NPC Natural Product Communications

2011
Vol. 7
No. 5
551 - 560

On-line (HPLC-NMR) and Off-line Phytochemical Profiling of the Australian Plant, *Lasiopetalum macrophyllum*

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Received: July 18th, 2011; Accepted: September 22nd, 2011

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On-line (HPLC-NMR) and off-line (HPLC, NMR and MS) methodologies were used to profile the constituents present in the crude extract of *Lasiopetalum macrophyllum*. On-flow and stop-flow HPLC-NMR supported the presence of *trans*-tiliroside and permitted partial identification of *cis*-tiliroside and 4′-methoxy-*trans*-tiliroside. Off-line isolation led to the unequivocal identification of four flavanoid glycosides including a new structural derivative, 4′-methoxy-*cis*-tiliroside. This is the first report of flavonoid glycosides occurring in this plant genus. In addition, a number of structure revisions have been proposed for previously reported flavonoid glycosides that were incorrectly assigned.

Keywords: HPLC-NMR, natural products, dereplication, flavonoid glycosides.

The plant kingdom has been an indispensable source of many of the most recognized drugs, including the analgesic morphine and the anti-cancer drug taxol, as well as providing a source of important drug leads [1,2]. Although combinatorial chemistry allows for the production of a large range of compounds [3], the sheer biodiversity of the plant kingdom has led to a unique range of metabolites [4-6]. Plant extracts are often complex mixtures that contain a vast array of compounds. Any possible insight into the range of secondary metabolites present is highly desirable so that lengthy isolation procedures can be circumvented. This process is commonly known as dereplication [7,8]. HPLC-NMR has been demonstrated to be effective in the chemical profiling of natural product extracts, as it not only allows for structural information to be obtained without the need for an isolation, but owing to the fact that it is a non-destructive technique, the extract or enriched fraction can be fully recovered for subsequent analyses [9]. However, this hyphenated spectroscopic technique does have limitations in that only a limited number of secondary metabolites have been studied by HPLC-NMR, making it difficult, at times, to unequivocally identify compounds [10,11]. Despite this, the greatest benefits of HPLC-NMR include the ability to separate compounds *in situ* and that it can potentially provide structure class information for complex mixtures [12,13]. In this way it can also quickly establish which HPLC chromatographic peaks in a complex mixture are due to structurally related secondary metabolites. One final benefit of HPLC-NMR is that it is the preferred NMR technique for the analysis and identification of unstable metabolites [14].

A major focus of the **M**arine **a**nd **T**errestrial **NA**tural Product (MATNAP) research group at RMIT University is the study of the chemical diversity and biological activities of Australian flora. The plant family Malvaceae, commonly referred to as the Mallow family, consists of 243 genera and 4225 species that are found worldwide [15]. In 1997, the taxonomy of the family Malvaceae was broadened to include the families Sterculiaceae, Tiliaceae and Bombacaceae [16]. Malvaceae is comprised of many well known genera including *Gossypium* (cotton) and *Hibiscus* (ornamental), and many of these genera are known to contain biologically active constituents [17,18]. The Malvaceae has afforded many classes of secondary metabolites, including terpenoids (**1-3**) [19], alkaloids (**4-5**) [20], steroids (**6-7**) [21], naphthalenes (**8-9**) [17], and flavonoids (**10-16**) (Figure 1) [17,21-25]. The genus *Lasiopetalum*, belonging to this family, has not been studied widely in terms of its secondary metabolites, with only fatty acid analyses of seed oils being reported to date [26,27]. An extract of the Australian plant *L. macrophyllum* was selected for chemical investigation on the basis that essentially no previous chemistry had been reported for this genus, together with the fact that this family is known to produce a wide array of structure classes with bioactive properties.

For the HPLC-NMR chemical profiling, the plant material was extracted and subjected to the fractionation methodology as described in the 'Preparation of the extract of *L. macrophyllum* for on-flow and stop-flow HPLC-NMR analysis' section. An off-line HPLC method for the separation of the methanol extract was developed prior to conducting the HPLC-NMR profiling studies. On-flow HPLC-NMR analysis of the extract supported the presence of a major aromatic glycoside. Diagnostic ^{1}H NMR signals were observed at δ 7.88 (d, J = 9 Hz), δ 7.21 (d, J = 8.5 Hz), δ 6.83 (d, J = 9 Hz) and δ 6.76 (d, J = 8.5 Hz) for the aromatic proton resonances; δ 7.24 (d, J = 16 Hz) and δ 5.92 (d, J = 16 Hz), which indicated the presence of a *trans* double bond; and protons at δ 5.03 (d, J = 7.5 Hz), δ 4.11 (d, J = 5.5 Hz), δ 3.38 (m), 3.32 (m) and 3.21 (m), all supportive of a sugar moiety. On the basis of this NMR data and the fact that this family is known to produce flavonoid glycosides [17,21,23,28,29], this compound was suggested to be *trans*-tiliroside (**16**), as illustrated in the 2D HPLC-NMR contour plot and in the extracted WET-1D ^{1}H NMR spectrum (Figure 2). Given the relatively poor signal-to-noise obtained in the on-flow HPLC-NMR analysis, together with the fact that the other components observed in the HPLC chromatogram could not be detected, it was necessary to carry out stop-flow HPLC-NMR analyses. This enabled longer acquisition times to be utilized for the detection of other compounds. The methanol extract

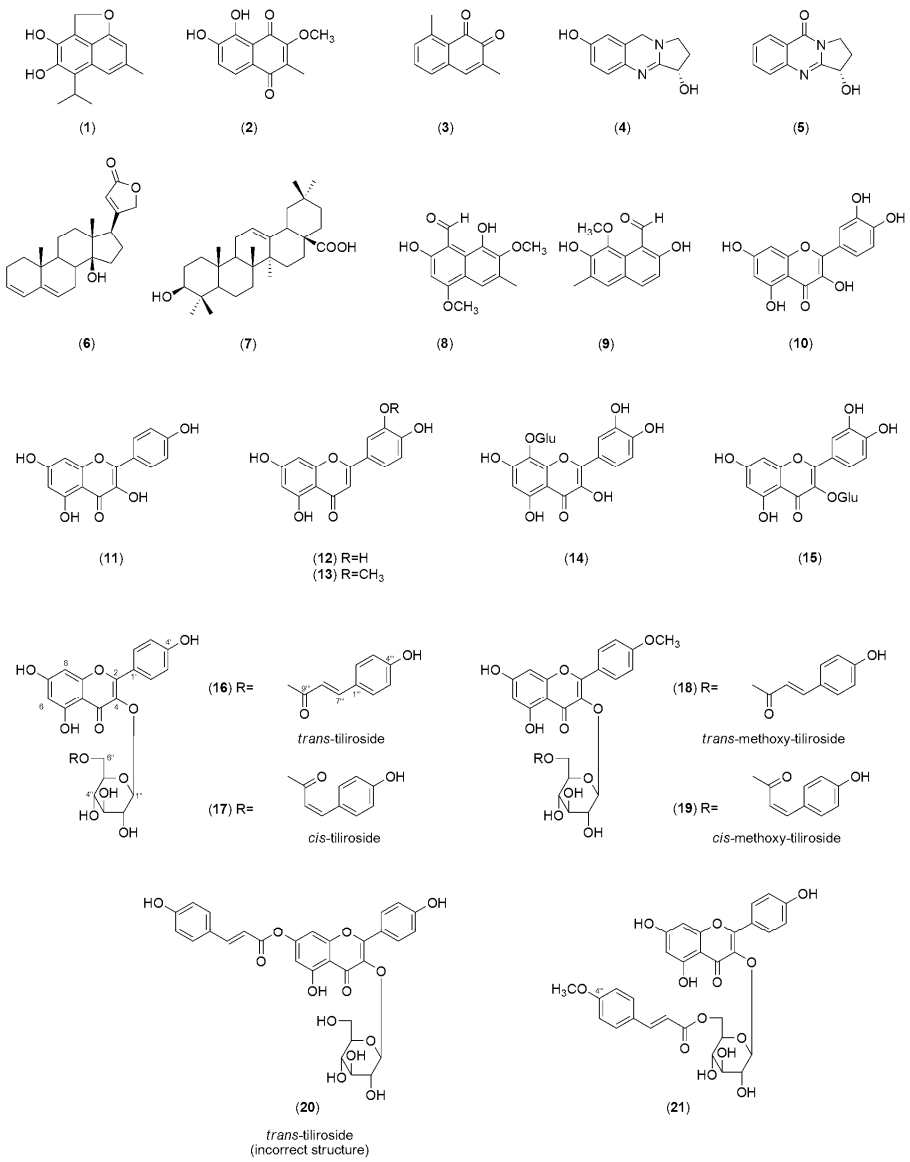

Figure 1: Secondary metabolites from species of Malvaceae.

