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Phytochemistry 64 (2003) 1285–1293
PHYTOCHEMISTRY
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2-Hydroxyflavanones from *Leptospermum polygalifolium* subsp. *polygalifolium* Equilibrating sets of hemiacetal isomers

Kamarul'Ain Mustafa[a], Nigel B. Perry[b], Rex T. Weavers[a,*]

[a]*Department of Chemistry, University of Otago, PO Box 56, Dunedin, New Zealand*
[b]*Plant Extracts Research Unit, New Zealand Institute for Crop and Food Research Limited, Department of Chemistry, University of Otago, PO Box 56, Dunedin, New Zealand*

Received 25 June 2003; received in revised form 2 September 2003

Abstract

2,5-Dihydroxy-6-methyl-7-methoxyflavanone and its isomer 2,5-dihydroxy-8-methyl-7-methoxyflavanone have been identified as the principal components in the ethanol extract of *L. polygalifolium* subsp. *polygalifolium*. The former compound has been separated from the latter for the first time by crystallisation. Other flavonoids identified include an equilibrating mixture of novel 2,3,5-trihydroxyflavanones; the first discovery of this type of compound from a natural source. This study has also confirmed the presence of the triketones, flavesone and leptospermone, along with the closely related isoleptospermone as anti-microbial components.

Keywords: *L. polygalifolium* subsp. *polygalifolium*; Myrtaceae; Flavonoids; Hemiacetals; 2,5-Dihydroxyflavanones; 2,3,5-Trihydroxyflavanones; Antimicrobial activity

1. Introduction

Leptospermum J. R. *et* G. Forst. is a genus in the Myrtaceae family consisting of 83 species that is widely distributed throughout Australia (Wrigley and Fagg, 1993). One tropical species (*L. amboinense*) extends to South-east Asia, and *L. scoparium*, a species common in Tasmania, is widespread in New Zealand. Three species, *L. javanicum*, *L. parviflorum* and *L. recurvum*, are endemic to South-east Asia, the latter being found only on Mt. Kinabalu in the state of Sabah, Malaysia (Lee and Lowry, 1980).

Chemical studies on this genus have concentrated on volatile oils, with little work having been published on non-volatiles. To the best of our knowledge, the flavonoids of only two species, *L. scoparium* (Haberlein and Tschiersch, 1994a, b, 1998; Haberlein et al., 1994; Mayer, 1990, 1993, 1996) and *L. laevigatum* (Wollenweber et al., 1996), have been examined. These species yielded flavonoids characterised by C-methylation. In the case of *L. scoparium* this feature was coupled with the absence of oxygenation in the B-ring. Our interest in the chemistry of this genus originated in the anti-microbial activity of the volatile oil of *L. scoparium*, due to triketones (**1**)–(**4**) (Perry et al., 1997; Porter and Wilkins, 1998).

We previously chose to study *L. recurvum* Hook. f. because its ethanol extract also showed anti-microbial activity in addition to some anti-viral action (Mustafa et al., 2003). This study established the presence of the dihydrochalcones (**5**) and (**6**), flavone (**7**) (but not the isomeric (**8**)) accompanied by the corresponding flavanone (**9**), and an inseparable mixture of 2,5-dihydroxy-6-methyl-7-methoxyflavanone (**10**) and its isomer 2,5-dihydroxy-8-methyl-7-methoxyflavanone (**11**).

We extended our study to include the Australian plant, *L. polygalifolium* subsp. *polygalifolium* Salisb., because the HPLC trace of its ethanol extract suggested a similar compound profile. This ethanol extract also showed anti-microbial, anti-viral and anti-fungal activity, and we had no more *L. recurvum* foliage to study the 2,5-dihydroxyflavanones (**10**) and (**11**). Previous reports described the volatile components of *L. polygalifolium* subsp. *polygalifolium* as α-pinene and the α, β and γ-eudesmols (Brophy and Goldsack, 1993; Brophy

* Corresponding author. Tel.: +64-34797925; fax: +64-34797906.
E-mail address: rweavers@alkali.otago.ac.nz (R.T. Weavers).

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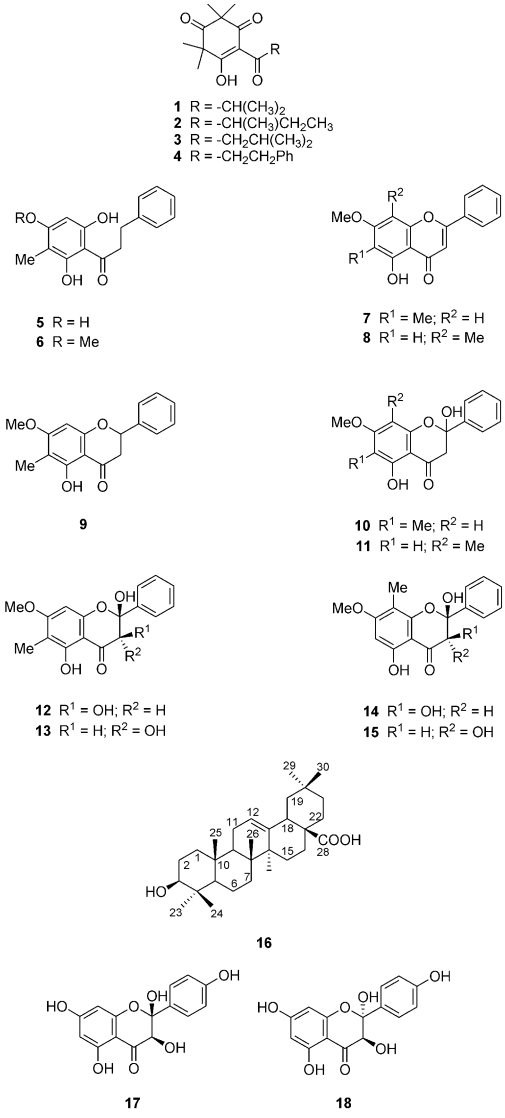
doi:10.1016/j.phytochem.2003.09.006



et al., 2000). The only reports of non-terpenoid metabolites stated that the leaf oils contain the triketones, flavesone (**1**) and leptospermone (**3**) (Briggs et al., 1938, 1945; Penfold, 1921), although Brophy found no evidence of these two compounds (Brophy et al., 2000).

