13-Dihydroreynosin (**12**)	>128	>128
Triol derivative of reynosin (**13**)	>128	>128
Triol derivative of 11,13-dihydroreynosin (**14**)	>128	>128
Triol derivative of santamarine (**15**)	>128	>128
Tamaulipin A angelate (**16**)	>128	>128
Rifampin[a]	0.25–0.125	—
Clarithroycin[a]	—	1–2

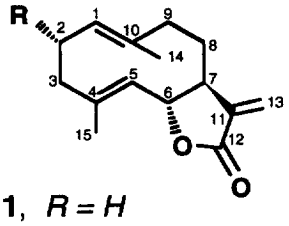
[a] Rifampin was used as a positive control against *M. tuberculosis* and clarithroycin against *M. avium*.

molecular arrangements which can undergo facile transannular cyclizations, processes proposed to be involved in the biosynthesis of biogenetically advanced SLs [9, 17, 18]. In these cyclizations, the cationic intermediates represent nucleophile receptors which might be involved in the biological actions of these compounds. Therefore, we tested a series of different germacranolide-type SLs and derivatives for their activity against *M. tuberculosis* and *M. avium* to learn about the functional groups essential for biological activity. The results of these chemical and biological studies are presented below.

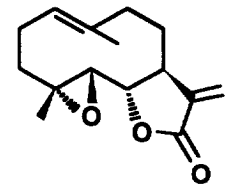
RESULTS AND DISCUSSION

The MICs of costunolide (**1**) [14], parthenolide (**3**) [14, 15] and 1(10)-epoxycostunolide (**5**) [14] as well as their semisynthetic derivatives [14–19] and references therein] are listed in Table 1. Costunolide (**1**), the most lipophilic lactone among this set of SLs, showed MICs against *M. tuberculosis* and *M. avium* of 32 and 128 $\mu g\,ml^{-1}$, respectively. Its 4,5-epoxide derivative, parthenolide (**3**), was the most active germacrolide against both *M. tuberculosis* and *M. avium*, with MICs of 16 and 64 $\mu g\,ml^{-1}$, respectively. The 1(10)-epoxycostunolide (**5**) was less active than **1** and **3**, with respective MICs of 64 and 128 $\mu g\,ml^{-1}$ against *M. tuberculosis* and *M. avium*. Except for tamaulipin A angelate (**16**) [20] and michelenolide (**7**) [19], α-methylene-γ-lactone-bearing SLs **1, 3, 5, 9** and **11** are moderately active against *M. tuberculosis* with MICs at 64 $\mu g\,ml^{-1}$ or below. In contrast, their 11βH,13-dihydroderivatives (**2, 4, 6, 10** and **12**) as well as the sesquiterpenes obtained by reductive opening of the lactone ring (**13–15**) [14] showed no activity against *M. tuberculosis* at concentrations below 128 $\mu g\,ml^{-1}$, suggesting that the presence of the exocyclic α-methylene-γ-lactone moiety is essential for activity. The data summarized in Table 1 and Fig. 1 also suggest that, besides the presence of the α-methylene-γ-lactone moiety, their lipophilicity values seem to play a role in antimycobacterial activity. Since the chemical composition of the cell walls of mycobacteria is highly lipophilic, they generally represent large barriers for the penetration of hydrophobic compounds and the transport of polar compounds through the outer lipid layer of mycobacteria is retarded [21, 22]. Contrary to lipophilicity considerations, the more polar epoxide parthenolide (**3**) exhibited higher antituberculosis activity than costunolide (**1**). Furthermore, in spite of the presence of the α-methylene-γ-lactone moiety in both, the 1(10)-epoxycostunolide (**5**) and the diepoxide michelenolide (**7**) [19], **5** and **7** are significantly less active than parthenolide (**3**).

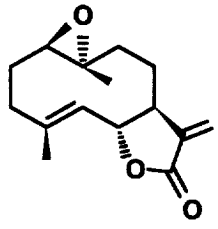
One possible explanation for the increase of biological activity of parthenolide (**3**) and related germacrolide monoexpoxides could be their unique property to undergo facile transannular cyclizations [17, 18]. Under mild Lewis acid conditions, 4,5-epoxides give guaianolides [14–18] and 1 (10)-epoxides form eudesmanolides [14, 18], processes which are proposed for the biogenesis of guaianolide- and eudesmanolide-type SLs [17, 18]. As outlined in Scheme 1, parthenolide (**3**) and structural analogs undergo a facile transannular cyclization to give a guaianolide-type cationic intermediate **A**, from which a number of