was re-injected and stop-flow HPLC-NMR analysis employed, enabling the HPLC separation to be stopped and the chromatographic peak of interest to be trapped within the NMR flow-cell. This led to extended acquisition times for the WET-1D ^{1}H NMR experiments (typically 20 mins - 16 h) and resulted in a significantly improved signal-to-noise WET-1D ^{1}H NMR spectrum for the major flavonoid glycoside *trans*-tiliroside (**16**), as well as allowing for the detection of two additional flavonoid glycosides suggested to be *cis*-tiliroside (**17**) and 4′-*O*-methoxy-*cis*-tiliroside (**18**), as illustrated in Figure 2. All WET-1D ^{1}H NMR spectra exhibited resonances consistent with the presence of olefinic and aromatic methines, along with signals associated with a sugar moiety. For compound (**17**), which eluted closely with compound (**16**), diagnostic ^{1}H NMR chemical shift differences for **17** were observed at δ 6.62 (d, J = 14.5 Hz) and δ 5.41 (d, J = 14.5 Hz), supporting the presence of a *cis* double bond geometry and suggesting this component to be *cis*-tiliroside. The signal to noise for compound **18** was relatively poor in comparison with compounds **16** and **17**, which meant that chemical shifts were not as well resolved from the baseline. Despite this, several diagnostic features could be extracted from the WET-1D ^{1}H NMR spectrum for compound **18**. In particular, the stop-flow HPLC-NMR analysis indicated that this compound also had a *trans* double bond [δ 7.33 (d, J = 14.5 Hz) and δ 6.01 (d, J = 14.5 Hz)] together with a methoxy moiety [δ 3.72, s]. When comparing the NMR data of compound **18** with that of compounds **16** and **17**, it was suggested that this compound was in fact the methoxy derivative of *trans*-tiliroside (**16**). This was further supported by the fact that the proton NMR chemical shift variations between **16** and **18** were minor. However, the coupling constant of the double bond in **18** could not be measured accurately due to overlapping resonances. This prolonged analysis time was inadequate in detecting the fourth component observed in the off-line HPLC chromatogram, but the presence of characteristic UV chromophores at 269 nm and 317 nm indicated that this compound was also a flavonoid glycoside. Off-line separation was necessary in order to determine the identity of this fourth component [compound **19**], as well as to complete the unequivocal structure identification of the other three flavonoid glycosides (**16-18**). In the case of compounds **16-18**, solvent suppression did not allow for a complete identification and in the case of compound **19** insufficient detection limits meant that even a partial identification by HPLC-NMR was not possible.

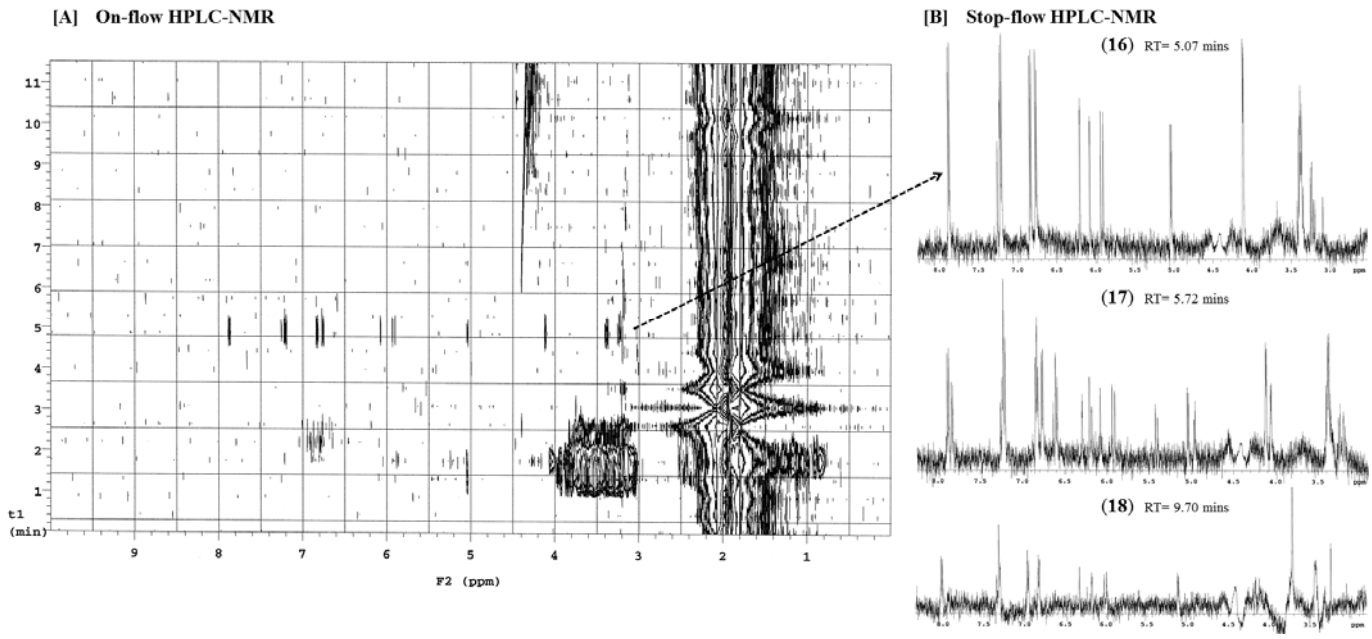

Figure 2: **[A]** 2D HPLC-NMR contour plot showing the major compound (**16**) (on-flow HPLC-NMR) and **[B]** Extracted WET-1D ^{1}H NMR spectra of compounds (**16-18**) (stop-flow HPLC-NMR).

HPLC-NMR, as well as other hyphenated spectroscopic techniques such as HPLC-MS and GC-MS, can be extremely useful in chemically profiling crude extracts and/or enriched fractions. However, combining off-line methodology with these hyphenated techniques is often essential, especially in the identification of previously unrecognized secondary metabolites. All hyphenated methodologies have specific limitations. In the case of HPLC-NMR these include firstly, the difference in the limit of detection that arises when both techniques are combined (i.e. HPLC often requires overloading which effects peak separation, in order to reach the detection limits required for NMR analysis), as well as the fact that analyte signals can often be completely or partially diminished when solvent signals are suppressed. In addition, chemical shifts for the solvents used for HPLC-NMR (typically CH_3CN/D_2O) generally differ from chemical shifts reported in deuterated NMR solvents, making comparisons with literature data difficult [9,12]. To unequivocally identify the four secondary metabolites (**16-19**), off-line purification employing reversed phased HPLC was undertaken.

