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Antituberculosis activity of natural and semisynthetic azorellane and mulinane diterpenoids

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ABSTRACT

The antituberculosis activity of 14 natural azorellane and mulinane diterpenoids isolated from *Azorella compacta*, *Azorella madreporica*, *Mulinum crassifolium*, and *Laretia acaulis*, together with eight semisynthetic derivatives, was evaluated against two *Mycobacterium tuberculosis* strains. The natural azorellanes azorellanol (**3**) and 17-acetoxy-13-α-hydroxyazorellane (**6**), and the semisynthetic mulinanes 13-hydroxy-mulin-11-en-20-oic-acid methyl ester (**13**) and mulinenic acid methyl ester (**23**), showed the strongest activity, with MIC values of 12.5 μg/mL against both strains. The methylated derivatives 13-hydroxy-mulin-11-en-20-oic-acid methyl ester (**13**), mulin-11,13-dien-20-oic acid methyl ester (**15**) and mulinenic acid methyl ester (**23**) proved to be more active than the parent compounds.

1. Introduction

The *Azorella*, *Bolax*, *Laretia* and *Mulinum* genus are recognized for producing unique diterpenoid structures having the novel mulinane and azorellane skeletons (Fig. 1). These diterpenoids have displayed a wide variety of interesting biological activities, including antiprotozoal [1–4], antibacterial [5], antiviral [6], spermicidal [7], cytotoxic [8,9], antihyperglycemic [10], and antiinflammatory and analgesic [11,12]. Additionally, at least one mulinane diterpenoid, 9,12-ciclomulin-13-ol (**1**), has been reported as having antituberculosis activity [13]. As part of a project directed towards the search for natural antituberculosis agents, we wish to report herein on the *in vitro* antituberculosis activity of a series of natural and semisynthetic azorellane and mulinane diterpenoids, when tested against two strains of *Mycobacterium tuberculosis*.

2. Experimental

2.1. Isolation of natural azorellane and mulinane diterpenoids

The various natural azorellane and mulinane diterpenoids were isolated from the aerial parts of *Azorella compacta*, *Azorella*

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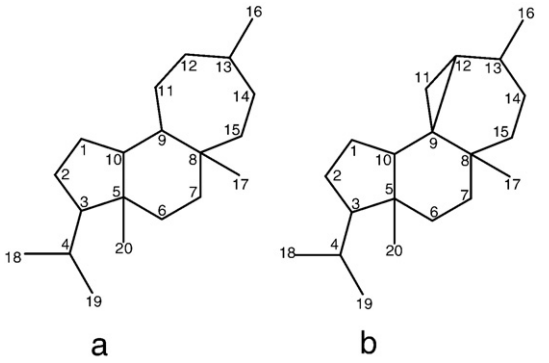

Fig. 1. Mulinane (a) and azorellane (b) diterpenoid skeletons.

madreporica, *Mulinum crassifolium* and *Laretia acaulis* following procedures described previously: 13-β-hydroxy-azorellane (**2**) [2]; azorellanol (**3**) [14]; 17-acetoxy-13α-hydroxy-azorellane (**6**) [15]; 7-deacetyl-azorellanol (**7**) [3]; azorellanone (**8**) [7]; 13-epi-azorellanol (**10**) [12]; yaretol (**11**) [16]; 13-hydroxy-mulin-11-en-20-oic acid (**12**) [17]; mulin-11,13-dien-20-oic acid (**14**) [18]; 13,14-dihiydroxy-mulinic-11-en-20-oic acid (**17**) [4]; mulinic acid (**18**) [19]; 17-acetoxy-mulinic acid (**19**) [20]; 13,20-dihydroxymulin-11-en (**21**) [21]; mulinenic acid (**22**) [22].