1, *R = H*
2, *11βH, 13-dihydro, R = H*
16, *R = OAng*



3
4, *11βH, 13-dihydro*



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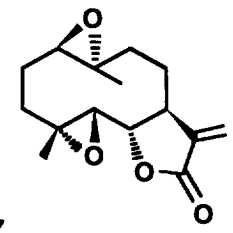
6, *11βH, 13-dihydro*



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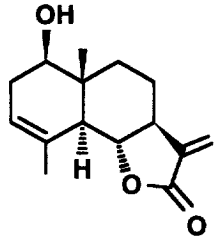
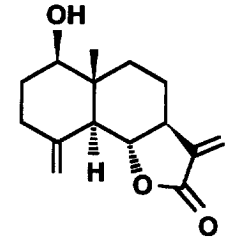
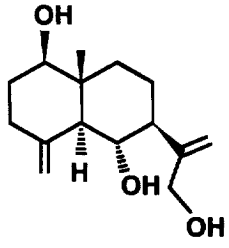
8, *11βH, 13-dihydro*



9
10, *11βH, 13-dihydro*



11
12, *11βH, 13-dihydro*



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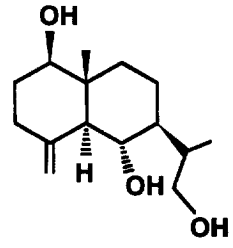

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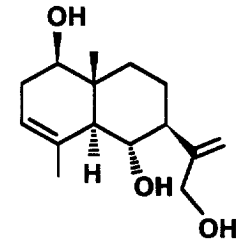
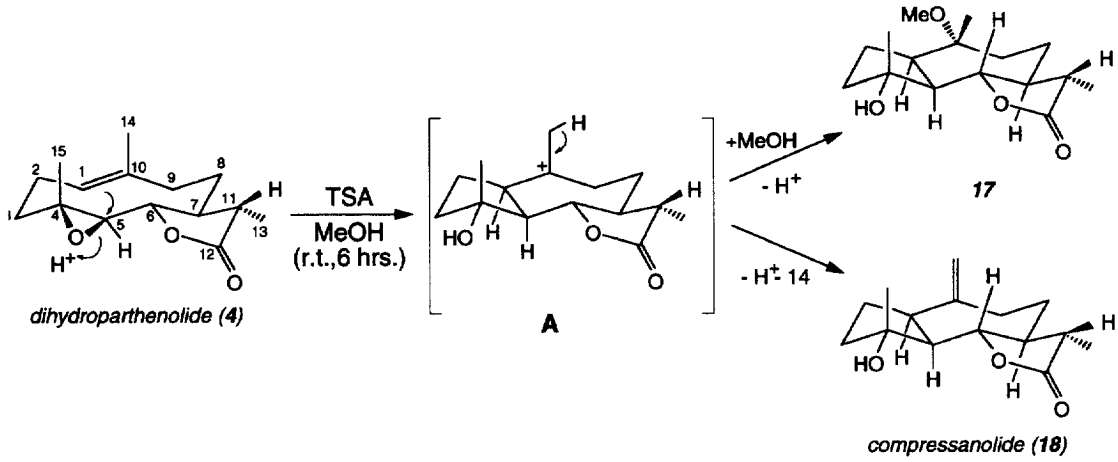


15

products can be formed [15, 17, 18] and references therein]. Therefore, in addition to the α-methylene-γ-lactone group, the electron-deficient site at C-10 of cation **A** could act as a second alkylation center to covalently bond essential nucleophiles at an active site of an enzyme causing irreversible inhibition.

Support for the above proposal was provided by an acid-catalysed transannular cyclization of dihydroparthenolide (**2**) in a nucleophilic protic solvent, methanol. Treatment of dihydroparthenolide (**2**) with toluenesulfonic acid (TSA) in methanol at room temperature afforded the new guaianolide derivative **17** as the major product and the known lactone compressanolide (**18**) [24] as a minor constituent (Scheme 1). Formation of compounds **17** and **18** involves initial transannular cyclization to give cation **A** which by loss of a proton from C-14 gives **18** and by nucleophilic addition of MeOH to C-10 and loss of a proton provides **17**. Compressanolide (**18**) was identified by comparison of its spectral data (^{1}H and ^{13}C NMR and MS) with reported values [24]. The mass spectrum of **17** showed a molecular ion at *m/z* 282 and further peaks



Scheme 1.