For the off-line HPLC purification, the plant material was extracted and subjected to the fractionation methodology as described in the 'Preparation of the extract for off-line isolation of secondary metabolites from *L. macrophyllum*' section. Analytical HPLC and ^{1}H NMR analysis of the methanol soluble extract confirmed the presence of the flavonoid glycosides that were previously observed in the HPLC-NMR analysis. Flash C18 Vacuum Liquid Chromatography (VLC) was carried out on the methanol crude extract and afforded 13 fractions. On the basis of the subsequent ^{1}H NMR analyses conducted, four of these fractions displayed characteristic proton NMR resonances of the flavonoid glycosides and so these fractions were combined. This combined fraction was filtered and subjected to semi-preparative reversed phased HPLC, which resulted in the isolation of the four flavonoid glycosides (**16-19**).

The structure of **16** was determined on the basis of 1D and 2D NMR spectroscopy (Table 1) and mass spectrometry. The HR-ESI-MS of **16** displayed a *m/z* at 593.1308 $[M-H]^-$ (calcd. for $C_{30}H_{25}O_{13}$: *m/z* 593.1295) and a *m/z* at 595.1440 $[M+H]^+$ (calcd. for $C_{30}H_{27}O_{13}$: *m/z* 595.1373) consistent with 18 degrees of unsaturation and a molecular formula $C_{30}H_{26}O_{13}$. The ^{13}C NMR spectrum of **16** showed the presence of 26 discrete signals [13 methines (4 of these being overlapped and each accounting for 2 aromatic methines), 1 methylene and 12 quaternary carbons], as supported by the gHSQCAD 2D NMR experiment. The ^{1}H NMR spectrum and the 2D gCOSY NMR spectrum identified the presence of a set of *meta* coupled aromatic methines [δ 6.15, d, J = 2 Hz (H6) and δ 6.38, d, J = 2 Hz (H8)]; two 1,4-disubstituted aromatic rings [δ 7.98, dd, 2H, J = 1.5, 9 Hz (H2′/H6′) and δ 6.85, dd, 2H, J = 1.5, 9 Hz (H3′/H5′)] and [δ 7.37, d, 2H, J = 8.5 Hz (H2‴/H6‴) and δ 6.78, d, 2H, J = 8.5 Hz (H3‴/H-5‴)]; two olefinic methines [δ 7.34, d, J = 16 Hz (H7‴) and δ 6.12, d, J = 16 Hz (H8‴)] with *trans* coupling and finally evidence of a sugar moiety including five deshielded methines [δ 5.45, d, J = 7.5 Hz (H1″) and those occurring between δ 3.16 and δ 3.37, (H2″, H3″, H4″, H5″)] as well as one deshielded methylene [δ 4.02, dd, J = 6.5, 12 Hz (H6a″) and δ 4.26, dd, J = 1.5, 12 Hz (H6b″)].

In combination with the ^{13}C NMR spectrum, the 2D HMBC NMR experiment allowed for the complete assignment of **16**. The sugar moiety was concluded to be disubstituted at both the anomeric and methylene carbon on the basis of HMBC NMR correlations being observed for both of these residues. This included the deshielded methylene resonances at δ 4.02 (H6a″) and δ 4.26 (H6b″) showing a HMBC NMR correlation to 166.2 ppm (C9‴), supporting an ester linkage to this side of the sugar. Further HMBC NMR correlations observed to this ester carbon 166.2 ppm (C9‴) from the olefinic methines at δ 7.34 (H7‴) and δ 6.12 (H8‴) unambiguously placed the *trans* double bond adjacent to the ester. This structure fragment could be extended further by considering the HMBC NMR correlations observed from the aromatic protons at δ 7.37 (H2‴/H6‴) to the methine carbon at 144.6 ppm (C7‴). A further HMBC NMR correlation from the methine at δ 6.12 (H8‴) to the quaternary carbon at 124.9 ppm (C1‴) suggested a connection to one of the two 1,4-disubstituted aromatic rings, which was

Table 1: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data of *trans*-tiliroside (**16**) in DMSO-d_6.

Position	δ_H, *J*	δ_C [a]	gCOSY	gHMBC (4 Hz)	gHMBC (8 Hz)	gHMBC (10 Hz)
1	-	-	-	-	-	-
2	-	156.4, C	-	-	-	-
3	-	133.1, C	-	-	-	-
4	-	177.4, C	-	-	-	-
5	-	161.2, C	-	-	-	-
6	6.15 d, *J* = 2 Hz	98.8, CH	H8	C5, C7, C8, C10	C5, C7, C8, C10	C5, C7, C8, C10
7	-	164.2, C	-	-	-	-
8	6.38 d, *J* = 2 Hz	93.7, CH	H6	C6, C7, C9, C10	C6, C7, C9, C10	C6, C7, C9, C10
9	-	156.3, C	-	-	-	-
10	-	103.9, C	-	-	-	-
1'	-	120.8, C	-	-	-	-
2'	7.98 dd, *J* = 1.5, 9 Hz	130.9, CH	H3'	C2, C4', C6'	C2, C4', C6'	C2, C3, C4', C6'
3'	6.85 dd, *J* = 1.5, 9 Hz	115.1, CH	H2'	C4', C5'	C1', C4', C5'	C1', C4', C5'
4'	-	160.0, C	-	-	-	-
5'	6.85 dd, *J* = 1.5, 9 Hz	115.1, CH	H6'	C3', C4'	C1', C3', C4'	C1', C3', C4'
6'	7.98 dd, *J* = 1.5, 9 Hz	130.9, CH	H5'	C2, C2', C4'	C2, C2', C4'	C2, C2', C4', C5'
1''	5.45 d, *J* = 7.5 Hz	101.0, CH	H2''	C3, C2'', C3''	C3	C3
2''	3.21[b] m	74.1, CH	H1'', 2''-OH	C1'', C3''	C1'', C3''	C1'', C3''
3''	3.24[b] m	76.2, CH	3''-OH	C1'', C2'', C4''	C1'', C4''	C1'', C2'', C4''
4''	3.16[b] m	69.9, CH	4''-OH	C3'', C5'', C6''	C3''	C3'', C5'', C6''
5''	3.37[b] m	74.2, CH	H6a'', H6b''	C1''	C4''	C1'', C3'', C4''
6a''	4.02 dd, *J* = 6.5, 12 Hz	63.0, CH_2	H5'', H6b''	C5'', C9'''	C4'', C5'', C9'''	C5'', C9'''
6b''	4.26 dd, *J* = 1.5, 12 Hz		H5'', H6a''	C4'', C5'', C9'''	C4'', C5'', C9'''	C4'', C9'''
1'''	-	124.9, C	-	-	-	-
2'''	7.37 d, *J* = 8.5 Hz	130.2, CH	H3'''	C4''', C7'''	C3''', C4''' C6''', C7'''	C3''', C4''', C7'''
3'''	6.78 d, *J* = 8.5 Hz	115.8, CH	H2'''	C1''', C4''', C5'''	C1''', C4''', C5'''	C1''', C4''', C5'''
4'''	-	159.8, C	-	-	-	-
5'''	6.78 d, *J* = 8.5 Hz	115.8, CH	H6'''	C1''', C3''', C4'''	C1''', C3''', C4'''	C1''', C3''', C4'''
6'''	7.37 d, *J* = 8.5 Hz	130.2, CH	H5'''	C4''', C7'''	C2''',C4''', C5''', C7'''	C4''', C5''', C7'''
7'''	7.34 d, *J* = 16 Hz	144.6, CH	H8'''	C2'''/C6''', C8''', C9'''	C2'''/C6''', C8''', C9'''	C2'''/C6''', C8''', C9'''
8'''	6.12 d, *J* = 16 Hz	113.6, CH	H7'''	C1''', C9'''	C1''', C9'''	C1''', C9'''
9'''	-	166.2, C	-	-	-	-
5-OH	12.58 s	-	-	C5, C6, C7, C10	C5, C6, C10	C5, C6, C7, C10
7-OH	10.88 bs	-	-	-	-	-
4'-OH	10.18 bs	-	-	-	-	-
2''-OH	5.48 d, *J* = 4.5 Hz	-	H2''	C1'', C2'', C3''	C1'', C2'', C3''	C1'', C2'', C3''
3''-OH	5.19 d, *J* = 5.5 Hz	-	H3''	C2'', C3'', C4''	C2'', C3'', C4''	C2'', C3'', C4''
4''-OH	5.24 d, *J* = 4.5 Hz	-	H4''	C3'', C4'' C5''	C3'', C4'' C5''	C3'', C4'' C5''
4'''-OH	10.05 bs	-	-	-	-	-

