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Bioactive metabolites from the Andean flora. Antituberculosis activity of natural and semisynthetic azorellane and mulinane diterpenoids

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Abstract Natural products are recognized as an important source of new and better pharmaceuticals for the treatment of diseases such as tuberculosis. The azorellane and mulinane diterpenoids represent an interesting group of bioactive metabolites produced by Andean plants belonging to the *Azorella*, *Mulinum*, *Laretia* and *Bolax* genus. Testing of natural and semisynthetic azorellanes and mulinanes against two *Mycobacterium tuberculosis* strains showed that while most changes in the structure of the natural metabolites result in the loss of antituberculosis activity, methylation of the C-20 carboxyl group improves the biological activity of the corresponding derivatives.

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Introduction

Tuberculosis is a highly contagious and mortal disease caused primarily by *Mycobacterium tuberculosis* (Ducati et al. 2006; Cataldi and Romano 2007). It is considered a health problem around the world, with 9.24 million new cases and 1.75 million deaths reported in 2007 (Bermejo et al. 2007; WHO 2009). Factors such as inappropriate administration practices, lengthy treatments and adverse secondary effects of currently available antituberculosis pharmaceuticals have favoured the development of multidrug-resistant (MDR) strains of *M. tuberculosis* (Basso et al. 2005; Del Olmo-Fernández et al. 2005), and in 2006 the WHO recognized the existence of extensively drug-resistant (XDR) strains of mycobacterium (Wright et al. 2006; WHO 2009). Because the disease represents a threat to world health, it is important to search and develop new antituberculosis agents with better activity, with novel chemical structures and/or with different mechanisms or sites of action to ensure efficacy and reduce secondary effects.

Presently, the importance of natural products as new and more efficient pharmaceuticals is well recognized (Newman and Cragg 2007). At the same time, the plant kingdom is also recognized as a prime source of natural products with a wide variety of chemical structures and biological activities (Verpoorte 1998). Some of the plant species that have been reported to posses antituberculosis activity include *Allium sativum, Borrichia frutescens, Ferula communis, Heracleum maximus*, *Karwinskia humboldtiana*, *Leucas volkensii*, *Monesses uniflora*, *Oplopanax horridus*, *Salvia multicaulis* and *Strobilanthus cusia* (Newton et al. 2000; Gautam et al. 2007); on the other hand, natural products with antituberculosis activity include lactones, phenols, quinones, alkaloids, peptides, terpenoids and steroids (Cantrell et al. 2001; Copp 2003; Okunade et al. 2004; Copp and Pearce 2007; De Souza 2009; Negi et al. 2009).

One plant family well known for producing bioactive metabolites is the Apiaceae family, which includes Andean plants belonging to the *Azorella*, *Bolax*, *Laretia* and *Mulinum* genus, recognized for producing unique diterpenoid structures having the novel mulinane and azorellane skeletons (Fig. 1) (Loyola et al. 1990a, b, 1991, 1996, 1997a, b, 1998, 2002). These diterpenoids have displayed a wide variety of interesting biological activities, including antiprotozoal (Neira et al. 1998; Loyola et al. 2001a, b, 2004), antibacterial (Wächter et al. 1999), antiviral (Abdel-Malek et al. 1996), spermicidal (Morales et al. 2003), cytotoxic (Mongelli et al. 1997, 2000), antihyperglycemic (Fuentes et al. 2005), antiinflammatory and analgesic (Delporte et al. 2003; Bórquez et al. 2007), and at least one mulinane diterpenoid, 9,12-cyclomulin-13-ol (**1**), has been reported as having antituberculosis activity (Wächter et al. 1998). As a result of this, and as part of a project directed towards the search for natural antituberculosis agents, we have recently investigated the in vitro antituberculosis activity of a series of natural and semisynthetic azorellane and mulinane diterpenoids, when tested against two strains of *Mycobacterium tuberculosis* using the Alamar-blue assay (Molina-Salinas et al. 2006, 2010a, b).