2.2. Preparation of azorellane and mulinane derivatives

2.2.1. Dehydration of azorellanol (**3**)

A solution of azorellanol (**3**, 230 mg) in acetone was cooled at 0 °C and treated with 0.5 mL of Jones reagent (CrO_3 in H_2SO_4); the mixture was stirred for 30 min at room temperature and then evaporated *in vacuo*. The residue was partitioned between EtOAc and H_2O and the EtOAc fraction was dried over anh Na_2SO_4; evaporation of the solvent produced a residue, which was purified by silica gel column chromatography (*n*-hex:EtOAc 98:2) to produce **4** (188 mg, 86.3%) in pure form. GC–MS: t_R 14.00 min; *m/z* 330.3 [M^+]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.38 (bd, *J* = 8 Hz; H–12), 4.93 (dd, *J* = 5.8, 10.6; H–7), 2.00 (s; Me–CO–), 1.56 (s; Me–16), 1.01 (s; Me–20), 0.97 (d, *J* = 6.4 Hz; Me–18), 0.93 (s; Me–17), 0.86 (d, *J* = 6.4 Hz; Me–19). ^{13}C NMR ($CDCl_3$, 100 MHz): 170.7 (s, –OCOMe), 142.0 (s, C–9), 136.6 (s, C–13), 131.8 (s, C–10), 121.4 (d, C–12), 25.8 (q, Me–16), 24.9 (q, Me–20), 22.9 (q, Me–18), 22.7 (q, Me–19), 19.8 (q, Me–17) ppm.

2.2.2. Hydroxylation of 7-acetoxy-mulin-9, 12-diene (**4**)

A portion (100 mg) of **4** was dissolved in a mixture of acetone:H_2O:t-BuOH (5:5:2, 12 mL); the solution was treated with 4-methylmorpholine N-oxide (48.5 mg) and a catalytic amount of OsO_4 and left to stir at room temperature for 24 h. The reaction mixture was neutralized with $NaHSO_4$ and partitioned between EtOAc and H_2O; the EtOAc fraction was washed with brine, dried over anh Na_2SO_4, and concentrated to give a residue which was further purified by silica gel column chromatography (*n*-hex:EtOAc 9:1) to yield 86.4 mg (78.3%) of pure **5**. GC–MS: t_R 17.31 min; *m/z* 364.3 [M^+]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.74 (dd, *J* = 6.4, 11.6 Hz; H–7), 3.10 (dd, *J* = 2.4, 11.2 Hz; H–12), 2.00 (s; Me–CO–), 1.22 (s; Me–13), 0.99 (d, *J* = 6.4 Hz; Me–18), 0.95 (s; Me–17), 0.94 (s; Me–20), 0.88 (d, *J* = 6.4 Hz; Me–19). ^{13}C NMR ($CDCl_3$, 100 MHz): 170.8 (s, –OCOMe), 147.2 (s, C–9), 127.2 (s, C–10), 76.6 (d, C–12), 72.1 (d, C–7), 71.7 (s, C–13), 54.1 (d, C–3), 29.0 (q, Me–16), 23.3 (q, Me–20), 23.0 (q, Me–19), 22.3 (q, Me–18), 21.6 (q, Me–17), 21.3 (q, MeCOO–) ppm.

2.2.3. Dehydration of azorellanone (**8**)

A solution of **8** (90 mg) in acetone was cooled at 0 °C and then treated with 0.5 mL of Jones reagent (CrO_3 in H_2SO_4); the mixture was stirred for 30 min at room temperature and then evaporated *in vacuo*. The residue was partitioned between EtOAc and H_2O and the EtOAc fraction was dried over anh Na_2SO_4; evaporation of the solvent produced a residue, which was purified by silica gel column chromatography (*n*-hex:EtOAc 98:2) to produce **9** (65 mg, 76.6%) in pure form. GC–MS: t_R 13.23 min; *m/z* 286.2 [M^+-C_3H_8]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.47 (bd, *J* = 8 Hz; H–12), 2.82 (d, *J* = 14.4, Hz; H–6), 2.21 (d, *J* = 14.4, Hz; H–6), 1.60 (s; Me–16), 1.19 (s; Me–17), 0.94 (d, *J* = 6.8 Hz; Me–18), 0.91 (d, *J* = 6.8 Hz; Me–19), 0.84 (s; Me–20). ^{13}C NMR ($CDCl_3$, 100 MHz): 218.8 (s, CO), 143.2 (s, C–13), 137.7 (s, C–10), 131.6 (s, C–9), 122.2 (d, C–12), 25.8 (q, Me–16), 24.9 (q, Me–20), 22.9 (q, Me–18), 22.7 (q, Me–19), 19.8 (q, Me–17) ppm.