In this paper, we report isolation of several flavonoids from *L. polygalifolium* subsp. *polygalifolium*, including the desired (**10**) and (**11**). We describe the first isolation of hemiacetal (**10**) from its position isomer (**11**), along with a study of its solution behaviour. In addition, we report a more complex system of hemiacetal isomers, some novel 2,3,5-trihydroxyflavanones.

2. Results and discussion

Extraction of the dried foliage of *L. polygalifolium* subsp. *polygalifolium* with ethanol followed by a sequence of chromatographic separations (Fig. 1) afforded separate mixtures of 2,5-dihydroxyflavanone isomers (**10**) and (**11**) and triketones (**1**)–(**3**). Base extraction of one chromatography fraction yielded further samples of (**1**)–(**3**) and further chromatography on the neutral fraction gave a mixture of the isomeric flavones (**7**) and (**8**). Chloroform extraction of the residue from the ethanol extract gave an extract that was partitioned into neutral and acid fractions. The latter yielded further samples of the triketone mixture (**1**)–(**3**). Compounds isolated to this point were identified by comparison with authentic samples; by HPLC using co-injection, and by comparison of ^{1}H NMR spectra. Chromatography of the neutrals gave fractions containing further samples of isomeric mixtures (**7**)/(**8**) and (**10**)/(**11**), but also a fraction from which oleanolic acid (**16**) could be crystallised. Data for this compound matched those reported previously (Mahato and Kundu, 1994). The filtrate from this crystallisation, and also another chromatography fraction, yielded a mixture of four new isomeric compounds, the *cis* and *trans* isomers of 2,3,5-trihydroxy-6-methyl-7-methoxyflavanone (**12**) and (**13**) and those of its position isomer 2,3,5-trihydroxy-8-methyl-7-methoxyflavanone (**14**) and (**15**).

2,5-Dihydroxyflavanones (**10**) and (**11**) were first reported from *Friesodielsia enghiana* (Annonaceae), as an approximate 1:1 mixture (Fleischer et al., 1997). Given the propensity for such cyclic hemiacetals to open in solution, it seems likely that these two compounds would inter-convert as in Fig. 2. This pathway also provides a mechanism for racemisation. Therefore it surprised us to find that the previous workers found non-zero optical rotation ($[\alpha]_D^{21}$ +20.7°). Our samples of (**10**) and (**11**), isolated from *L. recurvum* (Mustafa et al., 2003), and from *L. polygalifolium* subsp. *polygalifolium*, were also 1:1 mixtures as shown by paired peaks in NMR spectra. These also had significant non-zero optical rotations, but of opposite signs ($[\alpha]_D^{21}$ +24.2° and $[\alpha]_D^{20}$ −15.9° respectively). Vapour diffusion crystallisation (ethanol/water) on a sample of the 1:1 mixture of (**10**) and (**11**) from *L. polygalifolium* subsp. *polygalifolium* gave a crystalline material, in a yield of greater than 50% of the original mass. ^{1}H NMR experiments on a freshly prepared benzene-d_6 solution of this solid showed the presence of only one isomer that proved to be the 6-methyl derivative (**10**). In the NMR tube, equilibrium was established after about 390 min, giving approximately equal amounts of (**10**) and (**11**). 2D NMR methods on this equilibrated mixture enabled assignment of the ^{1}H and ^{13}C resonances for both (**10**) and (**11**). Compound (**10**) showed a correlation in the CIGAR-HMBC spectrum between the C-5 hydroxyl proton (δ 12.65) and the oxygenated carbon resonating at δ 160.2 (C-5). The latter in turn showed correlation to the methyl proton signal (δ 2.24), thereby establishing that the methyl group was at C-6. Further verification was obtained by correlation between the methyl proton signal and a further oxygenated carbon