[a] Carbon assignments based on gHSQCAD and gHMBC NMR experiments. [b] Signals overlapped

substituted with a hydroxy group, supported by the HMBC NMR correlation from the aromatic protons δ 7.37 (H2‴) and δ 6.78 (H3‴) to the carbon at 159.8 ppm (C4‴). These HMBC NMR correlations permitted this first structure fragment, attached to a central sugar moiety, to be identified as being *trans*-*p*-coumaroyl. A second ether linkage from the sugar moiety was identified on the basis of the HMBC NMR correlation observed from the anomeric proton at δ 5.45 (H1″) to the quaternary carbon at 133.1 ppm (C3). No further HMBC NMR correlations were observed to C3, suggesting a high degree of substitution in the structure at this point. The second 1,4-disubstituted aromatic ring was also established to be substituted with a hydroxy moiety on the basis of the correlations observed from the aromatic methines δ 7.98 (H2′) and δ 6.85 (H3′) to the deshielded carbon at 160.0 ppm. A further HMBC NMR correlation observed from δ 7.98 (H2′) to 156.4 ppm (C2), suggested that the 1,4-disubstituted aromatic ring was connected to a deshielded olefinic resonance. The remaining aromatic protons at δ 6.15 (H6) and δ 6.38 (H8) were *meta* coupled (*J* = 2 Hz) and concluded to be part of a highly substituted aromatic ring with correlations to the quaternary carbons 103.9 ppm (C10) and 156.3 ppm (C9). In addition to the carbon at 133.1 ppm (C3), a further quaternary carbon at 177.4 ppm (C4) was observed in the ^{13}C NMR spectrum, confirming that this substructure, attached to the anomeric proton of the sugar moiety, was the flavonol, kaempferol. Various 2D gHMBC NMR experiments were conducted using a range of coupling constants (*J* = 2 Hz, 4 Hz, 6 Hz, 8 Hz, 10 Hz and 12 Hz) in order to determine which experiment would result in more HMBC NMR correlations being observed. On the basis of these experiments it was concluded that the optimized coupling constant for sugars was 4 Hz, while for aromatics it was 10 Hz. The complete NMR data (as given in Table 1) unequivocally confirmed the structure of **16** to be *trans*-tiliroside, a well known flavonol glycoside first isolated from *Tilia argenta* [30]. *Trans*-tiliroside was initially reported as compound **20** before structural revisions conducted in 1964 [31], which ultimately led to the revised structure **16**. Prior to this structural revision of *trans*-tiliroside a compound named tribuloside was reported in recognition of its difference to structure **20** [32]. It became evident that tribuloside had the same structure as that of the revised structure of *trans*-tiliroside (**16**) [33]. A comparison of the NMR data acquired (Table 1 and experimental) to those reported in the literature (DMSO-d_6 and CD_3OD) confirmed the structure of **16** to be *trans*-tiliroside [33-35]. While the complete 2D NMR assignment of **16** is well documented, it is worth noting that on one occasion several of the carbon NMR assignments (namely positions C5, C6, C8 and C9) have been incorrectly assigned [36]. The NMR data as given in Table 1, and in particular the carbon chemical shift assignments, were confirmed on the basis of gHSQCAD and gHMBC NMR experiments and is consistent with all other literature NMR reports for **16** [30-35]. The sugar moiety in compound **16** was concluded to be a β-D-glucopyranoside on the basis of a comparison of the NMR chemical shifts for this moiety to the literature data [34]. The absolute configuration of the sugar moiety in compound **16** had been previously established by both acid and alkali hydrolyses [31,37]. *Trans*-tiliroside (**16**) is known to occur in Malvaceae species [25,28,29,38-41].

Table 2: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data of *cis*-tiliroside (**17**) in DMSO-d_6.

Position	Δ_H, *J*	δ_C[a]	gCOSY	gHMBC
1	-	-	-	-
2	-	156.5, C	-	-
3	-	133.1, C	-	-
4	-	NCH	-	-
5	-	161.1, C	-	-
6	6.17 d, *J* = 2 Hz	98.5, CH	H8	C5, C7, C8, C10
7	-	164.3, C	-	-
8	6.32 d, *J* = 2 Hz	93.5, CH	H6	C6, C7, C9, C10
9	-	156.4, C	-	-
10	-	103.7, C	-	-
1'	-	120.6, C	-	-
2'	7.95 d, *J* = 9 Hz	130.6, CH	H3'	C2, C4', C6'
3'	6.84 d, *J* = 9 Hz	115.0, CH	H2'	C1', C4', C5'
4'	-	159.7, C	-	-
5'	6.84 d, *J* = 9 Hz	115.0, CH	H6'	C1', C3', C4'
6'	7.95 d, *J* = 9 Hz	130.6, CH	H5'	C2, C2', C4'
1''	5.40 d, *J* = 7.5 Hz	101.5, CH	H2''	C3''
2''	3.20[b] m	73.8, CH	H1''	-
3''	3.24[b] m	75.9, CH	3''-OH	-
4''	3.15[b] m	69.7, CH	4''-OH	-
5''	3.37[b] m	74.2, CH	H6a''	-
6a''	4.07 dd, *J* = 6.0, 11.0 Hz	63.0, CH_2	H5'', H6b''	C9'''
6b''	4.15 dd, *J* = 2.0, 11.0 Hz		H6a''	-
1'''	-	125.4, C	-	-
2'''	7.55 d, *J* = 8.5 Hz	132.4, CH	H3'''	C4''', C6''', C7'''
3'''	6.69[b] d, *J* = 8.5 Hz	114.6, CH	H2'''	C1''', C4''', C5'''
4'''	-	158.8, C	-	-
5'''	6.69[b] d, *J* = 8.5 Hz	114.6, CH	H6'''	C1''', C3''', C4'''
6'''	7.55 d, *J* = 8.5 Hz	132.4, CH	H5'''	C2''',C4''', C7'''
7'''	6.67[b] d, *J* = 12.5 Hz	143.5, CH	H8'''	C2''', C6''', C9'''
8'''	5.46[b] d, *J* = 12.5 Hz	114.3, CH	H7'''	C1'''
9'''	-	165.6, C	-	-
5-OH	12.55 s	-	-	-
4'-OH	10.16 bs	-	-	-
2''-OH	5.45[b] d, *J* = 4.5 Hz	-	H2''	-
3''-OH	5.17 d, *J* = 5 Hz	-	H3''	-
4''-OH	5.22 d, *J* = 5.5 Hz	-	H4''	C5''

[a] Carbon assignments based on gHSQCAD and gHMBC NMR experiments
[b] Signals overlapped
ND indicates signals were not detected

