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Phytochemistry 62 (2003) 1125–1131
PHYTOCHEMISTRY
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Taxonomic significance of flavonoid variation in temperate species of *Nothofagus*

Eckhard Wollenweber[a,*], Jan F. Stevens[b,1], Marion Dörr[a], Andrew C. Rozefelds[c]

[a]*Institut für Botanik der Technischen Universität, Schnittspahnstrasse 3, D-64287 Darmstadt, Germany*
[b]*Department of Chemistry, Oregon State University, 153 Gilbert Hall, Corvallis, OR 97331, USA*
[c]*Tasmanian Herbarium, College Road, Sandy Bay, Hobart, Tax 7001, Australia*

Received 9 September 2002; received in revised form 13 November 2002

Abstract

Forty-two flavonoids and a stilbene (pinosylvin) were identified in the leaf exudates of 11 temperate species of *Nothofagus* from South America, Australia and New Zealand. The flavonoid profiles demonstrate significant taxonomic value at the subgeneric level. Most species of subgenus *Fuscospora* are characterized by the presence of pinosylvin, galangin and galangin methyl ethers. Kaempferol-type flavonols are abundant in subgenus *Lophozonia* while these flavonols are largely absent from species of subgenus *Fuscospora*. The flavonoid patterns are largely in agreement with a recent subgeneric classification of *Nothofagus*.

Keywords: *Nothofagus*; Fagaceae; Lipophilic leaf exudates; Flavonoids; Stilbene; Mass spectrometry; Chemotaxonomy

1. Introduction

Nothofagus (southern beeches) occurs in Australia, Tasmania, New Zealand, South America, New Guinea and New Caledonia. It is essentially a Southern Hemisphere genus with both temperate and tropical species, although this study is examining flavonoid chemistry in a number of temperate species. Hill and Jordan (1993), Martin and Dowd (1993), Manos (1997), Setoguchi et al. (1997) and Jordan and Hill (2000) have all supported the circumscription of four clades in *Nothofagus*, each given subgeneric status by Hill and Read (1991). These subgenera are consistent with the informal pollen groups (*menziesii, brassii, fusca* "a" and "b") that were recognized by Dettmann et al. (1990). The subgeneric groupings of Hill and Read (1991) for temperate *Nothofagus* species, viz. *Lophozonia*, *Fuscospora*, and subgenus *Nothofagus*, are used throughout this paper.

Conspicuous exudates in *Nothofagus* result from the colleters at the base of the stipules, which were illustrated by Van Steenis (1953), and may also be derived from glands which occur on the leaves (Hill and Read, 1991), or from glands on subtending bracts (ACR pers. obs.). In a previous paper (Wollenweber et al., 1997), we reported on the presence of flavonoid aglycones and flavonol glycosides in the lipophilic leaf exudate of *Nothofagus antarctica* (G.Forst.) Oerst. (subgenus *Nothofagus*). In this study we examine the leaf exudate flavonoids from another 10 *Nothofagus* species that were either growing wild in Australia and Tasmania or were from cultivated plants. The species of subgenus *Fuscospora* examined were *N. alessandri* Espinosa from South America, *N. gunnii* (Hook. f.) Oerst. from Tasmania, and *N. fusca* (Hook. f.) Oerst., *N. solandri* (Hook. f.) Oerst. and *N. truncata* (Colenso) Cockayne which are New Zealand species. In subgenus *Lophozonia*, we analyzed *N. cunninghamii* (Hook. f.) Oerst. from Tasmania, *N. menziesii* (Hook. f.) Oerst. from New Zealand, *N. moorei* (F. Muell.) Krasser from Australia and *N. nervosa* (Phil.) Dim. & Mil. [=*N. alpina* (Poepp. & Endl.) Oerst.][2] and *N. obliqua* (Mirb.) Oerst

* Corresponding author. Tel.: +49-6151-163602; fax: +49-6151-164630.
E-mail address: wollenweber@bio.tu-darmstadt.de (E. Wollenweber).
[1] Present address: Leibniz Institut für Pflanzenbiochemie, Weinberg 3, D-06120 Halle/Saale, Germany.