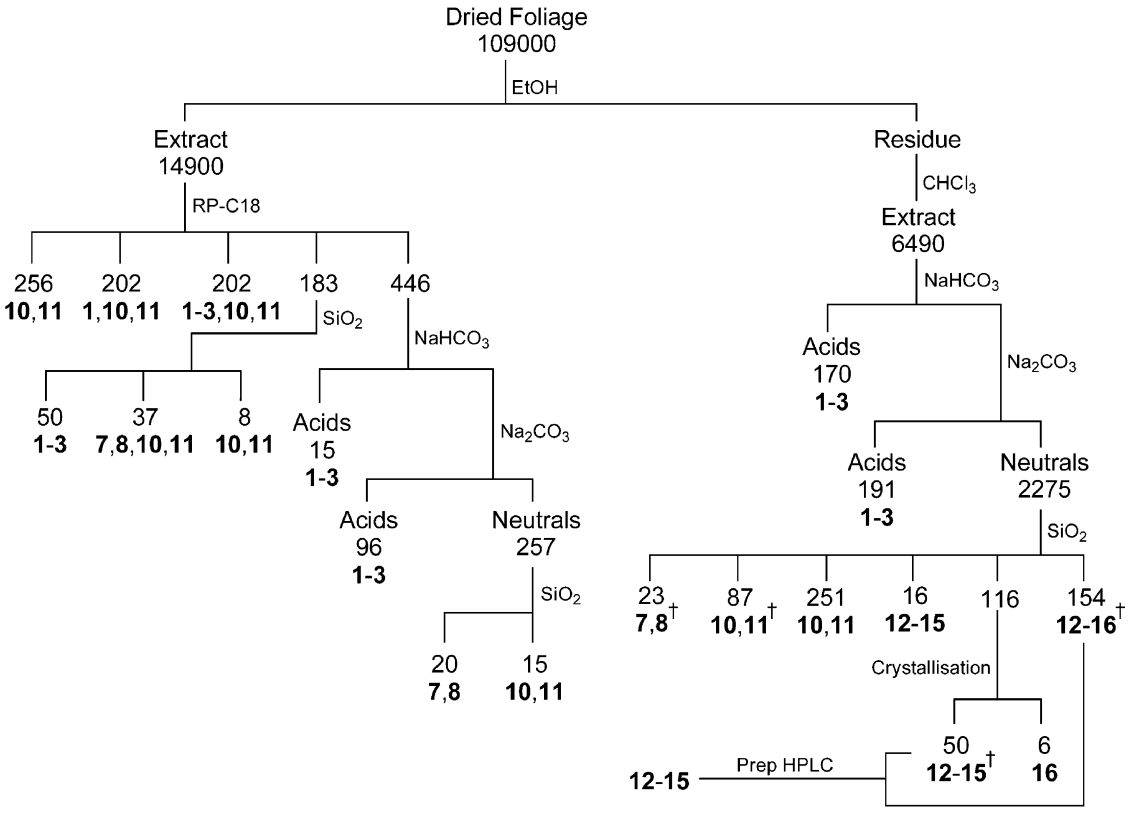


Fig. 1. Separation Tree for *L. polygalifolium* subsp. *polygalifolium* showing masses (mg) and compounds identified from each fraction. †Samples contaminated with uncharacterised materials.

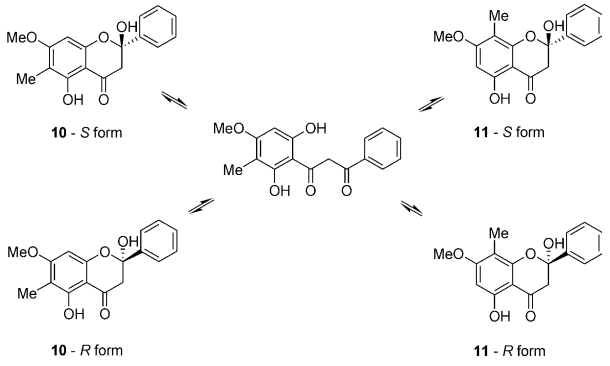


Fig. 2. Solution equilibria of 2-hydroxyflavanones (**10**) and (**11**).

peak at δ 165.7 (C-7) that was further linked to the methoxyl proton signal (δ 3.24). Similar correlations established that (**11**) was methyl substituted at C-8 (Fig. 3). The signals in the ^{1}H NMR spectrum of the freshly prepared sample of the selectively crystallised compound matched those assigned to (**10**). In more acidic solvents such as $CDCl_3$ equilibration between (**10**) and (**11**) was attained much more rapidly (<5 min), although equilibrium composition did not vary appreciably over the solvents examined (benzene-d_6, $CDCl_3$ and acetone-d_6). The results of the vapour diffusion crystallisation indicated that, as the less soluble (**10**) crystallised from the 1:1 mixture, equilibration resulted

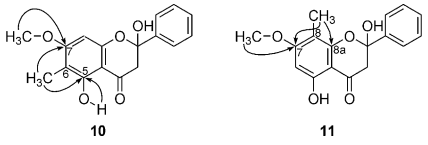


Fig. 3. CIGAR correlations establishing the methyl group positions in (**10**) and (**11**).

in the formation of more (**10**) yielding a higher than 50% return.

The average optical rotation of five equilibrated solutions prepared from the crystallised sample in chloroform ($[\alpha]_D^{21} = +8^\circ$ $\{\pm 3\}$) was significantly lower in magnitude than that of the parent mixture, but rotation values at longer wavelengths were very substantial ($[\alpha]_{405\ nm}^{20.9}$ -324° $\{\pm 4\}$). Because of the proposed solution behaviour (Fig. 2), the optical activity seems incompatible with the structures (**10**) and (**11**), and may be due to the presence of trace impurities. These would necessarily have very large rotations as the sample of (**10**) was analytically pure, and solutions showed only one peak in an HPLC trace (detection at 206, 254, 280 and 325 nm) and only signals for (**10**) and (**11**) in the ^{1}H NMR spectrum. Optical activity in samples of similar 2-hydroxyflavanones has been reported on at least three other occasions (Fleischer et al., 1997; Lee et al., 1995; Wang et al., 1999). Wang also isolated a single position

isomer from one of these equilibrating systems by selective crystallisation, a 6-methoxy analogue of (**10**) (Wang et al., 1999). The X-ray structure proved the compound to be racemic.

Mixture (**12**)–(**15**) showed similarity to the hemiacetal mixture (**10**) and (**11**) in that pairing of the dominant peaks was observed in both the ^{1}H and the ^{13}C spectra (Table 1). It showed one spot on silica TLC (cyclohexane:EtOAc; 3:1), at lower R_F (0.13) than that obtained for the mixture of (**10**) and (**11**) (R_F 0.28), consistent with further hydroxylation. Reversed-phase HPLC on C-18 showed a single peak of shorter retention time (21.2 min) than that for the mixture of (**10**) and (**11**) (26.4 min). This peak was present at low level in the HPLC trace of the crude ethanol foliage extract. The HR-EIMS gave a single molecular ion peak at m/z 316.0947, corresponding to $C_{17}H_{16}O_6$ (one more oxygen than (**10**) and (**11**)). UV analysis showed a strong absorption at 294 nm and a shoulder at 316 nm, which is typical of flavanones, (Markham and Mabry, 1975) while the IR spectrum indicated hydroxyl (3385 cm^{-1}) and conjugated carbonyl (1636 cm^{-1}) functionality.

