Note

Potent Limonoid Insect Antifeedant from *Melia azedarach*

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Received January 7, 2002; Accepted April 12, 2002

Systematic fractionation of a fruit extract from Argentine *Melia azedarach* L., which was monitored by an insect antifeedant bioassay, led to the isolation of meliartenin, a limonoid antifeedant, which existed as a mixture of two interchangeable isomers. At 4 μg/cm² and 1 μg/cm², the isomeric mixture was as active as azadirachtin in strongly inhibiting the larval feeding of *Epilachna paenulata* Germ. (Coleoptera: Coccinellidae) and the polyphagous pest, *Spodoptera eridania* (Lepidoptera: Noctuidae), respectively.

Key words: *Melia azedarach*; Meliaceae; meliartenin; insect antifeedant; limonoid

The continuous search for effective and more economical methods for pest control has turned attention to environmentally safe alternatives such as natural plant products which are now being investigated for their possible use in integrated management programs.

Limonoids from Meliaceae have attracted considerable interest because of their insect antifeedant activity and other insect control properties.1) Many limonoids have been isolated from *Azadirachta indica*,2–3) *Melia azedarach*,4–22) and *M. toosendan*,23–26) among others.22–26)

*M. azedarach* is native to India and China, but currently grows in a number of continents including Africa, Australia and the Americas. Particularly in Argentina, this tree is widespread and is used for timber and ornamental purposes. The root and stem bark of this tree have yielded sendanins,4–8) trichilins,8–10) azadirachins,8,9) meliacarpins,5–11) nimboalins12) and other types of limonoids12,13) that have been found to have cytotoxic and antifeedant activities. Moreover, sendanin,14) trendimexin,15) trichilins,16) meliacarpins,17–19) and others20,21) have also been obtained from the fruit, while meliacarpins22) have also been reported in the leaves, most of these compounds showing antifeedant properties.

Ripe fruit extracts of Argentine *M. azedarach* have shown a strong inhibitory effect on the feeding of *Xanthogalleruca luteola* (Coleoptera: Chrysomelidae) larvae and adults,27) and on several species affecting crops in central Argentina.28) We have, therefore, focussed our present study on the isolation and identification of the most active antifeedant compound present in the fruit.

The kernel of ripe fruit was defatted with hexane, extracted with ethanol, and finally partitioned with CH₂Cl₂. After removing the solvent, the residue was found to be a deterrent against *Epilachna paenulata* larvae in a leaf disk assay [antifeedant index (AI) = 99.5%].29) The viscous residue from the CH₂Cl₂ partition was twice flash-chromatographed, and radial chromatography provided a highly active fraction (AI = 93%, 400 ppm). This fraction was a mixture of two compounds, A (HPLC $t_R = 32.8$ min) and B (HPLC $t_R = 41.8$ min) in the ratio $A/B = 80–70:20–30$. An unsuccessful attempt to separate these compounds by column chromatography made it necessary to use preparative HPLC separation. Compounds A and B were collected in separate fractions identified as I and II, respectively. However, when the purity of each fraction was tested after separation, compounds A and B were found to be present in both fractions in the same ratio ($A/B = 80–70:20–30$) as that in the original sample. When fraction II was run immediately after collection, a ratio of $A/B = 20–30:80–70$ was observed, but in a few hours, it had changed back to a ratio of $A/B = 80–70:20–30$. The behavior of compounds A and B led us to conclude that they were interchangeable compounds. In order to identify these compounds, we explored the behavior of the $A-B$ equilibrium in CH₂CN and CHCl₃. Compound A was favored in CH₂CN ($A/B = 80–70:20–30$) while the molar fraction of B increased in CHCl₃ ($A/B = 53:47$). In subsequent bioassays, the mixture showed the highest activity among the limonoids present in the fruit extract.

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Abbreviation: AI, antifeedant index
Isomers A and B (1) were isolated as a white crystalline solid (17 mg). The IR spectrum showed absorption bands of hydroxyl groups (3450 cm⁻¹) and of two different carbonyl groups, one of ester (1725 cm⁻¹) and the other of ketone (1707 cm⁻¹). The El mass spectrum exhibited a molecular ion peak at m/z 532 [M⁺].

NMR spectra were acquired in CD₃CN. Extensive ¹H- and ¹³C-NMR studies on 1, including 1D and 2D experiments by DEPT, COSY, J-resolved, NOESY, COLOC and HETCOR, allowed us to assign all the peaks in the complex spectra.