The negative ESI-MS of **17** was identical to that of **16** in that it showed the presence of an ion at *m/z* 593 $[M-H]^-$, while the positive mode showed the presence of ions at *m/z* 595 $[M+H]^+$ and *m/z* 617 $M+Na]^+$. The ^{1}H NMR spectrum of **17** was also very similar to that of **16**. Comparison of the spectrum of **17** to **16** indicated that both compounds contained the same kaempferol and glycoside moieties (Table 2). Differences were evident in the olefinic methines of the coumaroyl moiety, with a shift from δ 7.34, d, *J* = 16 Hz (H7‴) and δ 6.12, d, *J* = 16 Hz (H8‴) in **16** to δ 6.67, d, *J* = 12.5 Hz (H7‴) and δ 5.46, d, *J* = 12.5 Hz (H8‴) in **17**. This change was consistent with a *cis* double bond geometry in the coumaroyl moiety of the structure of **17** to give *cis*-*p*-coumaroyl. In addition, the sugar methylene signals at δ 4.02, dd, *J* = 6.5, 12 Hz (H6a″) and δ 4.26, dd, *J* = 1.5, 12 Hz (H6b″) in **16** shifted to δ 4.07, dd, *J* = 6, 11 Hz (H6a″) and δ 4.15, dd, *J* = 2, 11 Hz (H6b″) in **17**, which was also consistent with a change in the double bond geometry. On this basis compound **17** was concluded to be *cis*-tiliroside, which had previously been reported occurring as a mixture with *trans*-tiliroside (**16**) in 1995 [42]. The first isolation and characterization of **17** was reported in 2004 [35]. A comparison of the NMR data for **17** with that in the literature reported in CD_3OD confirmed that this compound was *cis*-tiliroside [35].

The negative ESI-MS of **18** showed the presence of an ion at *m/z* 607 $[M-H]^-$, consistent with 18 degrees of unsaturation and a molecular formula of $C_{31}H_{28}O_{13}$. The ^{1}H NMR spectrum of **18** was very similar to that of **16**. Once again, the presence of the *p*-coumaroyl moiety could be confirmed, along with a *trans* double bond geometry [δ 7.35, d, *J* = 16 Hz (H7‴) and δ 6.17, d, *J* = 16 Hz (H8‴)]. Minor differences were evident in the ^{1}H NMR chemical shifts of the 1,4-disubstituted aromatic ring associated with the kaempferol moiety [δ 8.06, d, 2H, *J* = 8 Hz (H2′/H6′) and δ 6.99, d, 2H, *J* = 8 Hz (H3′/H5′)]. Also noted was the presence of a deshielded methyl resonance [δ 3.70, s, 3H, (4′-OCH_3)], indicating that **18** was a methoxy derivative of **16**. The methoxy moiety was positioned on the 1,4-disubstituted aromatic ring associated with the kaempferol moiety on the basis of the HMBC NMR correlations observed from the methoxy protons δ 3.70 (4′-OCH_3) and the aromatic protons δ 8.06 (H2′/H6′) to 161.3 ppm (C4′). In turn the aromatic protons δ 8.06 (H2′/H6′) showed a key correlation to 156.1 ppm (C2), thereby positioning the methoxy on the kaempferol moiety at position 4′. It was concluded that **18** was kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside, which was first reported in 2007 [24]. However the literature ^{1}H NMR chemical shifts reported for the *meta* coupled aromatic methines in the methoxy kaempferol moiety (δ 6.50, H6 and 6.68, H8) of this structure are not consistent with those typically observed for kaempferide glycosides (approximately δ 6.15 and 6.35) [43,44]. A closely related compound, 6′-*O*-(4‴-methoxy-*trans*-cinnamoyl)- kaempferol-3-β-D-glucopyranoside (**21**), was reported in 2009, for which 2D NMR correlations formed the basis of the structure elucidation argument [45], in particular, the positioning of the methoxy substitutent on the cinnamoyl correlation observed from the methoxy protons at δ 3.89 to the C4‴ position at 161.5 ppm [45]. The position of attachment of the methoxy moiety in this compound is not unequivocal since further HMBC NMR correlations from the associated aromatic ring to the remaining section of the cinnamoyl moiety were not reported [45]. On the basis of a comparison of the NMR data obtained for **18** in CD_3OD to that reported for these two compounds, it was concluded that both literature structures had been assigned incorrectly. The structure reported as 6′-*O*-(4‴-methoxy-*trans*-cinnamoyl)-kaempferol-3-β-D-glucopyranoside should be revised to kaempferol 4′-methyl ether 3-*O*-β-D-(6-O-*trans*-*p*-coumaroyl) glucopyranoside (**18**), on the basis of NMR chemical shift comparisons with the data obtained for **18**.

The compound reported in the literature as kaempferol 4′-methyl ether 3-*O*-β-D-(6-O-*trans*-*p*-coumaroyl) glucopyranoside also requires a structure re-assignment [24]. This structure revision was immediately recognized as being necessary on the basis of the downfield assignments allocated to the *meta* coupled aromatic protons (δ 6.50 and δ 6.68) of the flavonoid moiety. In comparison with literature NMR data for closely related metabolites, these ^{1}H NMR chemical shifts are not consistent with two hydroxy moieties being substituted at positions 5 and 7 (δ 6.15 and δ 6.40) [46,47]. Without an authentic sample of the compound incorrectly reported as kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside, only a *tentative* re-assignment can be proposed for this compound.

A ^{1}H NMR comparison of the *meta* coupled protons to literature NMR data for compounds **22**, **23**, **24**, **25** and **26**, indicated that a substitution at the C4' and C3 positions only has a small effect on the chemical shifts of the *meta* coupled aromatic protons, whereas substitution at the C7 position has a significant effect on these protons (see Figure 3) [44,46]. In considering the reported 2D NMR HMBC correlations for the compound reported as kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside, the anomeric proton of the sugar moiety showed a correlation to a quaternary carbon at 133.6 ppm. This correlation immediately eliminated the possibility of the glycoside being substituted at position C7. As shown in compound **27**, the quaternary carbon chemical shift would then reside at approximately 163 ppm [48]. This confirmed that the sugar moiety had to be attached to the

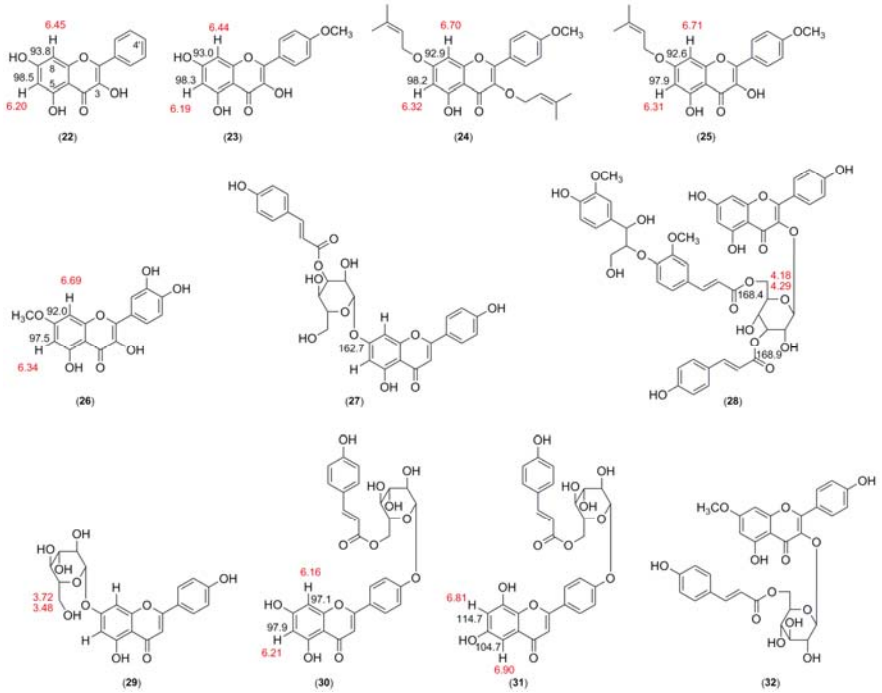

Figure 3: Comparsion of NMR data for structurally related flavonoids.