The ^{1}H NMR spectrum in $CDCl_3$ showed peaks consistent with two major isomers (**12**) and (**14**) in approximately equal proportions (Table 1), in particular the hydrogen-bonded proton singlets (5-OH) at δ 11.06/11.02, the methoxyl singlets (7-OMe) at δ 3.86/3.87, the methyl singlets (6-Me/8-Me) at δ 2.02 and the aromatic proton singlets (H-8/H-6) at δ 6.13/6.16 ppm. Furthermore, the aromatic multiplets at δ 7.45–7.47 and δ 7.76–7.80, which integrated in a 3:2 ratio, suggested an unsubstituted B-phenyl ring, which was supported by the fragment at m/z 77.0385 in the HR-EIMS. A feature of the spectrum of these new compounds, the presence of singlets at δ 4.65/4.59, had no analogue in the spectrum of (**10**) and (**11**). Also lacking were the 3-CH_2 signals of (**10**) and (**11**). Both features were consistent with hydroxylation at C-3. Assignment of individual peaks to a 6-methyl structure (**12**) and its 8-methyl isomer (**14**) was achieved by 2D NMR (HSQC, CIGAR-HMBC and NOESY). Two and three bond ^{1}H–^{13}C correlations (CIGAR-HMBC) confirmed that the signals at δ 4.65/4.59 belonged to H-3 for (**12**) and (**14**) respectively, consistent with the presence of a C-3 hydroxyl group (Fig. 4). Only in isomer (**12**) were correlations from the 5-OH proton signal (δ 11.06) observed. These linked to one oxygen bearing carbon (δ 159.4, C-5) and two others, a carbon with a peak at δ 106.9 (C-6), which also showed correlation to the methyl proton system resonating at δ 2.02 (8-Me), and to a carbon signal at δ 100.7, a chemical shift typical of C-4a of flavanone systems. This suggested a 6-methyl derivative. Correlations from the aromatic proton singlet at δ 6.13 (Fig. 4) were consistent with this deduction and showed that the contributing proton was located at C-8. Correlation between the methoxyl proton signal at δ 3.86 and the carbon signal at δ 166.5 (C-7), which in turn correlated to the H-8 signal, established C-7 methoxylation. A similar set of correlations (Fig. 4) established that (**14**) was methylated at C-8 and had a methoxyl group at C-7.

Table 1
^{1}H NMR and ^{13}C NMR data for (**12**) and (**14**) at 25 °C[a]

Position	^{1}H		^{13}C	
	(**12**)	(**14**)	(**12**)	(**14**)
2	–	–	103.4	102.6
3	4.65, *s*	4.59, *s*	75.4	75.4
4	–	–	194.0	194.3
4a	–	–	100.7	100.7
5	–	–	159.4	161.3
6	–	6.16, *s*	106.9	92.8
7	–	–	166.5	166.9
8	6.13, *s*	–	92.3	106.7
8a	–	–	157.4	154.9
1′	–	–	139.8	139.0
2′,6′	7.76, *m*	7.80, *m*	126.1	126.3
4′	7.47, *m*	7.47, *m*	128.5	128.5
3′,5′	7.45, *m*	7.45, *m*	129.5	129.6
2-OH	n.d.	n.d.	–	–
3-OH	n.d.	n.d.	–	–
5-OH	11.06, *s*	11.02, *s*	–	–
6-Me	2.02, *s*	–	6.9	–
8-Me	–	2.02, *s*	–	7.5
7-OMe	3.86, *s*	3.87, *s*	56.0	56.1

[a] Solvent $CDCl_3$; 500 MHz (^{1}H); 125 MHz (^{13}C); n.d. = not detected.

The next task was to assign the relative orientation of the 2-OH and 3-OH groups. The ^{1}H NMR spectrum of our sample was obtained in acetone-d_6 to compare with similar compounds reported in the literature where couplings had been reported between the 2-OH proton and H-3 (Hauteville et al., 1979) (2-OH signals were not observed in our spectra of the (**12**)–(**15**) mixture recorded in $CDCl_3$). The isomeric composition of the mixture proved to be solvent dependent. In $CDCl_3$ the major isomers (**12**) and (**14**) dominated over two minor isomers (**13**) and (**15**) to the extent of 97:3. In acetone-d_6 the ratio was about 7:3.

Conformational searching of the *cis*-isomer of the 8-methyl compound (**14**) generated several low energy conformations. These all had the O–H bond at C-2 in an *anti* relationship with the C–H bond at C-3 (Fig. 5). This *W* planar arrangement would favour long-range

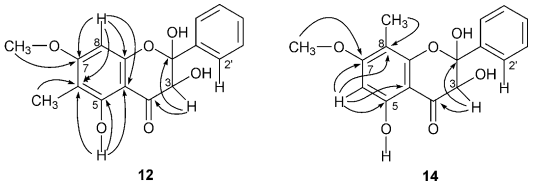


Fig. 4. Significant long-range (^{1}H–^{13}C) correlations (CIGAR) for isomers (**12**) and (**14**).

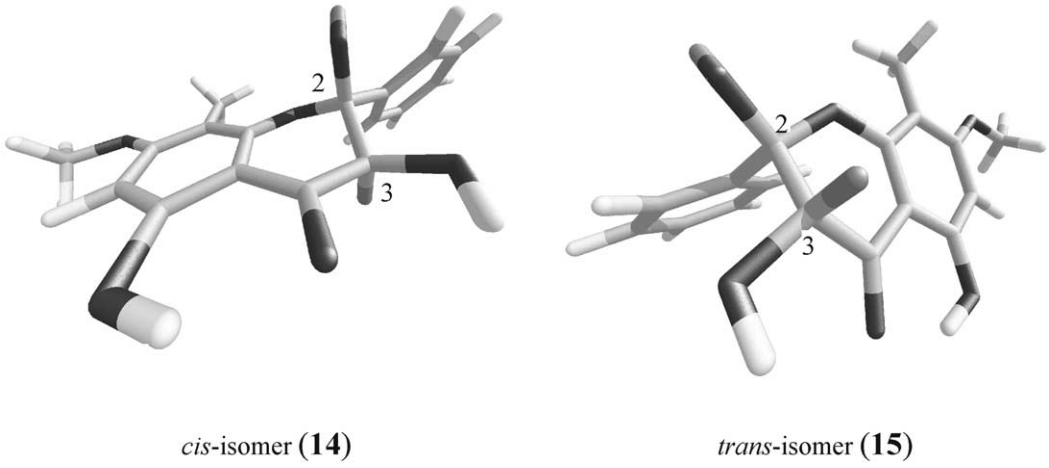


Fig. 5. Predicted most stable conformations of the *cis*- and *trans*-8-methyl-7-methoxy-2,3,5-trihydroxyflavanones, from molecular modelling, showing the spatial relationship between the 2-OH proton and H-3.