2.2.4. Methylation of 13-hydroxy-mulin-11-en-20-oic acid (**12**), mulin-11,13-dien-20-oic acid (**14**), 17-acetoxy-mulinic acid (**19**) and mulinenic acid (**22**)

A portion (100 mg) of each mulinane (**12**, **14**, **19**, and **22**) was dissolved in Et_2O and treated with an ethereal solution of CH_2N_2. Each reaction was allowed to take place for 2 h at 0 °C and then the solvent was evaporated. The reaction products were purified by silica gel column chromatography to afford **13** (98 mg, 95.8%), **15** (97 mg, 92.7%), **20** (95 mg, 92.5%) and **23** (96 mg, 95.8%) in pure form.

13-hydroxy-mulin-11-en-20-oic acid methyl ester (**13**). GC–MS: t_R 14.93 min; *m/z* 334.2 [M^+]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.58 (d, *J* = 12.6 Hz; H–11), 5.58 (d, *J* = 12.6 Hz; H–12), 3.70 (s; MeOCO–), 1.32 (s; Me–16), 1.04 (d, *J* = 6.5 Hz; Me–19), 0.94 (s; Me–17), 0.86 (d, *J* = 6.5 Hz; Me–18). ^{13}C NMR ($CDCl_3$, 100 MHz): 175.0 (s, –COOMe), 136.3 (d, C–12), 133.6 (d, C–11), 58.5 (s, C–5), 57.4 (d, C–3), 51.5 (d, C–10), 33.5 (q, Me–16), 27.3 (q, Me–17), 22.7 (q, Me–18), 22.3 (q, Me–19) ppm.

Mulin-11,13-dien-20-oic acid methyl ester (**15**). GC–MS: t_R 16.81 min; *m/z* 272.2 [M^+-C_3H_8]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.60 (dd, *J* = 0.9, 12.6 Hz; H–12), 5.50 (dd, *J* = 5.6, 12.6 Hz; H–11), 5.45 (bd, *J* = 6.5 Hz; H–14), 3.66 (s; MeOCO–), 1.90 (m; H–1), 1.80 (s; Me–16), 1.10 (d, *J* = 6.2 Hz; Me–19), 0.94 (d, *J* = 6.2 Hz; Me–18), 0.85 (s, Me–17). ^{13}C NMR ($CDCl_3$, 100 MHz): 174.7 (s, –COOMe), 132.6 (d, C–11), 131.5 (s, C–13), 127.7 (d, C–12), 125.2 (d, C–14), 50.7 (q, –COOMe), 27.3 (q, Me–17), 25.8 (q, Me–16), 22.9 (q, Me–18), 22.5 (q, Me–19) ppm.

17-acetoxy-mulinic acid methyl ester (**20**). GC–MS: t_R 20.44 min; *m/z* 364.3 [M^+-C_2H_2O]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.12 (m; H–12), 4.56 (ddd, *J* = 1.6, 4.6, 7.4, Hz; H–11), 4.40 (bs; H–14), 4.06 (d, *J* = 10.9 Hz; H–17), 3.94 (d, *J* = 10.9 Hz; H–17), 3.70 (s; MeOCO–), 2.00 (s; MeCOO–), 1.88 (s; Me–16), 1.06 (d, *J* = 6.4 Hz; Me–18), 0.85 (d, *J* = 6.5 Hz; Me–19). ^{13}C NMR ($CDCl_3$, 100 MHz): 174.6 (s, –COOMe), 171.2 (s, –OCOMe), 136.9 (s, C–13), 124.1 (d, C–

12), 79.6 (d, C–14), 77.0 (d, C–11), 71.1 (t, C–17), 22.3 (q, Me–18), 22.1 (q, Me–19), 20.8 (q, –OCOMe), 20.5 (q, Me–16) ppm.