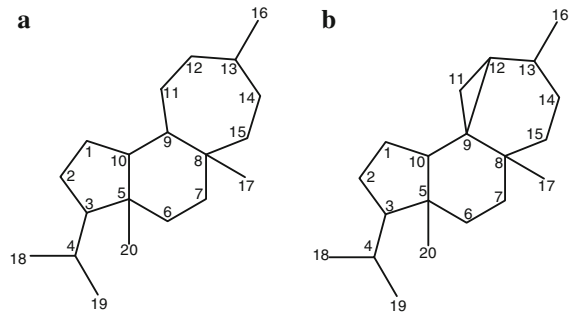

Fig. 1 Mulinane (**a**) and azorellane (**b**) diterpenoid skeletons

Results and discussion

The results of the antituberculosis activity evaluation of a first group of natural azorellane and mulinane diterpenes (Fig. 2), which are summarized in Table 1, showed that, in general, azorellanes appear to be more active than mulinanes, with **3** and **4**, followed by **2** and **6**, being the most active against both strains of *M. tuberculosis*, one susceptible to all current first-line antituberculosis drugs (SMtb) and a clinical multidrug-resistant isolate (RMtb). The only bioactive mulinane, **14**, displayed moderate antituberculosis activity. The antituberculosis activity of **2** is in agreement with that reported for its C-13 epimer,