coupling. Conformations calculated for the *trans*-arrangement (**15**) had a *gauche* relationship between the O–H bond at C-2 and the C–H bond at C-3, incompatible with a substantial long-range coupling. Published data for some synthetic 2,3-hydroxyflavanones (**17**) and (**18**) (Hauteville et al., 1979) (Table 2) are consistent with this conclusion. The fact that the major isomers showed significant long-range couplings between H-3 and the C-2 OH proton (1.5 Hz, Table 2) suggests that these both have the *cis* geometry as in (**12**) and (**14**).

The 2,3,5-trihydroxyflavanone mixture (**12**)–(**15**) gave a small optical rotation of $[\alpha]_D$ +4°. These compounds have two chiral centres and like (**10**)/(**11**) they will interchange C-2 stereochemistries readily (Fig. 6). Although, C-3 is not implicated in the tautomeric equilibria responsible for this interchange it too may lose its stereochemical integrity through enolisation in the ring-opened β-diketone state (the absolute stereochemistry has not been determined and the configuration shown at C-3 is arbitrarily chosen). These compounds have so far resisted attempts at separation, but it may be possible to isolate one or more components by selective crystallisation as was achieved for the mixture of (**10**) and (**11**). The limited amount of material available from this plant has precluded further investigation of this possibility.

Table 2
Key ^{1}H NMR signals of *cis* and *trans*-2,3-dihydroxyflavanones[a]

	(**17**)[b] *cis* (~70%)	(**18**)[b] *trans* (~30%)	(**12**)/(**14**) *cis* (~70%)	(**13**)/(**15**) *trans* (~30%)
2-OH	7.02 (*d*,2)	7.10 (*s*)	7.04/6.99 (*d*,1.5)	7.12/7.08 (*s*)
3-OH	5.23 (*d*,6.5)	5.90 (*d*,6.5)	5.13/5.11 (*d*,6.5)	5.88/5.85 (*d*,6.5)
H-3	4.80 (*dd*,2,6.5)	4.03 (*d*,6.5)	4.77/4.70 (*dd*,2.5,6.5)	4.00/3.97 (*d*,6.5)

[a] Solvent acetone-d_6; 500 MHz; −28 °C; expressed as chemical shift in ppm relative to TMS (multiplicity, coupling constant(s) in Hz).
[b] Hauteville et al. (1979).

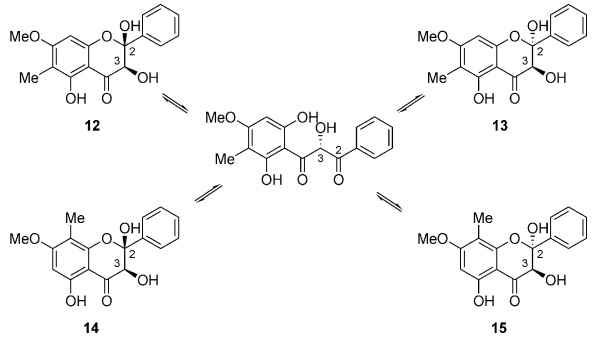


Fig. 6. Inter-conversion of hemiacetal isomers (**12**)–(**15**).

3. Conclusions

The presence of triketones (**1**) and (**3**) in this specimen of *L. polygalifolium* subsp. *polygalifolium* foliage oil, along with the closely related (**2**), agrees with early results on this sub-species (Briggs et al., 1938, 1945; Penfold, 1921), but contrasts with Brophy's findings (Brophy et al., 2000). It seems likely that this is another example of infraspecific variation of triketone levels, as found in *L. scoparium* (Perry et al., 1997). Oleanolic acid (**16**) is a common triterpene in plants and has been reported once previously from a *Leptospermum* species, from the bark of *L. scoparium* (Corbett and McDowall, 1958).

As in *L. recurvum* (Mustafa et al., 2003), the mixture of hemiacetal 2-hydroxyflavanones (**10**) and (**11**) dominates the flavonoid composition of the *L. polygalifolium* subsp. *polygalifolium* foliage. These compounds are accompanied by the the isomeric flavanones (**7**) and (**8**). However, the dihydrochalcones found in *L. recurvum* were not observed in this taxon. The fact that solution equilibria are rapidly established in 2-hydroxyflavanone systems such as (**10**)/(**11**) indicates

that reported optical rotations probably result from residual levels of co-occurring metabolites with high rotation values. Flavonoids with hydroxyl groups at both C-2 and C-3 are rare in nature. Of the four examples known prior to this work, two are biflavonoids (Arens et al., 1986). Compounds of this type have been generated in the laboratory by enzymic transformations of flavanols (Hosel and Barz, 1972).

Biological activities noted for the crude plant extract were not concentrated in the flavonoid fractions isolated and the anti-microbial activities were associated with the triketones (**1**)–(**3**) (Perry et al., 1997; Porter and Wilkins., 1998). No significant activities were noted for the 2-hydroxyflavanones reported in this study.