[2] Confusion exists over the appropriate species epithet for this taxon. It is variously referred to in the literature as either *N. nervosa* (Phil.) Dim. & Mil. (Lennon et al., 1987) or *N. alpina* (Poepp. & Endl.) Oerst. (Vázquez and Rodriquez, 1999).

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PII: S0031-9422(02)00666-0

from South America. The aim of this study was to document the flavonoid aglycones and flavonol glycosides in these additional 10 species and to determine whether the phenolic profiles were consistent with the subgeneric classification according to Hill and Read (1991).

2. Results and discussion

A survey of the leaf-surface phenolics of young leaflets of eleven *Nothofagus* was conducted using TLC. Forty-two flavonoids and one stilbene derivative were detected and identified by co-TLC with authentic markers and by spectroscopic techniques (MS, NMR). Ten species of *Nothofagus,* consisting of five species each from the two subgenera, *Fuscospora* and *Lophozonia*, were examined while the flavonoid data earlier recorded for *N. antarctica* (subgenus *Nothofagus*) (Wollenweber et al., 1997) were included in Table 1 for comparison. Two samples of *N. fusca* from different sources gave virtually identical results, with the exception that some flavonoids were conspicuous elements in one sample but present only in trace amounts in the other (Table 1).

2.1. Identification of flavonoids

Identifications were mainly made by TLC and MS–MS comparison with reference markers available in EW's laboratory. MS–MS analysis with an APCI (atmospheric pressure chemical ionization) source has proven to be a powerful method for characterization of flavonoid aglycones in our collaborative studies. Furthermore, MS–MS fingerprinting often allows identification of flavonoids by (on-line) comparison with MS–MS spectra recorded for known flavonoids (Stevens et al., 1999). Our MS–MS library of flavonoid aglycones counted 137 entries covering 97 flavonoid aglycones at the time that the present study was carrried out. Compounds **1** (xanthomicrol), **2** (4′-hydroxy-5,6,7,8-tetramethoxy flavone; Iinuma et al., 1980), **3** (cardamomin), and **4** (pinosylvin; Gorham, 1989), could not be identified unambiguously by TLC and MS–MS comparison alone (no library match found due to absence of standard spectra). These known phenolic compounds were therefore also examined by NMR spectroscopy.

2.2. Flavonoid patterns, phylogenetic implications

Exudate flavonoids of *Nothofagus* were reported some years ago for the first time. Wollenweber et al. (1997) found several flavonoid aglycones as well as some glycosides in the resinous layer present on young leaflets of *Nothofagus antarctica*. More recently, galangin, the 8-methyl- and the 3,8-dimethyl ether of 8-hydroxy-galangin as well as the stilbene pinosylvin were isolated from the methanol extract of *N. alessandri* leaves (Russell et al., 2000). In view of our present results, it can be taken for certain that these products are also constituents of *Nothofagus* leaf exudates. In our study, five additional flavonoids were identified in *N. alessandri*. The results of our flavonoid survey are compiled in Table 1. The flavonoid scores (present/absence) were examined by cluster and ordination analysis (see Section 3.4) with the aim to evaluate the taxonomic value of flavonoid variation in *Nothofagus*.