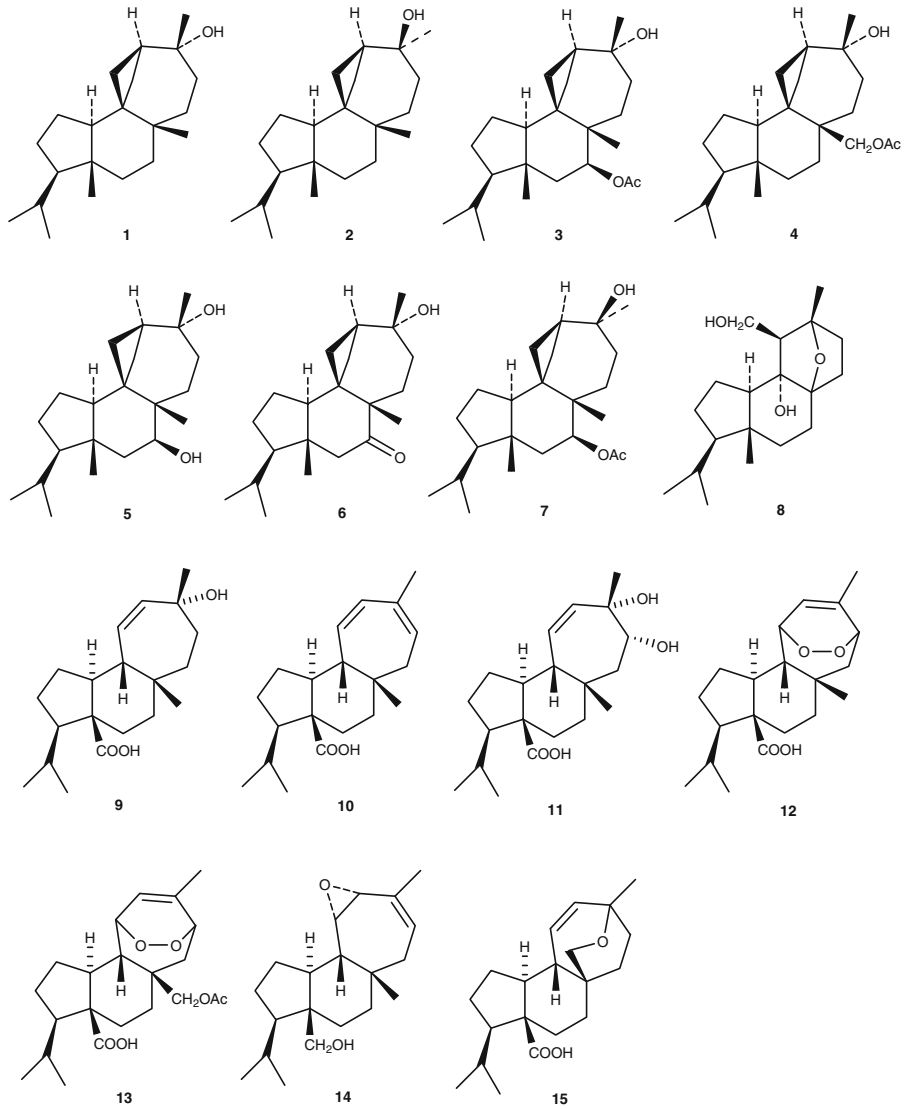

Fig. 2 Natural azorellanes and mulinanes evaluated for antituberculosis activity

13-α-hydroxy-azorellane (**1**), previously isolated from *A. madreporica* (Wächter et al. 1998). However, while **2** and its epimeric C-7-acetylated derivative **3** showed a similar level of activity, **7**, the C-7 acetylated derivative of **2**, was not active. Finally, the C-7 hydroxyl (**5**) and the C-7 oxo (**6**) azorellanes proved to be less active than the structurally related azorellanol (**3**) (Molina-Salinas et al. 2010a) (Fig. 3, 4).

Since it is known that chemical transformation of the functional groups in a molecule can improve its biological activity (Mascaretti 2003), a number of simple semisynthetic derivatives were prepared using the major azorellane (**3** and **6**) and mulinane (**10**, **9**, **13**, **15**) diterpenoids as starting materials. However, although natural mulinanes were easily derivatized, obtaining azorellane derivatives was hampered by the opening of the cyclopropane ring under weak acidic conditions; accordingly, azorellanol (**3**) and azorellanone (**6**) yielded the semisynthetic mulinanes **16** and **18**, respectively. Testing of the various semisynthetic derivatives for antituberculosis activity identified **19** and **23** as the most active mulinanes (Table 2). The results also confirmed the higher activity of azorellanes over mulinanes, when the opening of the cyclopropane ring in **3** and **6,** to produce **16** and **18**, resulted in the loss of antituberculosis activity. On the

Table 1 Antituberculosis activity of natural azorellane and mulinane diterpenoids

Diterpene	Name	Activity (MIC μg/ml)	
		SMtb[a]	RMtb[b]
2	13-*β*-hydroxy-azorellane	12.5	25
3	Azorellanol	12.5	12.5
4	17-acetoxy-13-α-hydroxy-azorellane	12.5	12.5
5	7-deacetyl-azorellanol	25	25
6	Azorellanone	12.5	25
7	13-epiazorellanol	100	50
8	Yaretol	100	50
9	13-α-hydroxy-mulin-11-en-20-oic acid	100	50
10	Mulin-11,13-dien-20-oic acid	50	25
11	13,14-*cis*-dihydroxy-mulin-11-en-20-oic acid	100	50
12	Mulinic acid	50	25
13	17-acetoxy-mulinic acid	100	50
14	Mulinol	25	12.5
15	Mulinenic acid	100	100
Rifampin	Positive control	0.062	100
Ofloxacin	Positive control	0.125	0.250

[a] SMtb: *Mycobacterium tuberculosis* ATCC 27294 a susceptible strain

[b] RMtb: *Mycobacterium tuberculosis* CIBIN/UMF15:99 a drug-resistant strain

other hand, while dihydroxylation of the C12–C13 double bond of **16** produced a more active diol (**17**), reduction of the C11–12 and C13–C14 double bonds of **10** led to a significant reduction in the antituberculosis activity of **21**. Finally, the results also showed that methylation of the natural products **9** and **15** yielded the corresponding derivatives **19** and **23** with a significantly higher activity. This last finding made the C-20 carboxyl group a potential target for the preparation of alkyl derivatives with improved antituberculosis activity.