Mulinenic acid methyl ester (**23**). GC–MS: t_R 15.91 min; m/z 332.2 [M^+]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.66 (dd, J = 2.5, 10.5 Hz; H–12), 5.52 (dd, J = 2.0, 10.9 Hz; H–11), 3.78 (d, J = 10.0 Hz; H–17), 3.64 (s; –COOMe), 3.46 (dd, J = 2.0, 10.0 Hz; H–17), 1.20 (s; Me–16), 1.00 (d, J = 6.4 Hz; Me–18), 0.82 (d, J = 6.5 Hz; Me–19). ^{13}C NMR ($CDCl_3$, 100 MHz): 174.6 (s, –COOMe), 136.9 (d, C–12), 129.1 (d, C–11), 73.6 (t, C–17), 70.2 (s, C–13), 28.5 (q, Me–16), 22.4 (q, Me–19), 22.3 (q, Me–18), 20.8 (q, –COOMe) ppm.

*2.2.5. Reduction of mulin-11,13-dien-20-oic acid (***14***)*

A solution of **14** (477.5 mg) in 150 mL of methanol was subjected to catalytic hydrogenation using a constant pressure of 70 psi of H_2 and Pd/C as catalyst; the mixture was allowed to stir for 24 h at room temperature, filtered and evaporated *in vacuo*. The residue was purified by silica gel column chromatography to produce 474.5 mg of **16**, (98.7%) in pure form. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.39 (d, J = 6.5 Hz; Me–16), 0.95 (s; Me–17), 0.87 (d, J = 8.0 Hz; Me–18), 0.80 (d, J = 7.9 Hz; Me–19). ^{13}C NMR ($CDCl_3$, 100 MHz): 182.1 (s, –COOH), 49.6 (d, C–10), 31.7 (d, C–13), 30.7 (t, C–11),