The dendrogram resulting from the clustering divides the taxa into two main groups (Fig. 1). The first group consists of five taxa, with ((((*N. alessandri*, *N. fusca*,) *N. gunnii*,) *N. solandri*) and *N. antarctica*) in order of dissimilarity. The first four species that clustered together all belong to subgenus *Fuscospora*, with *N. antarctica* belonging to subgenus *Nothofagus*. *Nothofagus alessandri* from South America and *N. fusca* from New Zealand share eight flavonoids and only differ in the relative abundance of galangin-3-methyl ether (Table 1). They therefore have an identical ordination, and cluster together (Figs. 1 and 2). These two species cluster near to the Tasmanian endemic, *N. gunnii*, which share six flavonoids with these taxa (Table 1, Fig. 1). *Nothofagus solandri* links into this group of taxa at the next highest level, and this is also evident in the ordination. It shares five flavonoids, including galangin and its methyl derivatives, pinocembrin and pinosylvin with other species in this group (Table 1). It also has five unique flavonoids detected in the leaf exudates that do not occur in any of the other taxa studied and which contribute to the high level of dissimilarity between *N. solandri* and the other taxa (Table 1). *Nothofagus antarctica* (subgenus *Nothofagus*) links into this group at the next highest level and shares three flavonoids with the species in this group (Table 1). It also has some unique flavonoids (myricetin and myricetin glycosides) which were not detected in the other species of *Nothofagus* studied.

The second group consists of six species and has two distinct subgroups. *Nothofagus menziesii* and *N. nervosa* group together closely, which is evident from both the dendrogram and ordination (Figs. 1 and 2). These taxa are relatively isolated from other species in this group (Figs. 1 and 2). *Nothofagus menziesii* and *N. nervosa* share 12 compounds and differ only in that pinocembrin is absent from *N. menziesii*. They are separated at a high level of dissimilarity from the second sub-group that includes *N. moorei*, *N. obliqua* and *N. cunninghamii* (all subgenus *Lophozonia*), and *N. truncata* (subgenus *Fuscospora*). All of these species are highly dissimilar, and share few or no flavonoids in common. The high dissimilarity is also due, in part, to relatively few flavonoids produced from some species, e.g., only four flavonoids were identified from *N. moorei* and from *N. truncata*. The grouping of *N. truncata* (subgenus

Table 1
Flavonoid profiles of *Nothofagus* spp.

Subgenus	*Nothofagus*	*Fuscospora*						*Lophozonia*				
species	*anta*	*aless*	*fusc* (a)	*fusc* (b)	*gunn*	*sola*	*trun*	*cunn*	*menz*	*moo*	*nerv*	*obli*
Flavonols												
Galangin	X	X	X	X	X	X						
Gal-3-Me		X	(X)	(X)		X	X					
Gal-7-Me	X	(X)	(X)	X	X	X						
8-OH-Gal-3-Me		X	X	X								
8-OH-Gal-8-Me	X	X	X	X	X	X			X		X	
8-OH-Gal-3,7-Me						X						
8-OH-Gal-3,8-Me		X	X	X		X	X					
8-OH-Gal-7,8-Me						X						
8-OH-Gal-3,7,8-Me						X	X					
Kaempferol												X
Kae-3-Me							X	X	X		X	
Kae-7-Me								X				
Kae-4′-Me									X		X	
Kae-3,7-Me								X				
Kae-3,4′-Me									X		X	
Kae-3,7,4′-Me								X				
Herbacetin-3,8,4′-Me									X	X	X	
Herb-3,7,8,4′-Me									X		X	
5,7-OH-3,6,8,4′-OMe-flav.									X	X	X	
5-OH-3,6,7,8,4′-OMe-flav.									X		X	
Quercetin										(X)		X
Qu-3-Me										X		
Qu-7-Me												(X)
Qu-3′-Me												(X)
Qu-3,3′-Me									X		X	
Qu-7,3′-Me								X				
Qu-3,7,3′-Me								X				
Qu-3,7,3′,4′-Me								X				
Qu-glycosides	X											X
Myricetin	X											
Myr-glycosides	X											
Flavones												
5-OH-7,8-OMe-flavone						X						
Apigenin								X	X		X	
Ap-7-Me								X	X		X	
Ap-4′-Me									X		X	
5,4′-OH-6,7,8-OMe-flavone								X				
4′-OH-5,6,7,8-OMe-flavone								X				
Flavanones/flavanonols												
Pinocembrin	X	(X)	X	X	X	X					X	X
Pinoc-7-Me						X						
Pinobanksin-3-acetate						X						
Chalcone												
2′,4′-OH-6′-OMe-chalcone		(X)	X	X	(X)							
Stilbene												
Pinosylvin		X	X	X	X	(X)						

X = major constituents, (X) = minor constituents. Abbreviations: *anta* = *antarctica*, *ales* = *alessandri*, *fusc* = *fusca*, *gun* = *gunnii*, *sola* = *solandri*, *trun* = *truncata*, *cun* = *cunninghamii*, *men* = *menziesii*, *moo* = *moorei*, *nerv* = *nervosa*, *obli* = *obliqua*. For flavonoid names see structures. Me = methyl ether, OMe = methoxy substituent.