Preparation and testing of eighteen linear and branched alkyl esters of five natural mulinanes allowed the identification of three derivatives, 13-hydroxy-

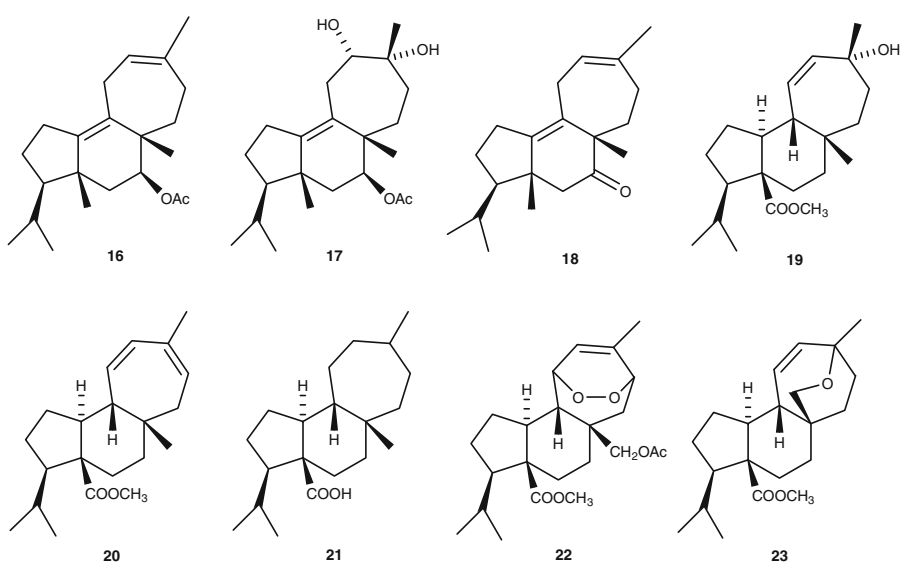

Fig. 3 Semisynthetic mulinanes evaluated for antituberculosis activity

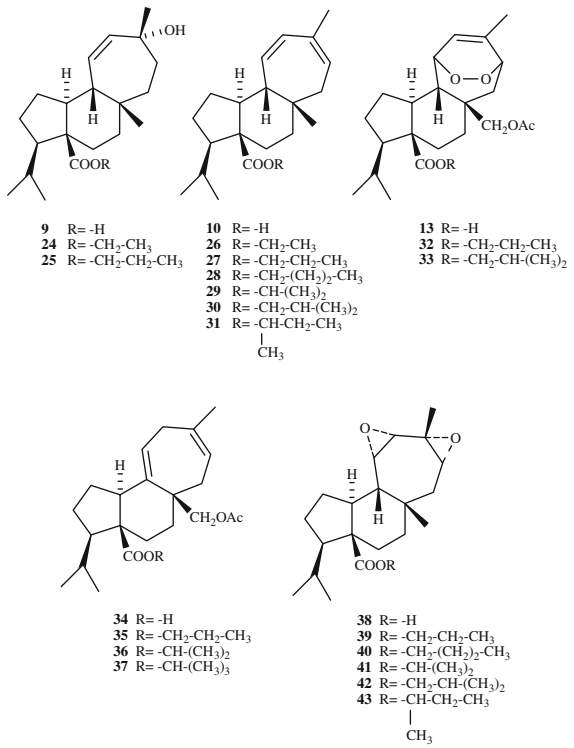

Fig. 4 Alkylated mulinanes evaluated for antituberculosis activity

mulin-11-en-20-oic acid-*n*-propyl ester (**25**) and the *n*-propyl (**39**) and *n*-butyl (**40**) esters of isomulinic acid, as the most active against the two strains of *M. tuberculosis* (Table 3). This first group of bioactive derivatives was followed by one including the ethyl ester of 13-hydroxy-mulin-11-en-20-oic acid (**24**) and the three alkyl-derivatives of mulin-11,13-dien-20-oic acid (**26**–**28**). Finally, a third group showing a good level of activity, but only against the resistant strain of *M. tuberculosis*, included the *n*-propyl ester of 17-acetoxy-mulinic acid (**32**), and the *iso*-propyl (**41**) and *iso*-butyl (**40**) esters of isomulinic acid (Molina-Salinas et al. 2010b).