flavonoid moiety at position C3. Also on the basis of the ^{1}H NMR data reported, the methylene protons of the sugar moiety occurred downfield, as in compound **28** (δ 4.29 and δ 4.18), compared with those occurring in a terminal sugar moiety, as in the case of compound **29** (δ 3.72 and δ 3.48) [49]. This, together with the 2D NMR HMBC correlations observed from the sugar methylene protons to the ester carbon at 168.3 ppm, confirmed that the *p*-coumaroyl moiety was indeed attached to the sugar moiety as depicted in compound **28** [47]. On the basis of the NMR data provided for the incorrectly assigned structure of kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside only a few possibilities remain which explain the significant differences observed for the *meta* coupled aromatic protons. One possibility considered was that of a 5,7-dihydroxy substituted flavonoid instead of a 6,8-dihydroxy substituted flavonoid. However, both ^{1}H and ^{13}C NMR chemical shifts of the *meta* coupled aromatic protons (δ 6.50, H6; δ 6.68, H8 and 99.4 ppm, C6; 95.0 ppm C8) reported were not in accordance with the NMR data reported for 5,7-dihydroxy substituted flavonoids, such as compounds **30** and **31** [50]. In 5,7-dihydroxy flavonoids the ^{1}H and ^{13}C NMR chemical shifts occur at δ 6.21 (H6)/97.9 ppm (C6) and δ 6.16 (H8)/97.1 ppm (C8), while in 6,8-dihydroxy flavonoids these are at δ 6.90 (H5)/104.7 ppm (C5) and δ 6.81 (H7)/114.7 ppm (C7). These large changes in both the ^{1}H and ^{13}C NMR chemical shifts do not support a 5,7-dihydroxy substituted flavonoid moiety in the revised structure being proposed.

In consideration of closely related flavonoid NMR data, it was concluded that the most likely revision to the structure originally reported as kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside is that of kaempferol 7-methyl ether 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside (**32**) [51]. This structure satisfactorily explains the differences observed in both the ^{1}H and ^{13}C NMR chemical shifts for the *meta* coupled aromatic protons on the flavonoid moiety and also supports all HMBC NMR correlations reported. This proposed structure revision corresponds to a known compound for which a direct NMR comparison was hampered by the fact that different NMR solvents had been used for the analyses [51]. For an unequivocal revision to the structure, a complete 2D NMR re-assessment of an authentic sample is required.

The HR-ESI-MS of **19** displayed a *m/z* at 607.1461 [M-H]$^{-}$ (calcd. for $C_{31}H_{27}O_{13}$: *m/z* 607.1452) consistent with 18 degrees of unsaturation and a molecular formula $C_{31}H_{28}O_{13}$. The ^{1}H NMR spectrum of **19** was very similar to that of **17**, with the only noticeable difference being the presence of a methoxy resonance [δ 3.77, s, 3H, (4′-OCH_3)] in **19**. Just like *cis*-tiliroside (**17**), compound **19** also has a *cis* double bond [δ 6.67, (H7‴) and δ 5.49, d, *J* = 13.5 Hz (H8‴)] supporting the presence of a *cis*-*p*-coumaroyl moiety. The coupling constant for the proton at δ 6.67 could not be measured accurately as it was overlapped with the H3’’’/H5’’’ aromatic protons. The location of the methoxy moiety was, once again, established on the basis of key HMBC NMR correlations observed from the methoxy protons δ 3.77 (4′-OCH_3) and the aromatic methines δ 8.03 (H2′/H-6′) to the carbon at 161.2 ppm (C-4′). The additional HMBC NMR correlation from the aromatic methines δ 8.03 (H2′/H-6′) to the carbon at 156.0 (C-2) allowed the methoxy to be positioned, once again, on the kaempferol moiety. As such, compound **19** was identified to be kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*cis*-*p*-coumaroyl) glucopyranoside. To the best of our knowledge, this represents a new flavonoid glycoside derivative.

A feature that was noted for both the purified *trans*-tiliroside (**16**) and *cis*-tiliroside (**17**) was that over a period of time both converted to an equilibrium mixture of the two compounds. This mixture was reminiscent of the initial ratio of the two compounds that occurred in the crude extract (approximately 4:1 *trans*-tiliroside to *cis*-tiliroside). The conversion was noted to be much more rapid in CD_3OD than in DMSO-d_6. A study was undertaken to determine the stability of *trans*-tiliroside (**16**) at different temperatures and various solvents. The conversion was monitored using analytical HPLC and it was found that in the presence of methanol, a sample of pure *trans*-tiliroside (**16**) converted to a mixture of both isomers. The presence of *cis*-tiliroside (**17**) could be detected in this conversion after about 3 weeks. In addition a mixture of *trans*-tiliroside **16**) and

cis-tiliroside (**17**) was dissolved in ethanol and subjected to UV light (254 nm and 365 nm) for 48 hours at each wavelength. This was carried out in order to determine if ethoxy derivatives of tiliroside would be formed. In this solvent the formation of ethoxy derivatives of *trans*-tiliroside (**16**) and *cis*-tiliroside (**17**) were not observed. It was also noted that the ratio of *trans*-tiliroside (**16**) to *cis*-tiliroside (**17**) isomers had increased from approximately 4:1 to almost 1:1 when subjected to prolonged UV light. Two separate small scale (5 g) extractions conducted in methanol and ethanol respectively showed the presence of both compounds **18** and **19** in a similar ratio to that detected initially in the crude extract obtained using 3:1 methanol: dichloromethane. This supported the notion that compounds **18** and **19** are natural products and not artefacts of the isolation procedure. Due to this rapid conversion, particularly in CD_3OD, we recommend DMSO-d_6 to be the optimum solvent choice for the NMR analysis of these compounds as the conversion is substantially slower in this solvent. It is worth noting that most of the literature NMR data for this class of compounds has been reported in CD_3OD.

Flavonoids and flavonoid glycosides are a class of secondary metabolites recognized for their important biological activities [17,52,53]. *Trans*-tiliroside (**16**) has been reported to display anti-oxidative properties [54], inhibits cAMP phosphodiesterase [36], exhibits anti-compliment, anti-inflammatory and free radical scavenging activities, potent activity towards d-GalN-induced cytotoxicity in hepatocytes, displays cytotoxicity against specific leukemia cell lines, and also exhibits moderate anti-bacterial activity [29,41]. It has also been observed that *trans*-tiliroside (**16**) can modulate the activity of known anti-bacterial agents, with a reduction of minimum inhibitory concentration (MIC) of at least 2 fold when *trans*-tiliroside (**16**) was incorporated into the growth medium at 32 μg/mL [41]. Mixtures of *trans*-tiliroside (**16**) and *cis*-tiliroside (**17**) show significant toxicity towards brine shrimp, as well as displaying potent inhibition towards CYP3A4 [35]. The anti-bacterial activity displayed by a mixture of the two compounds has been suggested to be due to the *cis* isomer. In a separate study it was demonstrated that compounds containing the *cis*-coumaroyl moiety are more active than those with the corresponding *trans*-coumaroyl moiety [47,54].

The crude extract of *L. macrophyllum* displayed modest cytotoxicity. Owing to the various interconversions and equilibrium mixtures observed for the isolated flavonoid glycosides, no cytotoxicity testing was conducted on the secondary metabolites isolated.