Fuscospora) with species of *Lophozonia* in the dendrogram is also likely to be an artifact of the analysis, because this species shares three flavonoids with *N. solandri* (Group 1), and only one flavonoid with *N. cunninghamii* in Group 2 (Table 1).

The exudate flavonoids exhibited by the various species shows that the species in *Fucsospora* and *Lophozonia* differ significantly in their overall chemical profiles (Table 1). All *Fuscospora* species have galangin or one of its methyl derivatives (Table 1). With the exception of

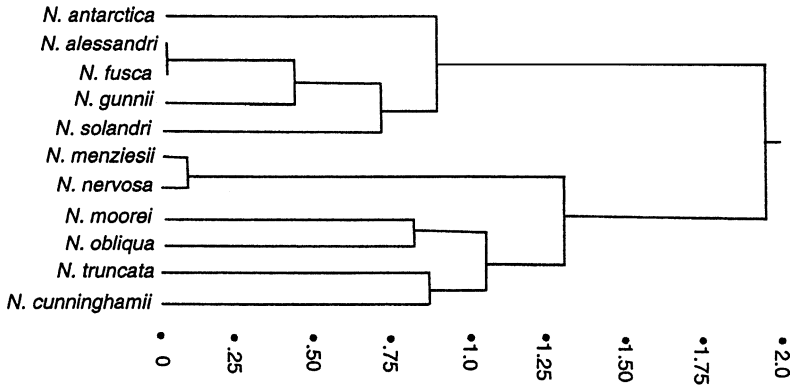


Fig. 1. A cluster analysis based on Jaccard coefficient distance calculated using the differences in flavonoid chemistry.

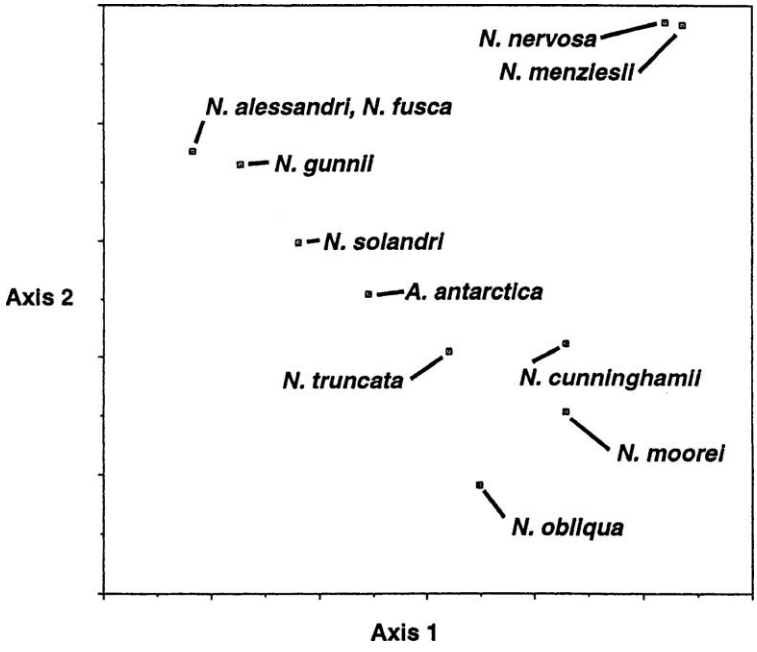
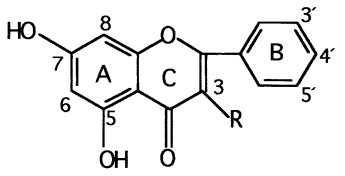
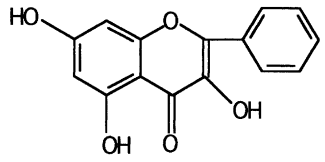


Fig. 2. Ordination in two dimensions summarising the differences in flavonoid chemistry among *Nothofagus* species.

