Antituberculosis activity of natural and semisynthetic azorellane and mulinane diterpenoids

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A B S T R A C T

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.

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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-ciclolulin-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 compacta.
madreporica, *Mulinum crassifolium* and *Laretia acaulis* following procedures described previously: 13β-hydroxy-azorellanol (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-hydroxymulin-11-en-20-oic acid (12) [17]; mulin-11,13-dien-20-oic acid (14) [18]; 13,14-dihydroxy-mulin-11-en-20-oic acid (17) [4]; mulinic acid (18) [19]; 17-acetoxy-mulinic acid (19) [20]; 13,20-dihydroxymulin-11-en (21) [21]; mulinic 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 (CrO3 in H2SO4); the mixture was stirred for 30 min at room temperature and then evaporated in vacuo. The residue was partitioned between EtOAc and H2O and the EtOAc fraction was dried over anh Na2SO4; 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: *m/z* 330.3 [M⁺].

1H NMR (CDCl3, 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). 13C NMR (CDCl3, 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:H2O:t-BuOH (5:5:2, 12 mL); the solution was treated with 4-methylmorpholine N-oxide (48.5 mg) and a catalytic amount of OsO4 and left to stir at room temperature for 24 h. The reaction mixture was neutralized with NaHSO4 and partitioned between EtOAc and H2O; the EtOAc fraction was washed with brine, dried over anh Na2SO4, 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: *m/z* 364.3 [M⁺]. 1H NMR (CDCl3, 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–19), 0.88 (d, *J* = 6.4 Hz; Me–19). 13C NMR (CDCl3, 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 (CrO3 in H2SO4); the mixture was stirred for 30 min at room temperature and then evaporated in vacuo. The residue was partitioned between EtOAc and H2O and the EtOAc fraction was dried over anh Na2SO4; 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: *m/z* 13.23 min; *m/z* 286.2 [M⁺].

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 mulinic acid (22)

A portion (100 mg) of each mulinane (12, 14, 19, and 22) was dissolved in Et2O and treated with an ethereal solution of CH2N2. 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: *m/z* 149.93 min; *m/z* 334.2 [M⁺]. 1H NMR (CDCl3, 400 MHz): δ 5.58 (dd, *J* = 12.6 Hz; H–11), 5.58 (dd, *J* = 12.6 Hz; H–12), 3.70 (s; MeOOC–), 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). 13C NMR (CDCl3, 100 MHz): 175.0 (s, –COOME), 136.5 (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: *m/z* 16.81 min; *m/z* 272.2 [M⁺]. 1H NMR (CDCl3, 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–13), 1.90 (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). 13C NMR (CDCl3, 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: *m/z* 20.44 min; *m/z* 364.3 [M⁺]. 1H NMR (CDCl3, 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; MeOOC–), 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). 13C NMR (CDCl3, 100 MHz): 174.6 (s, –COOME), 171.2 (s, –COOME), 136.9 (s, C–13), 124.1 (d, C–
Mulinenic acid methyl ester (23). GC–MS: \( t_R \) 15.91 min; \( m/z \) 332.2 [M⁺]. \(^1\)H NMR (CDCl₃, 400 MHz): 6 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 (CDCl₃, 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 H₂ 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 (CDCl₃, 400 MHz): 6 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 (CDCl₃, 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 (ATCC 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 o xo (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>
<thead>
<tr>
<th>Diterpene Name</th>
<th>Metabolite skeleton</th>
<th>Metabolite origin</th>
<th>Activity (MIC μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Azorellanol</td>
<td>A</td>
<td>H37Rv 12.5, CIBIN 99 12.5</td>
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<tr>
<td>4</td>
<td>7-Acetoxy-mulin-9, 12-diene</td>
<td>M</td>
<td>H37Rv 100, CIBIN 99 100</td>
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<tr>
<td>5</td>
<td>7-Acetoxy-12,13-cis-dihydroxy-mulin-9-ene</td>
<td>M</td>
<td>H37Rv 25, CIBIN 99 25</td>
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<tr>
<td>6</td>
<td>17-Acetoxy-13-α-hydroxyazorellane</td>
<td>A</td>
<td>H37Rv 12.5, CIBIN 99 12.5</td>
</tr>
<tr>
<td>7</td>
<td>7-Deacetyl-azorellanol</td>
<td>A</td>
<td>H37Rv 25, CIBIN 99 25</td>
</tr>
<tr>
<td>8</td>
<td>Azorellanolone</td>
<td>A</td>
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</tr>
<tr>
<td>9</td>
<td>7-Oxo-mulin-9,12-diene</td>
<td>M</td>
<td>H37Rv 100, CIBIN 99 50</td>
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<tr>
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<tr>
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<td>18</td>
<td>Mulinic acid</td>
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<td>23</td>
<td>Mulinic acid methyl ester</td>
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<td>H37Rv 0.062, CIBIN 99 0.250</td>
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</table>

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 lipophlicity 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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