The ¹H-NMR spectrum presented higher intensity signals together with similar ones at a lower intensity in a ratio of approximately 2:1, and in light of the behavior observed by HPLC, this characteristic of the spectrum was interpreted as signals belonging to two highly similar structures. The group of higher intensity signals was assigned as compound A, and that of lower intensity as compound B. Some protons in the latter could not be assigned because of overlapping with the signals of the major compound. In the decoupled ¹³C spectrum, larger signals were observed, each accompanied by another of lesser intensity, with a 0.03–3.65 ppm distance between them. Even though the intensity of the signals in the ¹³C spectrum was not directly proportional to the molar concentration of the atom/molecule it represents, the signals were assigned by the same criteria as those used for the ¹H spectrum.

¹³C resonances (Table 1) and DEPT data indicate that 1 contained 4 CH₃, 3 CH₂, 3 CH, 4 quaternary carbons in the region δc = 40–50, one primary and five secondary alcohol groups and a quaternary carbon in the region δc = 58–80, one hemiacetal signal at δc = 96, and four characteristic furane carbons and two carbons (1 acyloxy and 1 ketone) not bonded to hydrogen.

Compound A showed a signal at δH = 2.84 (1 H, dd, J = 11.0, 6.4 Hz, H^17) coupled to the protons of an adjacent methylene at δH 1.93 (1 H, m, H^16β) and at δH 2.21 (1 H, m, H^16α). Since the signal at δH = 2.84 showed W-type long range coupling to an axial methyl at δH = 1.07 (1H, s, Me^18), which was observed in the ¹H-¹H COSY experiment, the proton at δH = 2.84 was trans axial to the methyl, so it was assigned to H^17. The coupling of the proton at δH 3.73 (1H, br s, H^15) to the chemical shift at 1.93 and at 2.21 could not be clearly observed in the ¹H-NMR spectrum. It was visible, however, in the ¹H-¹H COSY spectra, although it was still not possible to measure the spin-spin coupling constants. This observation has also been found in the case of the dumsin limonoid.³⁰) The proton signals at δH 4.24 (1 H, br t, J = 4.0 Hz), δH 1.72 (1 H, dt, J = 16.0, 1.4 Hz), δH 2.70 (1 H, dt, J = 16.1, 4.8 Hz) and at δH 5.11 (1 H, dd, J = 1.3, 4.8 Hz), with their corresponding ¹³C-NMR signals, were assigned to H^1, H^2β, H^2α, and H^3, respectively. NOESY correlation of δH 3.52 (H^7) with δH 3.73 (H^15) and δH 1.01

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Fig. 1. Structures of 12-Hydroxyamoorastatin (A) and Meliartenin (B).
(MeA-30), of $\delta_{\text{H}}$ 4.47 (H-9) with $\delta_{\text{H}}$ 1.07 (MeA-18), and of $\delta_{\text{H}}$ 4.17 (H-19a) with $\delta_{\text{H}}$ 2.70 (H-2a) and MeA-30 ($\delta_{\text{H}}$ 1.01) support structure A.

The COLOC experiment enabled us to assign all the non-protonated carbons, showing that the protons of the 28-methyl group in A ($\delta_{\text{H}}$ 0.75) were coupled to C$^\text{A}$-4 ($\delta_{\text{C}}$ 40.15). Furthermore, since H-9 and MeA-30 were both coupled to the carbon at $\delta_{\text{C}}$ 42.70, this signal was assigned to C$^\text{B}$-8. Similarly, the COLOC data showed coupling between C$^\text{A}$-13 ($\delta_{\text{C}}$ 66.62) and the protons attached to both C$^\text{A}$-18 and C$^\text{A}$-30. The presence of a $\beta$-substituted furane was confirmed by comparing the chemical shifts with those of model compounds of azedarachs and by the coupling of signals observed in the $^1$H-$^1$H COSY data.

An analysis of the NMR data enabled us to conclude that isomer A was 12-hydroxyamoorastatin. This compound has previously been isolated from the root bark of M. azedarach, and its cytotoxic and antifeedant activity as azadirachtin, both compounds being more active than toosendanin.

Compound B showed similar characteristics to those of compound A, as can be seen from the chemical shifts of $^1$C (Table 1) and $^1$H. Most of the correlations in 2D described for compound A were also detected for the signals assigned to compound B. The differences between both structures lie in the fact that a doublet with a chemical shift at $\delta_{\text{H}}$ 4.44, assigned to H$^\text{B}$-9 (1 H, d, $J_{\text{H}}$ = 12.1 Hz), showed coupling to a secondary alcohol proton H$^\text{B}$-11 signal at $\delta_{\text{H}}$ 3.97 (1 H, d, $J_{\text{H}}$ = 12.1, 1.0 Hz), which was confirmed by the COSY correlation, and also coupling to an axial methyl at $\delta_{\text{H}}$ 1.04 (3 H, s, Me$^\text{B}$-30) which was observed in the COSY spectrum. The former observation strongly suggests that the methyl group at C-8 and the H-9 signal were trans to each other in W-type configuration. Moreover, the magnitude of the spin-spin coupling constant between H$^\text{B}$-9 and H$^\text{B}$-11 suggests that the latter was also axial. Since the chemical shift at $\delta_{\text{H}}$ 3.97 was not further coupled, the ketone group was assigned to position C$^\text{B}$-12. The H$^\text{B}$-11 signal then showed NOE connectivity with the MeA-30 signal at $\delta_{\text{H}}$ 1.04, this result being consistent with the assignment of the $\beta$-hydroxy group to C$^\text{B}$-11. We have named compound B meliartenin. Our observations indicate that the isomerism between A and B depended on the solvent. Similar isomerization (acid-induced) has been reported for trichilin A. As the presence of the isomer B could have been due to an artifact, we carefully analyzed the CH$_2$Cl$_2$-soluble extract by TLC and HPLC, and both isomers were always observed.