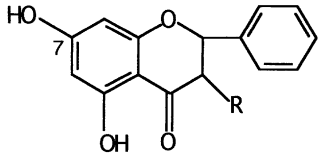
N. truncata, the other species in this subgenus share galangins (galangin, galangin-7-Me, 8-OH-Gal-8-Me), 2′,4′-dihydroxy-6′-methoxychalcone and the stilbene, pinosylvin. These chemicals are largely absent from most species in subgenus *Lophozonia* (Table 1). Russell et al. (2000) identified a similar pattern in *Nothofagus*, noting that galangin occurred in species of the subgenera *Fuscospora* and *Nothofagus* but not in subgenus *Lophozonia*, while pinosylvin occurred in species of *Fuscospora*, but was lacking in subgenera *Nothofagus* and *Lophozonia*. In our study pinosylvin was identified in the leaf exudates from all *Fuscospora* species, except *N. truncata* (Table 1). *Nothofagus menziesii* and *N. nervosa* (subgenus *Lophozonia*) have almost identical flavonoid profiles, while the other species of *Lophozonia* have relatively few flavonoids in common. The distribution of individual flavonoids within species in subgenus *Lophozonia* is not consistent, although all species in this subgenus have flavonols of the kaempferol type present (Table 1). Such flavonols are absent from all species in subgenus *Fuscospora*, except for *N. truncata*. Based upon the overall distribution of flavonoids it is possible to recognise two groups of species, which are largely congruent with the subgenera *Fuscospora* and *Lophozonia*, within *Nothofagus* (Manos, 1997; Jordan and Hill, 2000). This congruence is most evident in subgenus *Fuscospora* with taxa from South America, New Zealand and Tasmania having a number of galangin-based flavonols and other flavonoids in common.



Flavones
5,7,8-OH: Norwogonin
5,7,4′-OH: Apigenin
5-OH-7,8-diOMe
Compd. 1: 5,4′-diOH-6,7,8-triOMe
Compd. 2·4′-OH-5,6,7,8-tetraOMe

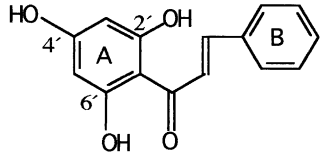


Flavonols
5,7-OH: Galangin
5,7,4′-OH: Kaempferol
5,7,3′,4′-OH: Quercetin
5,7,3′,4′,5′-OH: Myricetin
5-OH-3,7,8-triOMe
5,7-diOH-3,8,4′-triOMe
5,7-diOH-3,6,8,4′-tetraOMe