4. Experimental

4.1. General experimental procedures

UV and IR spectra were recorded on a Jasco 7800 UV–vis spectrometer and a Perkin-Elmer 1600 FTIR instrument respectively. NMR spectra were recorded on a Varian INOVA-500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. NMR spectra were recorded on ca 0.075 M solutions in $(CD_3)_2CO$ (referenced to solvent, δ 2.05 for ^{1}H and δ 29.9 for ^{13}C), C_6D_6 (referenced to solvent, δ 7.16 for ^{1}H and δ 128.6 for ^{13}C) or $CDCl_3$ (referenced to solvent, δ 7.25 for ^{1}H and δ 77.0 for ^{13}C) at 25 °C. Spectra were assigned with the aid of double-quantum filtered COSY (^{1}H–^{1}H correlations), HSQC (one bond ^{1}H–^{13}C correlations), and HMBC-CIGAR experiments (two and three bond ^{1}H–^{13}C correlations) (Hadden et al., 2000). Analytical HPLC was performed on HPLC equipment consisting of a Waters 717 auto-sampler, 600 pump and controller, and a 490E UV programmable multi-wavelength detector controlled by Millennium software. Samples were analysed using an RP-18 analytical column (Merck 100 RP-18, LichroCART 250×4 mm, 5 μm) fitted with a guard column (Merck 100 RP-18, LichroCART 4×4 mm, 5 μm). The mobile phase flow rate was 1.0 ml/min, with UV detection at 206, 254, 280 and 325 nm. The initial solvent mix was MeCN–H_2O (20:80) (both solvents containing 0.1% H_3PO_4), with a linear ramp up to 100% MeCN over 40 min, a return to the initial mix over 10 min, and 10 min equilibration before the next analysis. Semi-preparative HPLC used a Waters 717 auto-sampler, 600 pump and controller, a 2487 λ absorbance detector and fraction collector II. Samples were separated using an RP-18 semi-preparative column (Merck 100 RP-18, LichroCART 250×10 mm, 10 μm) fitted with a guard column (Merck 100 RP-18, LichroCART 4×4 mm, 5 μm). Silica gel 60, 200–400 mesh, 40–63 μm (Merck) was used in the column chromatography and octadecyl-functionalised silica gel (C-18, Aldrich) was used for reversed-phase (RP) chromatography. Melting points were determined on a hot bench Leica AG melting point apparatus first calibrated with standard samples of known melting points. Optical rotations were performed on a Perkin-Elmer 241 polarimeter using deuterium (Na, 589 nm) and mercury (Hg 577, 546, 435, 405 nm) lamps with specified filters. The instrument was first calibrated using cholesterol (20 mg/ml). Biological assays were performed as described previously (Lorimer et al., 1996). Molecular mechanics calculations were performed with PCModel version 7 incorporating π-calculations. Conformational searches used the MMX force field, with the mixed Monte Carlo coordinate movements/bond rotations strategy (Saunders et al., 1990) for the generation of initial structures. The default cut-off criteria were employed.

4.2. Plant material

L. polygalifolium subsp. *polygalifolium* was collected from Lincoln Landcare Research Garden, New Zealand in November 1997. A voucher specimen (code 971108-23) has been deposited at the Plant Extract Research Unit, University of Otago, Dunedin, New Zealand.

4.3. Extraction and isolation of flavonoids of *L. polygalifolium subsp. polygalifolium*

Details of the separation process are summarised in Fig. 1.

4.3.1. EtOH extract

Foliage (109 g) was dried, ground and extracted with EtOH (650 ml, then 350 ml). Removal of the solvent from the filtered extracts gave a green gum (14.9 g). In disk diffusion assays this crude extract showed activity against *Bacillus subtilis*, *Candida albicans* and *Trichophyton mentagrophytes* at 600 μg/disk. Fractionation by reversed-phase column chromatography (50 g, C-18) was carried out with gradient elution from H_2O through CH_3CN to $CHCl_3$. The anti-microbial active fractions were pooled into five groups: E1 (H_2O–CH_3CN; 1:3), E2 (H_2O–CH_3CN; 1:3), E3 (H_2O–CH_3CN; 1:3), E4 (H_2O–CH_3CN; 1:9) and E5 (H_2O–CH_3CN; 1:9 to 0:1) based on their UV-active spots on TLC silica (cyclohexane–EtOAc; 3:1). Compositions of fractions were determined by ^{1}H NMR and by HPLC. Fraction E1 (256 mg) contained only the isomeric 2,5-dihydroxyflavanones (**10**) and (**11**). Fraction E2 (202 mg) also contained (**10**) and (**11**) along with triketone (**1**). Fraction E3 (202 mg) consisted of compounds (**10**) and (**11**) and triketones (**1**)–(**3**). Fraction E4 (183 mg) was further fractionated on a silica gel column (5 g) using a cyclohexane–EtOAc gradient to give triketones (**1**)–(**3**) (59 mg, cyclohexane–EtOAc; 19:1), a mixture of com-

pounds (**7**), (**8**), (**10**) and (**11**) (37 mg, cyclohexane–EtOAc; 18:2) and a fraction of the isomeric (**10**) and (**11**) (8 mg, cyclohexane–EtOAc; 18:2, 15:1). Fraction E5 (446 mg) was dissolved in Et_2O and extracted with satd. $NaHCO_3$ (3×10 ml). The Et_2O phase was partitioned with 5% Na_2CO_3 (3×10 ml) dried ($MgSO_4$) and evaporated to give an oil (257 mg) that was fractionated on a silica gel column (6 g, cyclohexane–EtOAc; 19:1) yielding a mixture of isomers (**7**) and (**8**) (20 mg) and another of isomers (**10**) and (**11**) (15 mg). The Na_2CO_3 extracts from this work-up were acidified and extracted with Et_2O. The Et_2O extracts were dried ($MgSO_4$) and evaporated to give a mixture of triketones (**1**)–(**3**) (96 mg). The $NaHCO_3$ extracts were treated similarly to yield triketones (**1**)–(**3**) (15 mg).