It is interesting to point out that, in general, the bioactive C-20 alkyl-derivatives appeared to be more effective against the resistant strain of *M. tuberculosis* (RMtb) (Table 3). Additionally, it is worth mentioning that a linear C-20 alkyl ester group appears to be a required feature for the expression of activity in the majority of the derivatives, e.g. the antituberculosis activity of the *n*-propyl and *n*-butyl esters of isomulinic acid (**39** and **40**, respectively) is stronger than that of the branched ones (**41**-**43**), and the activity of the linear mulin-11,13-dien-20-oic acid alky-esters (**26**–**28**) is twice as strong as that of the branched ones (**29**–**31**), or the parent metabolite **10** (Molina-Salinas et al. 2010b).

The results presented here show that, although there doesn't seem to be a clear relationship between the structure of the various diterpenes and their antituberculosis activity, in general natural azorellane diterpenoids appear to be more active than mulinanes. Additionally, alkylation of the C-20 carboxyl group in the mulinane skeleton appears to improve the antituberculosis activity, with linear esters showing a better activity than their branched counterparts. Taken together, our observations

Table 2 Antituberculosis activity of semisynthetic mulinane diterpenoids

Diterpene	Name	Activity (MIC μg/ml)	
		SMtb[a]	RMtb[b]
16	7-acetoxy-mulin-9,12-diene	100	100
17	7-acetoxy-12,13-*cis*-dihydroxy-mulin-9-ene	25	25
18	7-oxo-mulin-9,12-diene	100	50
19	13-hydroxy-mulin-11-en-20-oic-acid methyl ester	12.5	12.5
20	Mulin-11,13-dien-20-oic acid methyl ester	25	12.5
21	Mulin-20-oic acid	100	50
22	17-acetoxy-mulinic acid methyl ester	100	50
23	Mulinenic acid methyl ester	12.5	12.5
Rifampin	Positive control	0.062	100
Ofloxacin	Positive control	0.125	0.250

[a] SMtb: *Mycobacterium tuberculosis* ATCC 27294 a susceptible strain

[b] RMtb: *Mycobacterium tuberculosis* CIBIN/UMF15:99 a drug-resistant strain

Table 3 Antituberculosis activity of alkylated mulinane diterpenoids

Semisynthetic mulinane	Name	Activity (MIC μg/ml)	
		SMtb[a]	RMtb[b]
24	13-hydroxy-mulin-11-en-20-oic-acid ethyl ester	25	12.5
25	13-hydroxy-mulin-11-en-20-oic-acid *n*-propyl ester	25	6.25
26	Mulin-11,13-dien-20-oic acid etyl ester	25	12.5
27	Mulin-11,13-dien-20-oic acid *n*-propyl ester	25	12.5
28	Mulin-11,13-dien-20-oic acid *n*-butyl ester	25	12.5
29	Mulin-11,13-dien-20-oic acid *iso*-propyl ester	50	25
30	Mulin-11,13-dien-20-oic acid *iso*-butyl ester	50	25
31	Mulin-11,13-dien-20-oic acid *sec*-butyl ester	50	25
32	17-acetoxy-mulinic acid *n*-propyl ester	50	12.5
33	17-acetoxy-mulinic acid *iso*-butyl ester	50	25
35	17-acetoxy-mulin-9(11),13(14)-dien-20-oic acid *n*-propyl ester	100	50
36	17-acetoxy-mulin-9(11),13(14)-dien-20-oic acid *iso*-propyl ester	50	25
37	17-acetoxy-mulin-9(11),13(14)-dien-20-oic acid *sec*-butyl ester	50	25
39	Isomulinic acid *n*-propyl ester	25	6.25
40	Isomulinic acid *n*-butyl ester	25	6.25
41	Isomulinic acid *iso*-propyl ester	50	12.5
42	Isomulinic acid *iso*-butyl ester	50	12.5
43	Isomulinic acid *sec*-butyl ester	100	50
Rifampin	Positive control	0.062	100
Ofloxacin	Positive control	0.125	0.250

[a] SMtb: *Mycobacterium tuberculosis* ATCC 27294 a susceptible strain

[b] RMtb: *Mycobacterium tuberculosis* CIBIN/UMF15:99 a drug-resistant strain

confirm the importance of esterification for improving activity and hint at the possibility of further increasing the potency of the natural acids by increasing the size of the ester substituent.

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