Experimental

General experimental procedures: For detailed information on the general experimental procedures please see reference [55]. Electrospray (ESI) mass spectra were obtained as in [56], and the HRESI mass spectra were obtained as outlined in reference [56]. Analytical HPLC analyses were performed using the gradient method as described in [13] on a Phenomenex Gemini ODS (3) C_{18} 100Å 250 × 4.6 mm (5 μm) column with a flow rate of 1.0 mL/min. All semi-preparative HPLC analyses were performed on a Varian Prostar 210 (Solvent Delivery Module) equipped with a Varian Prostar 335 PDA detector (monitored at λmax 254 and 300 nm) and STAR LC WS Version 6.0 software, a ramp solvent system (0 mins 30% CH_3CN/H_2O; 20 mins 50% CH_3CN/H_2O) and a Phenomenex Prodigy ODS (3) 100Å C_{18} 250 × 10 mm (5 μm) column with a flow rate of 3.5 mL/min. For general HPLC-NMR details see reference [12]. Both on-flow and stop-flow HPLC-NMR analysis was performed using gradient HPLC conditions (0-2 mins 30% CH_3CN/D_2O, 20-24 mins 50% CH_3CN/D_2O, 26 mins 30% CH_3CN/D_2O) on a Varian Microsorb-MV C_{18} 150 × 4.6 mm (5 μm) column at 1.0 mL/min with detection at λ_{max} 254 and 315 nm. Off-line ^1H, ^{13}C and 2D NMR spectra were acquired on a 500 MHz Varian INOVA NMR spectrometer in DMSO-d_6 and CD_3OD with referencing to solvent signals (δ 2.50 and 39.5 ppm and δ 3.30 and 49.0 ppm respectively).

Biological evaluation and details of assays: A 2 g portion of the *L. macrophyllum* specimen (stems and leaves) was extracted with 3:1 methanol:dichloromethane (40 mL) and evaluated in several assays (cytotoxicity and anti-microbial) at a concentration of 50 mg/mL at the University of Canterbury, Christchurch, New Zealand. For detailed information on the biological assays please see reference [55]. This crude extract displayed modest activity against the P388 (murine leukemia) cell line with an IC_{50} of 295,570 ng/mL, along with minimal anti-bacterial activity towards *Bacillus subtilis*. No activity was detected against *Eschericha coli*, *Pseudomonas aeruginosa*, *Candida albicans, Trichophyton mentagrophytes* and *Cladosporium resinae.*

Plant material: The plant specimen (stems and leaves) was collected from Troopers Creek in the Grampians National Park, Victoria, Australia on the 23rd September 2006 and identified as *L. macrophyllum* from "*The Grampians in flower*" textbook [57]. A voucher specimen, designated the code 2006-34, is deposited at the School of Applied Sciences (Discipline of Applied Chemistry), RMIT University.

Preparation of the extract of* L. macrophyllum *for on-flow and stop-flow HPLC-NMR analysis: The specimen of *L. macrophyllum* (50 g) was roughly chopped and extracted with 3:1 methanol: dichloromethane (1 L). This extract was then decanted and concentrated under reduced pressure and was then sequentially solvent partitioned into dichloromethane followed by methanol. The extracts were then evaporated to dryness. A portion of the methanol soluble fraction (72 mg) was re-solubilized in 1 mL 50:50 CH_3CN: D_2O and filtered through a 0.45 μm PTFE membrane filter (HP045 Advantec, Japan). For both the on-flow and stop-flow HPLC-NMR experiments, 50 μL (3,600 μg) of the methanol extract was injected and monitored at λ_{max} 254 and 315 nm. The HPLC-NMR analyses were performed using the conditions described in the 'General experimental procedures'.

Preparation of the extract for off-line isolation of secondary metabolites from* L. macrophyllum*:
A further extraction of *L. macrophyllum* (100 g) was carried out with 3:1 methanol: dichloromethane (2 L). This extract was decanted and concentrated under reduced pressure before sequential solvent partitioning into dichloromethane (0.9 g) and methanol (6 g) soluble extracts. These fractions were evaporated to dryness and stored at 4°C. C18 Vacuum Liquid Column (VLC) chromatography of the methanol extract was undertaken using a 25% stepwise elution from water to methanol and then to EtOAc and finally to DCM to afford 13 fractions. Analytical HPLC analysis of the fractions confirmed the presence of 3 dominant secondary metabolites (**16-18**) that could also be detected in the HPLC-NMR analyses, along with a fourth minor compound (**19**), which could only be observed in the off-line analytical HPLC chromatogram used to develop a method for HPLC-NMR analysis. A portion (800 mg) of the methanol extract was filtered through a 0.45 μm PTFE membrane filter (HP045 Advantec, Japan) and subjected to semi-preparative reversed phased HPLC as described in the 'General Experimental Procedures' to yield *trans*-tiliroside (**16**) (26 mg, 0.05%), *cis*-tiliroside (**17**) (6 mg, 0.01%), 4′-methoxy-*trans*-

tiliroside (**18**) (2 mg, 0.004%) and 4′-methoxy-*cis*-tiliroside (**19**) (1 mg, 0.002%).

Concluding remarks

As a result of this study it was demonstrated that an approach using both on-line and off-line chemical profiling techniques are complimentary for principle component analysis, as well as for the identification of minor components. On-line HPLC-NMR was utilized to chemically profile the crude methanol extract of the Australian plant *Lasiopetalum macrophyllum*, resulting in the partial identification of *trans*-tiliroside (**16**), *cis*-tiliroside (**17**) and 4′-methoxy-*trans*-tiliroside (**18**). Subsequent off-line purification permitted the complete structural elucidation of the four flavonoid glycosides (**16-19**). Compound **19** was established to be a new flavonoid glycoside structural derivative. This represents the first report of the isolation of flavonoid glycosides from the genus *Lasiopetalum*. The off-line purification of these compounds was particularly important since the NMR assignment of **18**, as reported in the literature, was found to be inconclusive in terms of the position of attachment for the methoxy moiety. Upon closer examination of the literature NMR data and that obtained for **18** it could be concluded that the structures of the two literature compounds reported as kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside and 6'-*O*-(4"-methoxy-*trans*-cinnamoyl)-kaempferol glucopyranoside should be revised. A series of stability studies undertaken concluded that compounds **18** and **19** are not artefacts of the extraction procedure with methanol. The use of HPLC-NMR was found to be particularly suited to the analysis of unstable compounds that convert to equilibrium mixtures, such as those investigated in this study.

HPLC-NMR characterization of compounds (16-18): HPLC-NMR assignment of **16** from stop-flow HPLC-NMR (500 MHz, gradient used as detailed in **Section 3.1**, δ, ppm): 7.88 (2H, d, *J* = 9 Hz, H2′/H6′), 7.24 (1H, d, *J* = 16 Hz, H7‴), 7.21 (2H, d, *J* = 8.5 Hz, H2‴/H6‴), 6.83 (2H, d, *J* = 9 Hz, H3′/H5′), 6.76 (2H, d, *J* = 8.5 Hz, H3‴/H5‴), 6.20 (1H, d, *J* = 1.5 Hz, H8), 6.07 (1H, d, *J* = 1.5 Hz, H6), 5.92 (1H, d, *J* = 16 Hz, H8‴), 5.03 (1H, d, *J* = 7.5 Hz, H1″), 4.11 (2H, d, *J* = 5.5 Hz, H6″), 3.38 (2H, m, H3″/H5″), 3.23 (1H, m, H2″), 3.21 (1H, m, H4″).