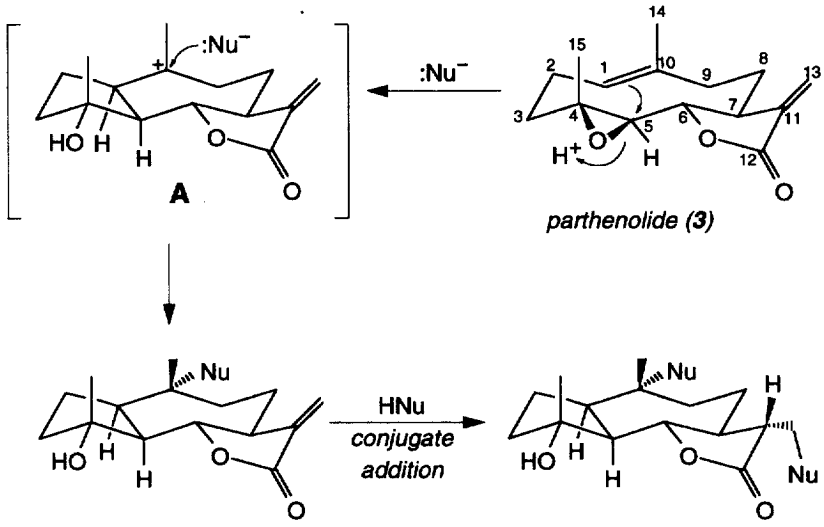
at m/z 264 $[M\text{-}H_2O]^+$, 250 $[M\text{-}MeOH]^+$, 232 $[M\text{-}H_2O\text{-}MeOH]$ and 217 $[232\text{-}Me]^+$, which were in agreement with the proposed structure. The ^{13}C NMR spectrum of **17** exhibited 16 carbon signals including one methoxy methyl signal at δ 48.1. Combined COSY, DEPT and inverse ^1H–^{13}C heteronuclear correlation methods allowed for the complete assignments of both the ^1H and ^{13}C NMR spectra of **17**. The ^1H NMR spectrum of **17** indicated the presence of four methyl signals with one three-proton singlet at δ 3.17 which was assigned to a methoxy methyl. When compared with the spectral data of guaianolide analogues [24, 25], the chemical shift of the C-10-Me singlet at δ 1.16 indicated that it is β-oriented, while the methoxy group is in the α-position. Inspection of molecular models of dihydroparthenolide (**2**) and the cationic intermediate **A** revealed that nucleophiles can approach the developing cationic center at C-10 only from the less hindered outer face resulting in the 10α-methoxy-substituted derivative **17** since the inner face is sterically inaccessible at C-10.

The antimycobacterial activities of other natural or semisynthetic germacranolide-type SLs (melampolide- and *cis, cis*-germacranolide-type α-methylene-γ-lactones) were generally lower with MIC values of 128 $\mu g\,ml^{-1}$ or higher. The following natural [17, 27] and references therein] and semisynthetic lactones [28] exhibited MICs of 128 $\mu g\,ml^{-1}$ against both *M. tuberculosis* and *M. avium*: cinerenin, its acetate, isobutyrate, hexanoate, palmitate and cinnamate ($> 128\,\mu g\,ml^{-1}$); melampodin B, its isobutyrate, hexanoate and palmitate [28]; enhydrin, leucanthin B, melampodin C acetate, melampodinin A and B, melampolidin, melcanthin A and B and melfusin [17, 27] and references therein]. The considerably more polar dilactones cinerenin and melampodin B and derivatives, which strongly inhibit release of serotonin from bovine platelets [29], showed no activity against *M. tuberculosis* and *M. avium* below 128 $\mu g\,ml^{-1}$. This suggests that the activity of these polar α,β-unsaturated dilactones is not as strongly influenced by their alkylating properties, but more likely by their low lipophilicity, which seems to significantly retard penetration of polar compounds through the outer lipid layer of mycobacteria [21].

In summary, qualitative structure-activity relationship studies with natural and semisynthetic SLs suggest that the α-methylene-γ-lactone moiety is an essential, but not sufficient, structural requirement for significant inhibition of *M. tuberculosis* and *M. avium*. The above data also suggest that the presence of a second alkylating site within a sesquiterpene lactone, together with a moderate to high lipophilicity [29] seems to enhance the *in vitro* antimycobacterial activity of SLs. In general, besides the essential α-methylene-γ-lactone group, further electron-deficient reaction sites, including α, β-unsaturated carbonyls, epoxides and/or carbocationic intermediates seem to enhance antimycobacterial activity.

As outlined in Scheme 2, we propose that the facile initial transannular cyclization of parthenolide (**3**) and germacrolide-4,5-epoxides, in general, give guaianolide-type cationic intermediates of type **A** as second alkylating sites. This could represent a general mechanism of action for antimycobacterial activity and for the broad spectrum of other biological activities of parthenolide and analogs [8–12, 29]. Although, transannular cyclizations are generally proposed to be involved in biosynthetic pathways from germacrolides to guaianolides and eudesmanolides [17, 18], this route has not been previously proposed as a path in the mechanisms of biological action of parthenolide and its analogs.