Compound I was tested for its antifeedant properties by a modified leaf disk method, showing a strong effect on the larvae of E. paenulata and S. eridania. Reference bioassays with azadirachtin and toosendanin were simultaneously made (Tables 2 and 3), in which the mixture exhibited the same level of activity as azadirachtin, both compounds being more active than toosendanin.

### Experimental

**General.** IR spectra (film) were recorded by a Shimadzu FTIR-8501 spectrometer. $^1$H- and $^13$C-NMR spectra were obtained by Serveis Cientifico-Tecnics-University of Barcelona with a Bruker AC 500 spectrometer operated at 500 MHz for $^1$H and at 125 MHz for the $^1$C nucleus in CD$_3$CN and CDCl$_3$, using tetramethylsilane as an internal standard. UV spectra were recorded in CH$_3$CN by a Shimadzu UV-260 spectrophotometer, and optical rotation angles were recorded with a Jasco DIP-370 polarimeter. HPLC was performed in a Phenomenex Prodigy 5 μODS (10 mm i.d. × 250 mm) column with UV detection at 210 nm. MS spectra were measured with a ZAB SEQ (BeqQ) instrument (VG Analytical, Manchester).

**Plant material.** Ripe fruits of M. azedarach L. were collected in Córdoba, Argentina in October, 1996. A voucher specimen has been deposited at the Botanical Museum of Córdoba (CORD 229, Córdoba, Argentina).

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### Table 2. Antifeedant Indices for I, Azadirachtin and Toosendanin against *Epilachna paenulata* Larvae

<table>
<thead>
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<th>Concentration (μg/cm$^2$)</th>
<th>Antifeedant index (%)</th>
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<tr>
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<td>I$^a$</td>
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<tr>
<td>10</td>
<td>90.7$^b$</td>
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<tr>
<td>8</td>
<td>96.1$^* b$</td>
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</table>

$^a$ dissolved in CH$_3$CN. $^b$ $p<0.05$, Wilcoxon signed rank test

### Table 3. Antifeedant Indices for I, Azadirachtin and Toosendanin against *Spodoptera eridania* Larvae

<table>
<thead>
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<th>Concentration (μg/cm$^2$)</th>
<th>Antifeedant index (%)</th>
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<tr>
<td></td>
<td>I$^a$</td>
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<td>14</td>
<td>87.8$^* a$</td>
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<td>95.5$^* b$</td>
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$^a$ dissolved in CH$_3$CN. $^b$ $p<0.05$; $^* p<0.01$, Wilcoxon signed rank test
Chemicals. Azadirachtin was purchased from Sigma Chemical Company, and toosendanin was presented by Dr. M. B. Isman (Department of Plant Science, University of British Columbia, Vancouver, British Columbia, Canada).

Extraction and isolation. Air-dried kernels of ripe fruits (290 g) of *M. azedarach* were extracted with ethanol after defatting with hexane to yield 10.5 g of an extract. This extract was concentrated under vacuum, dissolved in 320 ml of MeOH-CH₂Cl₂-H₂O (16:5:11) and then partitioned three times with CH₂Cl₂. The CH₂Cl₂-soluble extract (4.5 g) was flash-chromatographed on SiO₂, and eluted first with petroleum ether containing 1–9.5 % Et₂O and then with an Et₂O-Me₂CO-MeOH mixture in a gradient. The triterpenoid fractions, which were eluted with 1–10 % Et₂O-Me₂CO, showing antifeedant activity (AI = 81 %, 500 ppm) were rechromatographed in a flash column with a petroleum ether-etO-Me₂CO solvent system in a gradient. Those fractions with antifeedant activity above 90 % were then separated by successive radial chromatography with a gradient mixture of petroleum ether-Et₂O-Me₂CO. The resulting limonoid fraction was separated by HPLC, using reversed-phase column and eluting with 32 % acetonitrile in water, to yield 1 (17 mg).