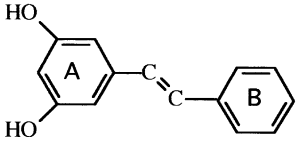


Flavanone: R = H
5,7-OH: Pinocembrin

Flavanonol: R = OH
5,7-OH: Pinobanksin



Chalcone
Compd. 3· 2′,4′,6′-triOH



Stilbene
Compd. 4· *trans*-Pinosylvin

3. Experimental

3.1. Plant material

Aerial parts of *Nothofagus* species were collected from cultivated trees or in the field. *N. alessandri* Espinosa, cultivated, near Smithton, Tasmania, Rozefelds 1865 & W.A. Gebert, 28 September 2000. *Nothofagus antarctica* (Forst.) Oerst. Botanical Garden Darmstadt. *N. cunninghamii* (Hook. f.) Oerst., Lake Dobson, Mt Field National Park, Rozefelds 1396, 23 February 1997. *N. fusca* (Hook. f.) Oerst. cultivated, (a) Pirianda Gardens, Dandenongs, Vic., Coll. Rozefelds s.n., 2 February 1997; (b) Wellington Botanical Garden, Coll. J. Dawson, Nov. 1997. *N. gunnii* (Hook. f.) Oerst. Mt. Field National Park, Tasmania, Coll. Rozefelds 487, 23 February 1997. *N. moorei* (F. Muell.) Krasser, Border Ranges, New South Wales, Rozefelds s.n. *N. menziesii* (Hook. f.) Oerst. cultivated, Pirianda Gardens, Dandenongs, Vic., Coll. Rozefelds s.n., 2 February 1997. *N. nervosa* (Phil.) Dim. & Mil. cultivated plants, private garden Ridgely, Tasmania, Coll. Rozefelds s.n. 2 February 1997. *N. obliqua* (Mirb.) Oerst. cultivated, Pirianda Gardens, Dandenongs, Vic., Coll. Rozefelds s.n., 2 February 1997. *N. solandri* (Hook. f.) Oerst. cultivated, Pirianda Gardens, Dandenongs, Vic., Coll., Rozefelds s.n., 2 February 1997. *N. truncata* (Colenso) Cockayne ex Pirianda Gardens, Dandenongs, Vic., Coll. Rozefelds s.n., 2 February 1997. Bulk material of *N. cunninghamii* and *N. menziesii* was collected in the Austral. Nat'l. Botanic Gardens, Canberra (B. Wallace, January 1998). Bulk material of *N. fusca*, *N. solandri* var. *cliffortioides* and *N. truncata* was collected in the Otari Native Botanic Garden, Wellington (J. Dawson, September 1997).

3.2. Isolation and chromatographic procedures

Air-dried plant material was briefly rinsed with acetone to dissolve the exudate materials from the leaves. After evapn of the solvent, the residues (except for *N. moorei*, where only a small sample was available) were redissolved in a small amount of boiling MeOH,

cooled to −10 °C and centrifuged to separate the MeOH soluble flavonoids from precipitated lipid material. The supernatants were subsequently chromatographed on Sephadex LH-20 with MeOH as the eluting solvent. Fractions were monitored by TLC on polyamide (DC 11, Macherey-Nagel) with solvents A (petrol$_{100-140}$–toluene–MeCOEt–MeOH 12:6:1:1), B (toluene–petrol$_{100-140}$–MeCOEt–MeOH 12:6:2:1) and C (toluene–MeCOEt–MeOH 12:5:3) and on silica gel with solvents D (toluene–MeCOEt 9:1) and E (toluene–dioxane–HOAc 18:5:1). Chromatograms were viewed under UV (366 nm) before and after spraying with "Naturstoffreagenz A" (a 1% methanolic solution of diphenyl-boric acid-ethanolamine complex). Terpenoids were visualized by spraying silica gel plates with $MnCl_2$ reagent, followed by heating (Jork et al., 1989). Fractions of similar flavonoid composition were combined and rechromatographed on polyamide SC-6 or on silica gel columns, both eluted with toluene and increasing amounts of MeCOEt and MeOH.

Some of the highly methylated flavones/flavonols were purified to homogeneity by prep TLC on silica (toluene–MeCOEt 9:1) and/or by semi-preparative HPLC on a 10 μm Econosil RP-18 (250×22 mm) column. Typically, linear gradient elution was used from 40 to 100% acetonitrile in 1% aq. HCOOH over 30 min at 11.2 ml min^{-1}. The UV trace was recorded at 280 nm. Peak fractions were collected manually, concentrated on a rotavapor and then lyophilized.

3.3. Spectroscopy

^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded in DMSO-d_6 at room temperature using the DMSO resonances (δ_H 2.50, δ_C 39.51) as internal shift references. ^{1}H–^{13}C HMBC experiments were performed using standard Bruker pulse sequences. APCI-MS–MS experiments were carried out as described earlier (Stevens et al., 1999). The MS–MS library was created using Multiview 1.3 software supplied with the API III Plus triple-quadrupole mass spectrometer (AB Sciex, Concord, Ontario). High-resolution mass spectrometry was performed on a Kratos MS-50 magnetic sector mass spectrometer.