The molecular target(s) of the active SLs is (are) not known. As we were previously able to show, parthenolide (**3**) and its analogs are potent PTK inhibitors [13]. Therefore, it is reasonable to suggest that they could also affect mycobacterial protein kinases, phosphofructokinase and other essential enzymes inhibited by alkylating agents [8].



Scheme 2.

EXPERIMENTAL

Isolation and synthesis of sesquiterpene lactones

Compounds **1–12** were isolated or prepared by methods previously described [14, 17].

Acid-catalysed transformation of 11βH, 13-*dihydroparthenolide* (**4**)

Lactone **4** (52 mg) was dissolved in MeOH (10 ml) and *p*-toluenesulfonic acid (TSA) (120 mg) was added. The mixture was stirred at r t and monitored by TLC. After 6 hrs the reaction was completed and the solvent removed in *vacuo* to give a purple oil (150 mg). VLC separation of the crude product on silica gel using hexane as a mobile phase, followed by mixtures of hexane and EtOAc with increasing polarity, provided **17** (25 mg). In addition, an oil (10 mg) was isolated, which was shown to be identical with compressanolide (**18**) based on ^{1}H and ^{13}C NMR spectral, as well as mass spectral data, and correlations with published data [24].

α-*Hydroxy*-10α-*methoxyguaian*-12,6-*olide* (**17**)

$C_{16}H_{26}O_4$, oil; IR ν_{max}^{KBr} cm^{-1}: 3467 (OH), 1769 (C=O), 1458, 1381, 1131, 1072, 994; EIMS (rel. int.): 282 [M]$^+$ (1), 264 [M-H_2O]$^+$ (6), 250 [M-MeOH]$^+$ (4), 232 [264-MeOH]$^+$ (9), 217 [232-Me]$^+$ (5), 192 (11), 177 (7), 85 (100), 72 (22), 55 (38), 43 (81); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.78 (H-1, *ddd*, J=3.2, 7.8, 12.1 Hz), 1.52 (H-2β, m), 1.83 (H-2α, m), 1.82 (H-3, m), 2.25 (H-5, *dd*, J=12.1, 11.7 Hz), 4.25 (H-6, *dd*, J=11.7, 10.3 Hz), 1.78 (H-7, m), 1.42 (H-8a, m), 2.00 (H-8b, m), 1.63 (H-9β, m) 1.93 (H-9α, m), 2.22 (H-11, m), 1.23 (H-13, *d*, J=7.1 Hz), 1.16 (H-14, s), 1.34 (H-15, m), 3.17 (-OCH_3, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 45.9 (C-1), 25.5 (C-2), 39.2 (C-3), 80.2 (C-4), 54.9 (C-5), 82.9 (C-6), 51.1 (C-7), 25.8 (C-8), 37.4 (C-9), 78.0 (C-10), 41.3 (C-11), 177.9 (C-12), 12.9 (C-13), 21.9 (C-14), 23.8 (C-15), 48.1 (O-CH_3).

Antimycobacterial assays

All compounds were solubilized at 10.24 mg/ml in DMSO, filter sterilized and stored at −80°C until used. Subsequent dilutions were performed in DMSO. Fifty microliters of solutions were added to 4 ml BACTEC 12B broth (Becton Dickinson, Towson, MD) to achieve the desired final concentrations.

Radiorespirometric bioassay of Mycobacterium tuberculosis *and* M. avium cultures

Drug susceptibility testing was performed in the BACTEC 460 essentially as described by Heifets [30]. *Mycobacterium tuberculosis* H_{37}Rv was cultured in 4 ml BACTEC 12B broth until a daily growth index (GI) of 400–999 was reached and one-tenth ml of this was used to inoculate 4 ml fresh BACTEC 12B broth containing test compounds. *Mycobacterium avium* ATCC 25291 was cultured in BACTEC 12B broth until a daily GI of 999 was reached. Cultures were then diluted 1:25 in 7H12 broth and frozen at −80°C until needed. One tenth-ml of this solution was used to inoculate 4 ml fresh BACTEC 12B media containing test compounds. For both mycobacteria, additional controls diluted 1:100 were included. Cultures were incubated at 37°C and the GI determined daily starting on the third day of incubation. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of test sample which effected a daily GI increase and final GI lower than the 1:100-diluted control vial readings when the 1:100 GI was >30. This corresponds to the concentration which inhibited the growth of 99% of the organisms.

Experiments were usually completed within 5 days for *M. avium* and 10 days for *M. tuberculosis*.

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