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Insect antifeedant compounds from *Nothofagus dombeyi* and *N. pumilio*

Odile Thoison [a], Thierry Sévenet [a,*], Hermann M. Niemeyer [b], Graeme B. Russell [c]

[a] *Institut de Chimie des Substances Naturelles, CNRS, Avenue de la Terrasse, Bat. 27, F-91198, Gif-Sur-Yvette Cedex, France*
[b] *Departmento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago, Chile*
[c] *Horticultural Research Institute, Palmerston North, New Zealand*

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Abstract

A bioassay-guided purification of the extracts of *Nothofagus dombeyi* and *N. pumilio* leaves yielded several triterpenes and flavonoids including 2-*O*-acetylmaslinic acid, 3-*O*-acetyl 20,24,25-trihydroxydammarane, and 3,20,24,25-tetrahydroxydammarane as new natural products. All the isolated compounds were assessed for antifeeding activity against the 5th instar larvae of *Ctenopsteustis obliquana*. 12-Hydroxyoleanolic lactone and pectolinarigenin from *N. dombeyi* and dihydrooroxylin A from *N. pumilio*, showed significant antifeeding activity.
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Keywords: *Nothofagus dombeyi*; *Nothofagus pumilio*; Fagaceae; Insect antifeeding activity; Flavonoids; Triterpenes

1. Introduction

The genus *Nothofagus* Bl. (Fagaceae) constitutes the most important forest cover in both Chile and New Zealand (Tanai, 1986) and its wide distribution, longevity and success in forest formation, make it eminently suitable for studying the phytochemical interactions that may have arisen as the genus co-evolved with invertebrates. In a recent investigation (Russell et al., 2000) of bioactivity and patterns of herbivory within the genus *Nothofagus*, we identified several species which showed significant antifeeding activity against the larvae of the leafrollers, *Ctenopsteustis obliquana* and *Eiphyas postvittana.* The antifeeding activity of leaf extracts of *N. alessandri* Espinosa, from Chile and of *N. fusca* (Hook f.) Oerst., from New Zealand was shown to be due to the combined presence of both the stilbene pinosylvin and the flavonoid galangin. We have now investigated two further Chilean species for antifeeding compounds, *Nothofagus dombeyi* (Mirb.) Oerst and *N. pumilio* (Pepp & Endl.) Oerst.

2. Results and discussion

The ethyl acetate extracts of dried leaves of *N. dombeyi* and *N. pumilio* were chromatographed on silica gel, eluting with a gradient mixture of *n*-heptane, EtOAc and MeOH. Three fractions in each plant extract were found to have antifeeding activity in the two-way choice bioassay using 5th instar larvae of *C. obliquana* and were subjected to further chemical analysis.

From the active fractions of *N. dombeyi*, six compounds were isolated. They were identified from their MS and NMR spectra and by comparison with literature data and included, lupeol, cabraleone (**1**), 2- and 3-*O*-acetylmaslinic acids (**2,3**), pectolinarigenin (**4**), and 12-hydroxyoleanolic lactone (**5**). The active fractions of *N. pumilio* gave, cabraleadiol monoacetate (**6**), β-sitosterol, 5-hydroxytetramethoxyflavone (**7**), betulonic acid, dihydrooroxylin A (**8**), oleanolic acid, ursolic acid, maslinic acid (**9**), 3-*O*-acetyl-20,24,25-trihydroxydammarane (**10**), and its 3-*O*-deacetyl derivative (**11**). Of these

* Corresponding author. Tel.: +33-169-82-31-03; fax: +33-169-07-72-47.
E-mail address: sevenet@icsn.cnrs-gif.fr (T. Sévenet).