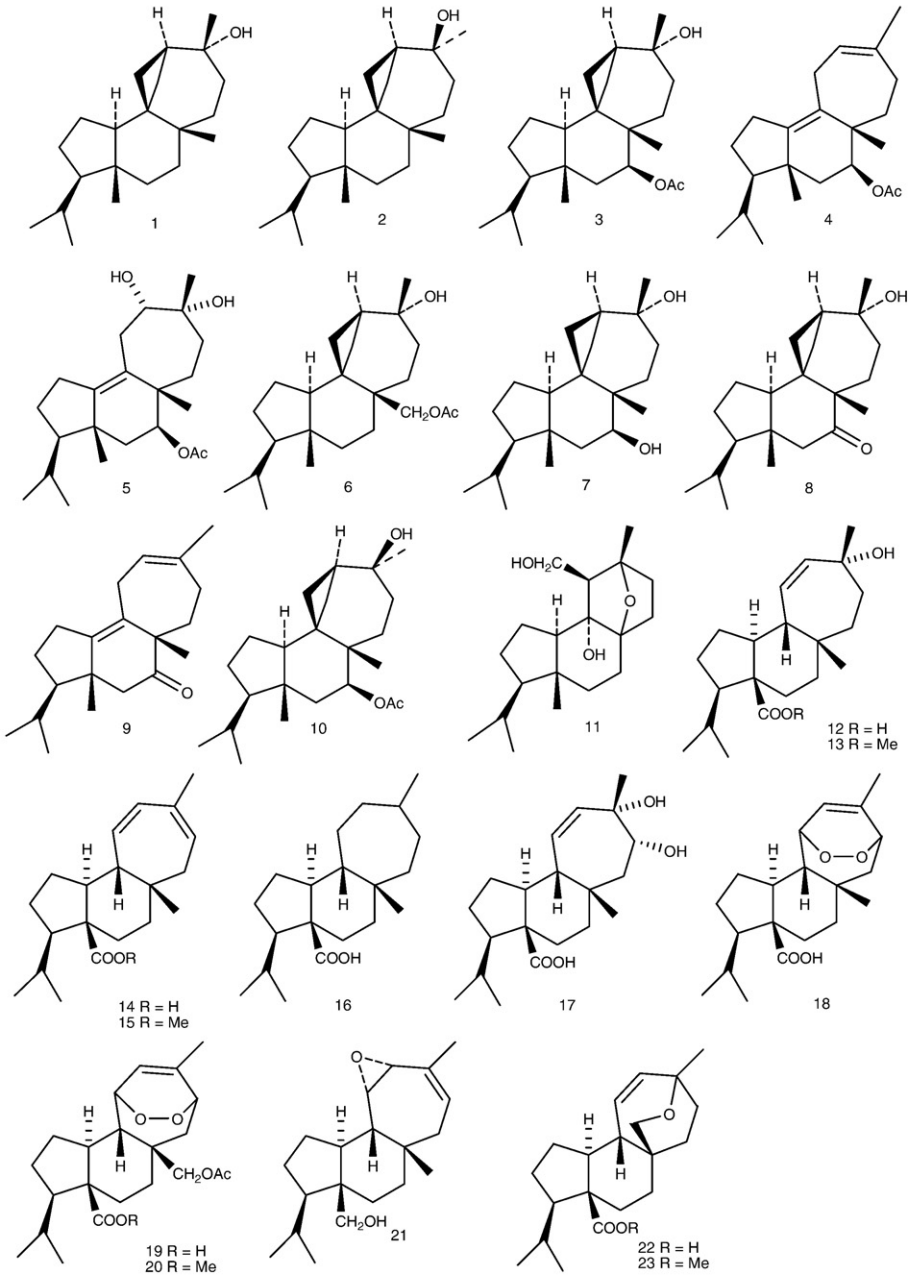

Fig. 2. Natural and semisynthetic azorellanes and mulinanes evaluated for antituberculosis activity.

30.5 (t, C–12), 28.5 (q, Me–17), 25.7 (q, Me–16), 24.7 (t, C–14), 22.7 (q, Me–19), 22.4 (q, Me–18) ppm.

2.3. Mycobacteria

Evaluation of antituberculosis activity was carried out using two strains: *M. tuberculosis* H37Rv (ATTC 27294) and a clinical isolate designated as CIBIN/UMF15:99. The first strain is susceptible to all five first-line antituberculosis drugs (streptomycin, isoniazid, rifampin, ethambutol, and pyrazinamide), while the second one is resistant to the same drugs.

2.4. In vitro antituberculosis activity

The antituberculosis activity of natural and semisynthetic products was determined by the Microplate Blue Alamar Assay [23]. Each product was tested using a concentration range of 200.00 to 3.13 μg/mL; results are reported as the minimal inhibitory concentration (MIC). In each microplate assay, rifampin and ofloxacin were included as positive internal controls, while culture medium and 2.5% (v/v) DMSO were included as negative and solvent controls, respectively. All evaluations were carried out in triplicate.

3. Results and discussion

The results of the antituberculosis activity evaluation of natural and semisynthetic diterpenes (Fig. 2) are summarized in Table 1. The evaluation of the antituberculosis activity of the various natural and semisynthetic products showed that the natural azorellanes **3** and **6**, and the semisynthetic mulinanes **13** and **23**, were the most active, with MIC values of 12.5 μg/mL, against both strains of *M. tuberculosis*; these were followed by azorellanes **2** and **8** which showed MIC values of 12.5 and 25 μg/mL against the drug sensitive and drug-resistant strains, respectively. Finally, diterpenes **5**, **7**, **15** and **21** displayed only moderate antituberculosis activity. The remaining natural and semisynthetic diterpenes proved inactive, with MIC values ≥50 μg/mL.