Compound 1. C₂₆H₃₆O₁₀; mp 243–244 °C from Me₂CO; [α]D = –43.7 °c (0.4, CH₃CN); UV λmax (CH₃CN) nm (ε) = 204 (6032); EIMS m/z: 532 (M⁺), 514 (M⁺-H₂O), 496 (M⁺-2 H₂O), 478 (M⁺-3 H₂O), 472 (M⁺-AcO), 454 (M⁺-H₂O-AcO), 408, 311, 239, 163, 94.

Isomer A (12-hydroxyamoorastatin). tR 32.8 min (by HPLC). ¹H-NMR (CD₃CN) δ: 0.75 (3H, s, Me-28), 1.01 (3H, s, Me-30), 1.07 (3H, s, Me-18), 1.60 (1H, dt, J = 14.3, 3.9 Hz, H-6o), 1.72 (1H, dt, J = 16.0, 1.4 Hz, H-2β), 1.85 (1H, dd, J = 14.1, 2.2 Hz, H-6β), 1.93 (1H, m, overlapping H-16β and CH₃CN), 1.99 (3H, s, COCH₃), 2.21 (1H, m, H-16a), 2.66 (1H, dd, J = 13.6, 5.1 Hz, H-5), 2.70 (1H, dt, J = 16.1, 4.8 Hz, H-2α), 2.84 (1H, dd, J = 11.0, 6.4 Hz, H-17), 3.52 (1H, dd, J = 5.5, 3.3 Hz, H-7), 3.73 (1H, br s, H-15), 3.91 (1H, d, J = 2.0 Hz, H-12), 4.16 (1H, d, J = 12.6 Hz, H-19b), 4.17 (1H, d, J = 12.5 Hz, H-19a), 4.24 (1H, br t, J = 4.0 Hz, H-1), 4.47 (1H, s, H-9), 4.70 (1H, dd, J = 5.1, 0.9 Hz, H-3 endo), 4.78 (1H, d, J = 4.3 Hz, H-29), 5.11 (1H, dd, J = 4.8, 1.3 Hz, H-3 exo), 6.50 (1H, dd, J = 1.85, 0.5 Hz, H-22), 7.26 (1H, q, J = 1.2 Hz, H-21), 7.34 (1H, t, J = 1.7 Hz, H-23). ¹³C-NMR data are presented in Table 1.

Isomer B (meliartinin). tR 41.7 min (by HPLC). ¹H-NMR (CD₃CN) δ: 0.82 (3H, s, Me-28), 1.04 (3H, s, Me-30), 1.06 (3H, s, Me-18), 1.70 (1H, dt, J = 16.0, 1.4 Hz, H-2β), 2.01 (3H, s, COCH₃), 2.57 (1H, dd, J = 13.6, 4.1 Hz, H-5), 2.72 (1H, dt, J = 16.1, 4.8 Hz, H-2α), 2.85 (1H, dd, J = 11.2, 6.0 Hz, H-17), 3.48 (1H, dd, J = 5.9, 3.6 Hz, H-7), 3.72 (1H, br s, H-15), 3.97 (1H, dd, J = 11.6, 1.0 Hz, H-11), 4.12 (1H, d, J = 11.9 Hz, H-19b), 4.13 (1H, d, J = 11.6 Hz, H-19a), 4.44 (1H, d, J = 12.0 Hz, H-9), 4.29 (1H, br t, J = 4.4 Hz, H-1), 4.66 (1H, d, J = 3.2 Hz, H-29), 6.51 (1H, dd, J = 2.0, 0.6 Hz, H-22). ¹³C-NMR data are presented in Table 1.

Antifeedant activity. The antifeedant potential of 1 was tested on *E. paenulata* larvae by a modified leaf-disk-choice test. Two cotyledon leaves from a *Cucurbita maxima* seedling were placed in a Petri dish, and a glass disk with two holes was placed on top. A larva III of *E. paenulata* was placed equidistant from both a treated and untreated (solvent control) leaf disk, and allowed to eat for 24 h. The relative amounts of the treated and untreated leaf area eaten by the larva were visually estimated (the leaf damage provoked by this insect did not allow an evaluation by a video camera) and then compared by using the Wilcoxon signed paired test. Fifteen replicates were made for each dose. A comparable technique was used for *S. eridania* larvae, but in this case the glass disk was not used, and true 1 cm² leaf disks cut from *C. maxima* leaves were used instead. The results were analyzed visually, and with a video camera. In both tests, the antifeedant index was calculated as AI = (1-T/C)×100, where T and C represent the consumption of treated and untreated disks, respectively.

Acknowledgments

Financial support for this work was provided by Centro de Excelencia en Productos y Procesos de la Provincia de Córdoba and by Secretaria de Ciencia y Técnica (Secty-UNC). MCC gratefully acknowledges receipt of a fellowship from CONICET. We are grateful to Prof. M. B. Isman, University of British Columbia, for the methodology of raising *Spodoptera* species.

References

Potent Insect Antifeedant from *M. azedarach*