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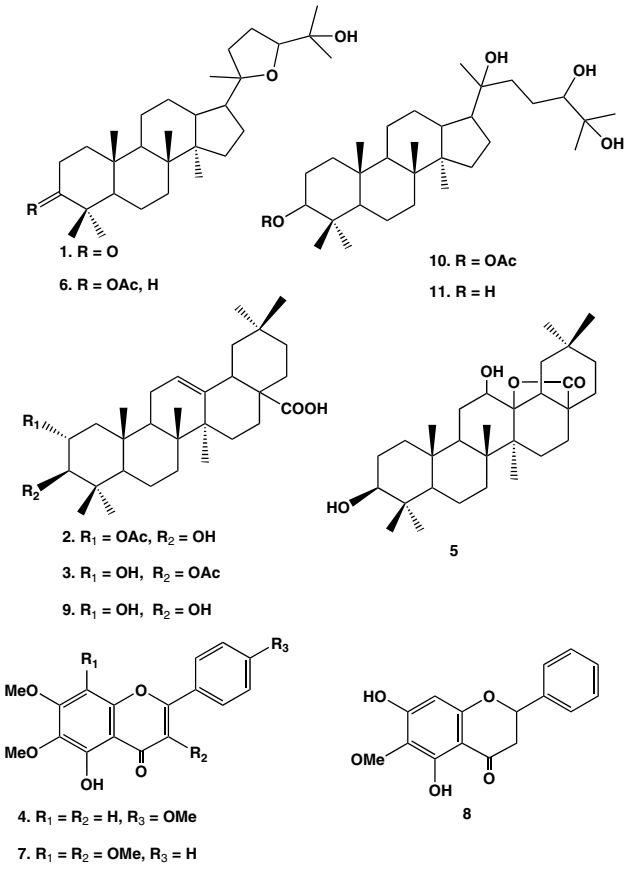
compounds, 2-*O*-acetylmaslinic acid (**2**) and the two dammarane derivatives (**10,11**) have not been described previously from natural sources.



The 2- and 3-*O*-acetylmaslinic acids (**2,3**) both exhibit a MH^+ peak at m/z 515 in the CIMS matching the molecular formula, $C_{32}H_{50}O_5$. The IR spectra of both compounds showed strong bands at 1724 and 1695 cm^{-1} for acetyl and carboxylic groups, respectively. The ^1H NMR spectrum of **2** exhibits seven methyl signals, an olefinic proton at δ 5.25 ppm (br *s*) and two methine signals at δ 4.93 ppm (1H, *ddd*, $J = 4.3$, 10.2, 10.2 Hz) and δ 3.18 (1H, *d*, $J = 10.2$ Hz), assigned to H-2 and H-3, respectively. The *O*-acetyl moiety was confirmed at C-2 from HMBC correlations that showed that the acetyl methyl signal at δ 2.06 ppm was connected to H-2. Compound **2** was acetylated to give a 2,3-di-*O*-acetyl derivative with identical ring A NMR data published for 2α,3β-di-*O*-acetylursenes (Kojima and Ogura, 1989). Thus compound **2** is 2α-*O*-acetylmaslinic acid and compound **3** is 3β-*O*-acetylmaslinic acid.

Compound **10** exhibits in FABMS a $[M+Na]^+$ at m/z 543 which is consistent with the molecular formula, $C_{32}H_{56}O_5$. The ^1H NMR spectrum shows eight methyl groups (δ 1.6–0.8 ppm) and one acetyl methyl at δ 2.06 ppm. The four signals occurring from δ 73.3 to 75.0 ppm in the ^{13}C NMR spectrum are attributed to two quaternary carbons (C–OH) and two methine carbons (CH–O). These data, together with HMBC correlations, indicate **10** is a dammarane-type triterpene similar to cabraleadiol acetate (**6**), but with a C-17 side chain like that of neoalsoside K1 from *Neoalsomitra integrifoliola* (Fujita et al., 1995). Attempts to compare this compound with cabraleadiol monoacetate by acid cyclization failed, and so stereochemistry of C-20 and C-24 cannot be confirmed. The stereochemistry at C-3 is deduced from the appearance at δ 4.83 ppm of a broad singlet due to a low coupling constant, consistent with H-3 being equatorial. An HMBC correlation study confirmed that the acetyl group was on C-3. Compound **10** is therefore 3α-*O*-acetyl-20,24,25-trihydroxydammarane. Compound **11** exhibits in the CIMS a MH^+ at m/z 478, which is consistent with the molecular formula, $C_{30}H_{54}O_4$. The ^1H NMR spectrum is similar to that of compound **10** but without a signal for an *O*-acetyl group. Compound **11** is 3α,20,24,25-tetrahydroxydammarane.

Ten compounds were tested at 1% concentration, in the two-way choice bioassay for antifeeding activity against 5th instar larvae of *C. obliquana.* The results are shown in Table 1. There was only enough material of 3-*O*-acetylmaslinic acid for testing at 0.5% concentration and its feeding index is not strictly comparable with those of the other ten compounds.