Compound **1** (5,4′-dihydroxy-6,7,8-trimethoxy flavone) was isolated from *N. cunninghamii*. APCI-MS, *m*/*z* 345 [MH]$^+$ (100%); ^{1}H NMR, δ_H 12.78 (*s*, OH-5), 10.44 (*s*, OH-4′), 7.95 and 6.96 (2H each, both *d*, *J* = 8.7 Hz, H-2′/6′ and H3′/5′), 6.89 (1H, *s*, H-3), 4.02, 3.92 and 3.82 (3H each, *s*, 3×OMe).

Compound **2** (4′-hydroxy-5,6,7,8-tetramethoxy flavone) was isolated from *N. menziesii*. APCI-MS, *m*/*z* 359 [MH]$^+$ (100%); ^{1}H NMR, δ_H 10.3 (br *s*, OH-4′), 7.89 and 6.94 (2H each, both *d*, *J* = 8.8 Hz, H-2′/6′ and H3′/5′), 6.67 (1H, *s*, H-3), 4.02, 3.96, 3.83 and 3.77 (3H each, *s*, 4×OMe).

Compound **3** (2′,4′-dihydroxy-6′-methoxychalcone) was isolated as a yellow substance from *N. fusca*. APCI-MS, *m*/*z* 271 [MH]$^+$ (100%); MS–MS, *m*/*z* 167 (A-ring fragment); ^{1}H NMR, δ_H 6.02 and 5.93 (1H each, *d*, *J* = 1.5 Hz, H-3′ and H-5′), consistent with an asymmetrical A-ring having 2′,4′-dihydroxy-6′-methoxy substitution.

Compound **4** (pinosylvin) was also isolated from *N. fusca*. HR-EI-MS, *m*/*z* 212.08365 [M]$^+$ ($C_{14}H_{12}O_2$ calculates for 212.08373); ^{1}H NMR, δ_H 9.27 (2H, *s*, 2×OH), 7.07 and 7.04 (1H each, both *d*, *J* = 16.4 Hz, olefinic protons), 6.47 (2H, *d*, *J* = 2.1 Hz, H-2′/6′), 6.19 (1H, apparent *t*, *J* = 2.1 Hz, H-4′), 7.57-7.25 (5H, *m*, phenyl protons).

3.4. Phenetic analysis

The phenetic relationships among *Nothofagus* species, in terms of flavonoid chemistry, are summarised using clustering and ordination. The data used the presence or absence of each compound as a binary character, and trace elements were treated in the analysis as present. Similarities in flavonoid chemistry among the species were computed using the Jaccard Coefficient, as this coefficient does not treat the shared absence of a character as evidence of similarity.

The classification approach used was a hierarchical agglomerative strategy (Lance and Williams, 1967), with the sorting strategy being the incremental sum of squares. A principal coordinate analysis was used to produce an ordination that allows the sets of results to be viewed as points on a set of coordinate axes. The distance apart in the ordination reflects the relative differences in chemistry of pairs of species.

Acknowledgements

J.F.S. wishes to thank Dr. Monika Ivancic and Prof. Victor L. Hsu for help with the NMR experiments, and Professor Max L. Deinzer for access to MS facilities at OSU. Thanks are also due to Lyn Craven (Austr. Nat'l. Herbarium, Canberra), John Dawson (Wellington, NZ), Sue Jennings (Forestry Tasmania, Smithton) and Ben Wallace (Austr. Nat'l. Botanic Gardens, Canberra) for valuable plant material (see Experimental). Dr David Ratkowsky (School of Agric. Sci., Univ. of Tasmania, Hobart) kindly undertook the ordination and phenetic analysis and commented on parts of the ms. E.W. further wishes to thank the Deutsche Forschungsgemeinschaft for financial support, and E. Merck, Darmstadt, for a generous gift of solvents.

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