4.3.2. $CHCl_3$ extract

The residue from the EtOH extraction was extracted with $CHCl_3$ (265 ml). The extract was dried ($MgSO_4$), dissolved in Et_2O and extracted with satd. $NaHCO_3$ and Na_2CO_3 as above. The $NaHCO_3$ and Na_2CO_3 extracts gave mixtures of triketones (**1**)–(**3**) (170 and 191 mg respectively). The neutral fraction was fractionated on a silica gel column (20 g) to give six flavonoid containing fractions, C1 (cyclohexane–EtOAc; 19:1), C2 (cyclohexane–EtOAc; 19:1), C3 (cyclohexane–EtOAc; 19:1), C4 (cyclohexane–EtOAc; 18:2), C5 (cyclohexane–EtOAc; 18:2) and C6 (cyclohexane–EtOAc; 17:3 and 16:4). Fraction C1 (23 mg) contained the isomeric flavones (**7**) and (**8**) along with unidentified terpene derivatives. Fraction C2 (87 mg) contained isomers (**10**) and (**11**) contaminated with fatty acid derivatives. Fraction C3 (251 mg) consisted of (**10**) and (**11**), while fraction C4 (16 mg) comprised a mixture containing the isomers (**12**)–(**15**). Fraction C5 (116 mg) consisted of a mixture of hemiacetals (**12**)–(**15**) and oleanolic acid (**16**). The composition of fraction C6 (154 mg) was similar to that of fraction C5. Further chromatography on C5 failed to separate the triterpene from the flavonoids, but careful addition of H_2O to a soln. of the mixture (60 mg) in a 1:1 mixture of MeOH and CH_3CN (1.0 ml) crystallised out the triterpene, (**16**) as white fluffy material (6.5 mg). A sub-sample of the filtrate (22 mg) was further purified by preparative RP HPLC (C-18) (CH_3CN:MeOH; 1:1) to give the isomeric flavonoids (**12**)–(**15**) (3 mg). A sample of C6 (154 mg) was passed through a short C-18 column which removed the major component (**16**), and the effluent was also subjected to preparative HPLC to give a further portion of the mixture of (**12**)–(**15**) (2 mg).

4.3.3. Triketones (1)–(3) (CAS No. (1)-22595-45-5; (2)-5009-05-2; (3)-567-75-9)

Identity was established by comparison with authentic samples by HPLC and NMR spectroscopy (van Klink et al., 1999).

4.3.4. Flavonoids (7), (8), (10) and (11) (CAS No. (7)-55969-57-8; (8)-14004-48-9; (10)-186906-54-7; (11)-186906-53-6)

Identity was established by comparison by HPLC and NMR spectroscopy with samples isolated from *L. recurvum* (Mustafa et al., 2003).

4.3.5. cis and trans-2,3,5-Trihydroxy-6-methyl-7-methoxyflavanone (12) and (13) and cis and trans-2,3,5-trihydroxy-8-methyl-7-methoxyflavanone (14) and (15)

The mixture of (**12**) (*cis*-2,3,5-trihydroxy-7-methoxy-6-methyl-2-phenylchroman-4-one), (**13**) (*trans*-2,3,5-trihydroxy-7-methoxy-6-methyl-2-phenylchroman-4-one), (**14**) (*cis*-2,3,5-trihydroxy-7-methoxy-8-methyl-2-phenylchroman-4-one) and (**15**) (*trans*-2,3,5-trihydroxy-7-methoxy-8-methyl-2-phenylchroman-4-one) was obtained as a yellow oil; $[\alpha]_D^{19.7}$ +4.1°, $[\alpha]_{577\ nm}^{20.0}$ −7.5°, $[\alpha]_{546\ nm}^{20.3}$ −24.0°, $[\alpha]_{435\ nm}^{20.6}$ −136.4°, $[\alpha]_{405\ nm}^{20.9}$ −156.5° ($CHCl_3$; *c* 0.20); UV λ_{max} MeOH nm (log ε): 294 (3.83), 316 (3.18) (shoulder); IR ν_{max} (film) cm^{-1}: 3385 *br*, 3015, 2923, 1636, 1585, 1451, 1143; ^1H NMR (acetone-d_6), (**12**)/(**14**) (*cis*): 11.54/11.49 (2×1H, *s*, 5-OH), 7.81 (4H, *m*, 2′-H and 6′-H), 7.41–7.43 (6H, *m*, 3′-H, 4′-H and 5′-H), 6.56/6.59 (2×1H, *d*, J=1.5 Hz, 2-OH), 6.20 (2H, s, H-6 and H-8), 4.74/4.68 (2×1H, *br*. *d*, J=4.5 Hz, H-3), 4.591/4.61 (2×1H, *d*, J=6.0 Hz, 3-OH), 3.90 (6H, *s*, 7-OMe), 1.96 (6H, *s*, 6-Me and 8-Me); (**13**)/(**15**) (*trans*): 11.80/11.78 (2×1H, *s*, 5-OH), 7.76 (4H, *m*, 2′-H and 6′-H), 7.41–7.43 (6H, *m*, 3′-H, 4′-H and 5′-H), 6.68/6.65 (2×1H, *s*, 2-OH), 6.24/6.18 (2×1H, *s*, H-6 and H-8), 4.07/4.05 (2×1H, *d*, J=6.5 Hz, H-3), 5.38/5.35 (2×1H, *d*, J=6.5 Hz, 3-OH), 3.92 (6H, *s*, 7-OMe), 2.03/1.96 (2×3H, *s*, 6-Me and 8-Me); ^{13}C NMR (acetone-d_6), (**12**)/(**14**) (*cis*): 197.8/197.6 (C-4), 167.1/166.8, (C-7), 162.3/160.2 (C-5), 159.1/156.7 (C-8a), 141.8/141.5 (C-1′), 129.6/129.5 (C-3′/C-5′), 128.7/128.6 (C-4′), 127.8 (C-2′/C-6′), 106.3/106.1 (C-6/C-8), 105.4/104.8 (C-2), 102.3/102.2 (C-4a), 93.1/93.0 (C-6 /C-8), 77.4 (C-3), 56.6 (7-OMe), 7.9/7.1 (8-Me/6-Me); (**13**)/(**15**) (*trans*): 197.5/197.3 (C-4), 167.0/166.7 (C-4), 163.7/161.4 (C-5), 159.2/156.6 (C-8a), 141.4/141.0 (C-1′), 129.6/129.5 (C-3′/C-5′), 128.8/128.7 (C-4′), 127.8/127.7 (C-2′/C-6′), 105.9/105.8 (C-6/C-8), 103.7/103.4 (C-2), 101.6/101.5 (C-4a), 93.0 (C-6 /C-8), 75.4 /75.2 (C-3), 56.6 (7-OMe), 8.0 (8-Me/6-Me); ^1H and ^{13}C NMR ($CDCl_3$) for (**12**) and (**14**), see Table 1. HR-EIMS: *m*/*z* (rel. int.): 316.0947 [M^+] (14) (calc. for $C_{17}H_{16}O_6$ 316.0947), 298.0849 (20), 181.0509 (100), 105.0347 (81), 77.0385 (39).