The fractions of *N. dombeyi* and *N. pumilio* that showed antifeeding activity, contained several common

Table 1
Antifeeding activity of *Nothofagus* compounds against 5th instar larvae of *Ctenopsteustis obliquana*

	Feeding index[a]	Yield (%)
From N. dombeyi		
2-*O*-Acetylmaslinic acid (**2**)	+22	0.021
3-*O*-Acetylmaslinic acid (**3**)[b]	+12	0.001
12-Hydroxyoleanolic lactone (**5**)	+26*	0.002
Lupeol	+14	0.167
Cabraleone (**1**)	−1	0.005
Pectolinarigenin (**4**)	+35*	0.003
From N. pumilio		
3-*O*-Acetylcabraleadiol (**6**)	+26	0.240
Sitosterol	0	0.012
Betulonic acid	−25	0.014
5-Hydroxy-3,6,7,8-tetramethoxy-flavone (**7**)	−1	0.013
Dihydrooroxylin A (**8**)	+36*	0.003
Maslinic acid (**9**)	Not tested	0.012
Acetyltrihydroxydammarane (**10**)	Not tested	0.006
Tetrahydroxydammarane (**11**)	Not tested	0.006

[a] $(C-T)/(C+T) \times 100$, where C and T are the amounts eaten of control and test squares, respectively.
[b] Tested at 0.5% concentration.
* Significant difference between treatment and control ($P < 0.05$). Wilcoxon matched pairs test.

and known triterpenes and flavonoids and in this respect the chemistry of these two species is unspectacular. However, it is likely that antifeeding activity toward some *Lepidoptera* may be due to the matrix of these compounds, consistent with a generalised resistance toward insect feeding of a type expected of long lived, highly apparent plants such as *Nothofagus* (Feeny, 1976; Fox, 1981). Most of the isolated compounds did not in themselves, show antifeeding activity at 1%, although fractions and plant extracts were highly active. This indicates it is the mixtures that are ecologically important as we discovered with *N. alessandrii* when a mixture of both pinosylvin and galangin demonstrated antifeeding activity at 1% whereas the individual compounds did not (Russell et al., 2000). Otherwise, it can be noted that in the active fractions, the minor compounds were the most active.

The flavanone (**8**) from *N. pumilio* and the flavone (**4**) from *N. dombeyi*, showed significant antifeeding activity but tetramethoxyflavone (**7**) was inactive. In previous work (Russell et al., 2000) we found the flavones, galangin, 8-methoxygalangin and 3-*O*-methyl-8-methoxygalangin from *N. alessandri* also did not have significant antifeeding activity at 1%. The three galangin flavones and flavone **7** all have a 3-oxy substituent and an unsubstituted Ring C and it seems likely when their activities are compared to flavone **4**, that the absence of a C-3 substituent and the presence an *O*-substituted Ring C may be important for antifeeding activity of flavones. Of the triterpenes tested, some were inactive (**1**, sitosterol, betulonic acid), others bordered on activity (**3**, lupeol) and 12-hydroxyoleanolic lactone (**5**) and 3-hydroxycabraleadiol (**6**) showed significant activity.

3. Experimental

Optical rotations were measured at 20 °C on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Perkin–Elmer Spectrum BX FT-IR spectrometer. HR-FAB mass spectra were recorded on a MALDI-TOF spectrometer (Voyager-DE STR; PerSeptive Biosystems) with a matrix of gentisic acid. CIMS and EIMS were recorded on Kratos MS-9 and MS50 mass spectrometers, respectively. NMR spectra were recorded in $CDCl_3$-pyridine-d_5 on Bruker AC 250, AC 300 or AMX 400 spectrometers. Chemical shifts (relative to TMS) are in ppm and coupling constants (in parentheses) in Hz. Column chromatography (CC) was performed using silica gel Merck H60. Preparative plates (PLC) were silica gel 60 F_{254} (1 cm).

3.1. Plant collection

Leaves of *N. dombeyi* and *N. pumilio* were collected from Altos de Lircay National Reserve, (35°37′S; 71°03′W) near Talca, Chile. Herbarium specimens are kept at the Laboratorio de Química Ecológica (NGS-6 and NGS-5, respectively).

3.2. Extraction and isolation from N. dombeyi

Dried and powdered leaves of *N. dombeyi* (0.9 kg) were extracted with CH_2Cl_2. After removal of the solvent by evaporation, the CH_2Cl_2 extract (150 g) was chromatographed on a column of silica gel by using gradient elution with heptane–EtOAc–MeOH (9.5/0.5/0 to 0/0/10). Only the bioactive fractions were subsequently worked up. Fraction 3 (2.5 g, heptane–EtOAc, 9:1) gave lupeol (1.5 g). Fraction 8 (1.1 g, heptane–EtOAc 85:15), was further purified by CC on silica gel with CH_2Cl_2–MeOH 95:5, and afforded cabraleone (**1**) (48 mg). Fraction 16 (1.4 g, EtOAc) was purified by CC on silica gel with CH_2Cl_2–MeOH (95:5), and afforded 2-*O*-acetylmaslinic acid (**2**) (200 mg), 3-*O*-acetylmaslinic acid (**3**) (8 mg), pectolinarigenin (**4**) (25 mg) and 12-hydroxyoleanolic lactone (**5**) (18 mg).