The antituberculosis activity of **2** is in agreement with that reported for its C–13 epimer, 13-α-hydroxy-azorellane (**1**, MIC 20 μg/mL), previously isolated from *A. madreporica* (Wächter et al. [5]). However, it is interesting to mention that while **2** and its epimeric C–7-acetylated derivative **3** show a similar level of activity (MIC = 12.5 μg/mL), **10**, the C–7 acetylated derivative of **2**, is not active (MIC = 100 μg/mL). Similarly, opening of the cyclopropane ring in **3** and **8**, to produce **4** and **9**, resulted in the loss of antituberculosis activity, while di-hydroxylation of the C12–C13 double bond of **4**, produced a diol (**5**) with improved antituberculosis activity. Finally, neither the C–7 hydroxyl (**7**) nor the C–7 oxo (**8**) derivatives showed a better activity than that of the corresponding parent metabolite, azorellanol (**3**).

On the other hand, evaluation of the various mulinane diterpenes showed that methylation of the natural products **12** and **22**, yielded methylated derivatives **13** and **23** that were eight-fold more active. Similarly, the methyl-derivative **15** showed an antituberculosis activity that was twice as potent as that of **14**. However **19** and its methyl-derivative **20** were both only moderately active. Finally, reduction of the C11–12 and C13–C14 double bonds of **14** led to a significant reduction in the antituberculosis activity of the reduced derivative **16**.

It is interesting to point out that, in general, the natural and semisynthetic diterpenes showed a stronger activity against the drug-resistant strain. The results obtained in this

Table 1
Antituberculosis activity of azorellane and mulinane diterpenoids.

Diterpene	Name	Metabolite skeleton [a]	Metabolite origin [b]	Activity (MIC μg/mL)	
				H37Rv	CIBIN 99
2	13-β-Hydroxyazorellane	A	N	12.5	25
3	Azorellanol	A	N	12.5	12.5
4	7-Acetoxy-mulin-9, 12-diene	M	SS	100	100
5	7-Acetoxy-12,13-cis-dihydroxy-mulin-9-ene	M	SS	25	25
6	17-Acetoxy-13-α-hydroxyazorellane	A	N	12.5	12.5
7	7-Deacetyl-azorellanol	A	N	25	25
8	Azorellanone	A	N	12.5	25
9	7-Oxo-mulin-9,12-diene	M	SS	100	50
10	13-Epiazorellanol	A	N	100	50
11	Yaretol	Y	N	100	50
12	13-Hydroxy-mulin-11-en-20-oic acid	M	N	100	50
13	13-Hydroxy-mulin-11-en-20-oic-acid methyl ester	M	SS	12.5	12.5
14	Mulin-11,13-dien-20-oic acid	M	N	50	25
15	Mulin-11,13-dien-20-oic acid methyl ester	M	SS	25	12.5
16	Mulin-20-oic acid	M	SS	100	50
17	13,14-Dihydroxy-mulin-11-en-20-oic acid	M	N	100	50
18	Mulinic acid	M	N	50	25
19	17-Acetoxy-mulinic acid	M	N	100	50
20	17-Acetoxy-mulinic acid methyl ester	M	SS	100	50
21	13,20-Dihydroxymulin-11-en	M	N	25	12.5
22	Mulinenic acid	M	N	100	100
23	Mulinenic acid methyl ester	M	SS	12.5	12.5
Rifampin	Positive control			0.062	100
Ofloxacin	Positive control			0.125	0.250

[a] A: azorellane, M: mulinane, Y: yaretane.
[b] N: natural, SS: semisynthetic.

evaluation show that, although there is no clear relationship between the structure of the various diterpenes and their antituberculosis activity, methylation of the C–20 carboxyl group of mulinanes **12**, **14**, and **22** results in a significant improvement on the activity of the corresponding methylated derivatives **13**, **15** and **23**. This finding makes the C–20 carboxyl group a suitable target for additional alkylation reactions. Presently, the formation of various long-chain esters, to improve lipophylicity of the resulting derivatives, is currently in progress in our laboratory; the results of these investigations will be published in due course.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fitote.2009.07.005.

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