4.3.6. Oleanolic acid (16) (CAS No. 508-02-1)

Compound (**16**) (Mahato and Kundu, 1994) was obtained as a white fluffy solid; mp 288 °C (Lit. 295–297 °C (Corbett and McDowall, 1958)) IR (KBr) ν_{max} 3500–3000, 3412, 2924, 2851, 1683, 1636, 1614, 1457 cm^{-1}; ESI (-ve): *m*/*z* 455 [M^+–H]; ^1H NMR and ^{13}C NMR as reported by Mahato and Kundu (1994).

4.4. 2,5-Dihydroxy-6-methyl-7-methoxyflavanone (10)

A soln. of a mixture containing compounds (**10**) and (**11**) (263 mg) in EtOH (2 ml) in a 4 ml vial was placed in a 16 ml capped-vial containing H_2O (2.5 ml). After cooling in a refrigerator for a week, the crystals were collected by removal of supernatant liquid and air-drying to give 2,5-dihydroxy-6-methyl-7-methoxyflavanone (2,5-dihydroxy-7-methoxy-6-methyl-2-phenylchroman-4-one) (**10**) as a milky-yellow crystalline powder (92 mg). The supernatant liquor was stored in the refrigerator for another week to give a further crop of (**10**) (50 mg). Mp 143°; IR ν_{max} (KBr) cm^{-1}: 3412, 3274, 3083, 2992, 2923, 1645, 1603, 1573, 1508, 1497, 1447, 1325, 1295, 1203, 1154, 1120, 769, 697; HR-EIMS: *m*/*z* (rel. int.): 300.0994 [M^+] (45) (calc. for $C_{17}H_{16}O_5$ 300.0998), 282.0834 (31), 181.0396 (25), 180.0333 (28), 154.0545 (33), 152.0412 (21), 105.0296 (100), 77.0365 (42); found: C, 67.9; H, 5.1, $C_{17}H_{16}O_5$ requires: C, 68.0; H, 5.4%. The remaining data were obtained on equilibrated mixtures of (**10**) and (**11**) derived from pure (**10**): optical rotations (average of 5 measurements) $[\alpha]_D^{24.2}$ 8 (±3)°, $[\alpha]_{577\ nm}^{25.1}$ −14 (±4)°, $[\alpha]_{546\ nm}^{25.5}$ −45 (±4)°, $[\alpha]_{435\ nm}^{25.9}$ −269 (±4)°, $[\alpha]_{405\ nm}^{26.2}$ −324 (±4)° ($CHCl_3$; *c* 0.10); UV λ_{max} MeOH nm (log ε): 290 (4.14), 335 (3.55) (shoulder); ^1H and ^{13}C NMR ($CDCl_3$) as reported by Mustafa et al. (2003): ^1H NMR (C_6D_6) δ 12.65 (1H, *s*, 5-OH), 7.35 (2H, *m*, H-2′/H-6′), 7.09 (3H, *m*, H-3′/H-4′/H-5′), 5.98 (1H, *s*, H-8), 3.24 (3H, *s*, 7-OMe), 2.24 (3H, *s*, 6-Me); ^{13}C NMR (C_6D_6): δ 194.2 (C-4), 165.7 (C-7), 160.2 (C-5), 157.7 (C-8a), 141.9 (C-1′), 129.2 (C-4′), 128.8 (C-3′/C-5′), 125.0 (C-2′/C-6′), 106.8 (C-6), 102.3 (C-4a), 101.1 (C-2), 91.6 (C-8), 55.8 (7-OMe), 48.4 (C-3), 6.9 (6-Me).

4.5. NMR study of equilibration of (10) and (11)

The pre-acquisition time delay was arrayed (0, 60 s×5, 120 s×5, 180 s×5, 240 s×5, 300 s×5, 360 s×5, 420 s×5, 480 s×5, 540 s×5, 600 s×5, 660 s×5) to create a set of time intervals for data collection. The spectrometer was shimmed on a blank sample of C_6D_6 (0.75 ml) immediately prior to conducting the experiment. C_6D_6 (0.75 ml) was added to a sample of 2,5-dihydroxy-6-methyl-7-methoxyflavanone (**10**) (1.0 mg, 3.4 μmol) in a 5 mm NMR tube that was immediately inserted into the NMR probe and the data collection commenced. In total, 56 experiments were run over a period of 20 h. Equilibrium was reached after ca 390 min when an approximately 1:1 mixture of (**10**) and (**11**) was obtained.

Acknowledgements

Thanks to W. Harris and J. van Klink for plant material, D. Orlovich for taxonomic identification, M. Thomas and W. Redmond for assistance with NMR experiments, B. Clark for MS analysis; G. Ellis for biological assays, and M. Dick for microanalysis. This research was supported in part by the New Zealand Foundation for Research, Science and Technology and by the New Zealand Official Development Assistance (NZODA)/New Zealand Agency for International Development (NZAID), Ministry of Foreign Affairs and Trade for a PhD scholarship.

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