3.2.1. 2-O-Acetylmaslinic acid (2)

Powder, $[\alpha]_D^{19}$ +1.4° ($CHCl_3$, *c* 2.3); EIMS *m/z* (rel. int.): 514 [M^+] (4%), 454 (4%), 248 (100%), 203 (62%); HR-FABMS: *m/z* 537.35800 $[M + Na]^+$ for $C_{32}H_{50}O_5Na$ (calcd. 537.35559); IR $[\nu]_{KBr}$ cm^{-1}: 2940, 1724, 1695; ^1H NMR ($CDCl_3$): δ 5.25 (1H, br *s*, H-12), 4.93 (1H, *ddd*, *J* = 10.2, 10.2, 4.3, H-2), 3.18 (1H, *d*, *J* = 10.2, H-3), 2.82 (1H, *dd*, *J* = 13.3, 3.4, H-18), 2.06 (3H, *s*, OAc), 1.11 (3H, *s*, Me-27), 1.03 (3H, *s*, Me-23), 1.01 (3H, *s*, Me-25), 0.91 (3H, *s*, Me-30), 0.89 (3H, *s*, Me-29), 0.83 (3H, *s*, Me-24), 0.72 (3H, *s*, Me-26); ^{13}C NMR: δ 185.0 (C-28), 170.4 ($O\underline{C}OCH_3$), 144.0 (C-13), 123.0 (C-12), 81.5 (C-3), 73.4 (C-2), 55.5 (C-5), 48.0 (C-9), 46.9 (C-17), 46.2 (C-19), 44.8 (C-1), 42.0 (C-14), 41.3 (C-18), 40.1 (C-4), 39.7 (C-8), 38.7 (C-10), 34.2 (C-21), 33.5 (C-29), 32.8 (C-7), 31.0 (C-20), 30.0 (C-22), 28.9 (C-23), 28.0 (C-15), 26.3 (C-27), 24.0 (C-30), 23.3 (C-16), 23.2 (C-11), 21.8 ($OCO\underline{C}H_3$), 18.6 (C-6), 17.4 (C-26), 17.0 (C-24), 16.7 (C-25).

3.2.2. 2, 3-Di-O-acetylmaslinic acid

Ac_2O was added to the pyridine solution (pyridine–Ac_2O, 1:2) of compound **2** (10 mg) and the mixture heated at 100 °C for 1 h to give a di-*O*-acetyl compound in high yield. The H-2 and H-3 shifts were observed in the ^1H NMR at 5.10 (*ddd*) and 4.75 ppm (*d*), corresponding to H-2α and H-3β, respectively, as observed for data published for 2α,3β-di-*O*-acetylursenes (Kojima and Ogura, 1989). The comparison with compound **2** shows clearly that in the di-*O*-acetyl compound, H-3 shifts from 3.18 to 4.75 ppm due to the *O*-acetyl group attached to C-3.

3.3. Extraction and isolation from N. pumilio

Dried and powdered leaves of *N. pumilio* (1 kg) were extracted with CH_2Cl_2. After removal of the solvent by evaporation, the CH_2Cl_2 extract (84 g) was chromatographed on a column of silica gel by using gradient elution with heptane–EtOAc–MeOH (10/0/0 to 0/0/10). Only the bioactive fractions were subsequently worked up. Fraction 8 (6 g, heptane–EtOAc, 8:2) gave, after purification by CC on silica gel with heptane–Et_2O (8:2) and repeated CC with CH_2Cl_2–EtOAc (1:1), cabraleadiol monoacetate (**6**) (2.4 g), β-sitosterol (0.12 g), and 5-hydroxy-3,6,7,8-tetramethoxyflavone (**7**) (0.13 g). Fraction 11 (6 g, heptane–EtOAc, 1:1), was purified by CC on silica gel with heptane–acetone (1:1) and afforded betulonic acid (144 mg), dihydrooroxylin A (**8**) (28 mg). Fraction 18 (1 g, EtOAc) was purified by CC on silica gel with toluene–acetone (3:1) then EtOAc–isopropanol (95:5) and afforded a mixture of ursolic and oleanolic acids (0.4 g), maslinic acid (**9**) (0.12 g), 3-*O*-acetyl-20,24,25-trihydroxydammarane (60 mg) (**10**) and its 3-*O*-acetyl derivative, 3,20,24,25-tetrahydroxydammarane (**11**) (60 mg).

3.3.1. 3-O-Acetyl-20,24,25-trihydroxydammarane (*10*)

Powder, $[\alpha]_D^{25}$ +37° (MeOH, *c* 0.9); HR-FABMS: *m/z* 543.40135 $[M+Na]^+$ for $C_{32}H_{56}O_5Na$ (calcd. 543.40254); IR $[\nu]_{KBr}$ cm^{-1}: 1740; ^1H NMR ($CDCl_3$): δ 4.83 (1H, br *s*, H-3), 3.80 (1H, *d*, $J = 8$, H-24), 2.06 (3H, *s*, OAc), 1.52 (3H, *s*, Me-26), 1.48 (3H, *s*, Me-27), 1.43 (3H, *s*, Me-21), 0.97 (3H, *s*, Me-18), 0.92 (3H, *s*, Me-29), 0.85 (3H, *s*, Me-30), 0.84 (3H, *s*, Me-28), 0.81 (3H, *s*, Me-19); ^{13}C NMR: δ 170.4 ($O\underline{C}OCH_3$), 80.5 (C-24), 78.6 (C-3), 75.0 (C-20), 73.3 (C-25), 51.8 (C-5), 51.5 (C-9), 51.4 (C-17), 51.2 (C-14), 43.1 (C-13), 41.4 (C-8), 39.8 (C-22), 37.9 (C-10), 37.5 (C-4), 36.0 (C-7), 35.1 (C-1), 32.2 (C-15), 28.6 (C-29), 28.8 (C-16), 27.3 (C-23), 26.7 (C-21), 26.7 (C-27), 26.4 (C-26), 26.0 (C-2), 24.5 (C-12), 22.4 (C-28), 22.3 (C-11), 21.7 ($CO\underline{C}H_3$), 19.0 (C-6), 17.4 (C-30), 17.0 (C-19), 16.3 (C-18).

3.3.2. 3,20,24,25-Tetrahydroxydammarane (*11*)

Powder, $[\alpha]_D^{25}$ +150° (MeOH, *c*0.11); HR-FABMS: *m/z* 501.39381 $[M+Na]^+$ for $C_{30}H_{54}O_4Na$ (calcd. 501.39198); IR $[\nu]_{KBr}$ cm^{-1}: 2931; ^1H NMR ($CDCl_3$): δ 3.80 (1H, *d*, $J = 8$, H-24), 3.60 (1H, br *s*, H-3), 1.52 (3H, *s*, Me-26), 1.49 (3H, *s*, Me-27), 1.43 (3H, *s*, Me-21), 1.21 (3H, *s*, Me-29), 1.00 (3H, *s*, Me-18), 0.92 (3H, *s*, Me-28), 0.90 (3H, *s*, Me-19), 0.83 (3H, *s*, Me-30); ^{13}C NMR: δ 80.5 (C-24), 76.1 (C-3) 75.0 (C-20), 73.4 (C-25), 51.7 (C-9), 51.5 (C-5), 51.3 (C-17), 51.2 (C-14), 43.3 (C-13), 41.5 (C-8), 40.0 (C-22), 38.7 (C-4), 38.2 (C-10), 36.3 (C-7), 35.0 (C-1), 32.2 (C-15), 30.0 (C-29), 28.7 (C-16), 27.3 (C-2), 27.1 (C-23), 27.0 (C-21), 27.0 (C-27), 26.3 (C-26), 26.0 (C-12), 23.2 (C-28), 22.4 (C-11), 19.2 (C-6), 18.2 (C-30), 17.3 (C-19), 16.4 (C-18).

3.4. Antifeeding test

In a two way test design (Blaney et al., 1990) 5th instar larvae of the brown headed leafroller (*C. obliquana*) were presented with diet squares incorporating the extracts or fractions or pure compounds from the plants to make a final concentration of 1% w/w on the moistened squares (Russell and Lane, 1993). The compounds or extracts were applied to the dry diet squares in MeOH or CH_2Cl_2 (10 μl) and the solvent evaporated. Control diet squares were treated with solvent only. Antifeeding activity was assessed by recording the weight of the test square after 24 h exposure to the larvae compared to a control square. A feeding index $[FI = (C - T/C + T) \times 100]$ was calculated from the amount eaten of control (*C*) and test (*T*) squares, respectively. Antifeedant activity shows as a positive index. The treatment versus control data of replicates were analyzed by the two sided, Wilcoxon matched-pairs test.

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