HPLC-NMR assignment of **17** from stop-flow HPLC-NMR present as a mixture with **16** in a ratio of 1:4 [compound **17**:compound **16**]: (δ, ppm): 7.83 (d, *J* = 9 Hz, H2′/H6′), 6.62 (d, *J* = 14.5 Hz, H7‴), 6.60 (d, *J* = 9 Hz, H3′/H5′), 6.45 (s, H8), 6.18 (s, H6), 5.41 (d, *J* = 14.5 Hz, H8‴), 4.96 (d, *J* = 7.5 Hz, H1″), 4.06 (d, *J* = 5 Hz, H6″), 3.43-3.20 (m, H2″/H3″/H4″/H5″), remaining signals overlapped with **16** or suppressed.

HPLC-NMR assignment of **18** from stop-flow HPLC-NMR: HPLC-NMR (δ, ppm): 8.02 (d, *J* = 8.5 Hz, H2′/H6′), 7.33 (d, *J* = 14.5 Hz, H7‴), 7.31 (d, *J* = 7.5 Hz, H2‴/H6‴), 6.97 (d, *J* = 8.5 Hz, H3′/H5′), 6.83 (d, *J* = 7.5 Hz, H3‴/H5‴), 6.33 (s, H8), 6.18 (s, H6), 6.01 (d, *J* = 14.5 Hz, H8‴), 5.13 (d, *J* = 7.5 Hz, H7‴), 3.72 (s, 4′-OCH_3), 3.44 (m), 3.25 (m), all other signals suppressed.

Off-line characterisation of compounds (16-19):

***Trans*-tiliroside** (**16**), also known as kaempferol 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside, was isolated as yellow fibrous crystals.

MP: 260-265°C.

$[\alpha]^{21}_{D}$: -33 (*c* 0.164, CH_3OH).

IR (film) ν_{max}: 3367, 1655, 1605, 1509, 1443, 1359, 1260, 1207, 1179 cm^{-1}.

UV (EtOH) λ_{max}: 205, 229 sh, 269, 300 sh, 317, 350 sh nm (ε = 3924, 2488, 2049, 2400, 2665, 1434 respectively).

^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) are detailed in Table 1.

^{1}H NMR (500 MHz, CD_3OD, δ, ppm) present as a mixture with **17**: 7.99 (2H, d, *J* = 9 Hz, H2′/H6′), 7.40 (1H, d, *J* = 16 Hz, H7‴), 7.32 (2H, d, *J* = 9 Hz, H2‴/H6‴), 6.82 (2H, d, *J* = 9 Hz, H3′/H5′), 6.80 (2H, d, *J* = 9 Hz, H3‴/H5‴), 6.32 (1H, s, H8), 6.13 (1H, s, H6), 6.07 (1H, d, *J* = 16 Hz, H8‴), 5.24 (1H, d, *J* = 7 Hz, H1″), 4.29 (1H, d, *J* = 11.5 Hz, H6b″), 4.19 (1H, m, H-6a″), 3.44* (1H, m, H2″), 3.43* (1H, m, H3″), 3.39* (1H, m, H5″), 3.31* (1H, m, H4″) * indicates overlapping signals.

^{13}C NMR (obtained from gHSQCAD and gHMBC NMR experiments, CD_3OD, ppm): 161.4 (C, C4′), 161.0 (C, C4‴), 159.1 (C, C2), 146.5 (CH, C7‴), 131.9 (CH, C2′/C6′), 130.9 (CH, C2‴/C6‴), 126.9 (C, C1‴), 116.4 (CH, C3‴/C5‴), 115.8 (CH, C3′/C5′), 114.4 (CH, C8‴), 103.7 (CH, C1″), 99.9 (CH, C6), 94.6 (CH, C8), 77.6 (CH, C3″), 75.5 (CH, C2″/C5″), 71.4 (CH, C4″), 63.9 (CH_2, C6″), all other carbons were not detected.

ESIMS (negative): *m/z* 593 $[M-H]^-$, (positive): *m/z* 595 $[M+H]^+$, 617 $[M+Na]^+$.

HR-ESI-MS displayed a *m/z* at 593.1308 $[M-H]^-$ (calcd. for $C_{30}H_{25}O_{13}$: m/z 593.1295) and a *m/z* at 595.1440 $[M+H]^+$ (calcd. for $C_{30}H_{27}O_{13}$: *m/z* 595.1373).

***Cis*-tiliroside** (**17**) also known as kaempferol 3-*O*-β-D-(6-*O*-*cis*-*p*-coumaroyl) glucopyranoside was isolated as a yellow powder.

^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) are detailed in Table 2.

^{1}H NMR (500 MHz, CD_3OD, δ, ppm) present as a mixture with **16**: 7.96 (2H, d, *J* = 9 Hz, H2′/H6′), 7.51 (2H, d, *J* = 8.5 Hz, H2‴/H6‴), 6.82 (2H, d, *J* = 9 Hz, H3′/H5′), 6.69 (1H, d, *J* = 12.5 Hz, H7‴), 6.67 (2H, d, *J* = 8.5 Hz, H3‴/H5‴), 6.32 (1H, s, H8), 6.19 (1H, s, H6), 5.50 (1H, d, *J* = 12.5 Hz, H8‴), 5.20 (1H, d, *J* = 7.5 Hz, H1″), 4.29 (1H, d, *J* = 11.5 Hz, H6b″), 4.19 (1H, m, H6a″), 3.44* (1H, m, H2″), 3.43* (1H, m, H3″), 3.39* (1H, m, H5″), 3.31* (1H, m, H4″) * indicates overlapping signals

^{13}C NMR (obtained from gHSQCAD NMR experiment present as a mixture with **16** in a ratio of 1:2 [compound **17**: compound **16**], CD_3OD, ppm): 145.0 (CH, C7‴), 133.5 (CH, C2‴/C6‴), 131.9 (CH, C2′/C6′), 115.8 (CH, C3′/C5′), 115.8 (CH, C8‴), 115.4 (CH, C3‴/C5‴), 103.6 (CH, C1″), 99.7 (CH, C6), 94.5 (CH, C8), 77.6 (CH, C3″), 75.4 (CH, C2″/C5″), 71.4 (CH, C4″), 63.9 (CH_2, C6″), all other carbons were not detected.

ESIMS (negative): *m/z* 593 $[M-H]^-$, (positive): *m/z* 595 $[M+H]^+$, 617 $[M+Na]^+$.

4′-Methoxy-*trans*-tiliroside (**18**) also known as kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*trans*-*p*-coumaroyl) glucopyranoside was isolated as a yellow powder.

^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 12.51 (brs, 5-OH), 8.06 (2H, d, *J* = 8 Hz, H2′/H6′), 7.40 (2H, d, *J* = 8 Hz, H2‴/H6‴), 7.35 (1H, d, *J* = 16 Hz, H7‴), 6.99 (2H, d, *J* = 8 Hz, H3′/H5′), 6.78 (2H, d, *J* = 8 Hz, H3‴/H5‴), 6.38 (1H, s, H8), 6.17 (1H, d, *J* = 16 Hz, H8‴), 6.15 (1H, s, H6), 5.46 (1H, m, 2″OH), 5.45 (1H, d, *J* = 8 Hz, H1″), 5.26 (m, 4″OH), 5.22 (m, 3″OH), 4.28 (1H, d, *J* = 11.5 Hz, H6b″), 4.02 (1H, dd, *J* = 5.0, 11 Hz, H6a″), 3.70 (3H, s, 4′-OCH_3), 3.37* (1H, m, H5″), 3.28* (1H, m, H3″), 3.25* (1H, m, H4″), 3.23* (1H, m, H2″) * indicates overlapping signals.

^{13}C NMR (125 MHz, DMSO-d_6, ppm): 166.2 (C, C9‴), 164.7 (C, C7), 161.3 (C, C4′), 161.1 (C, C5), 159.8 (C, C4‴), 156.4 (C, C9), 156.1 (C, C2), 144.4 (CH, C7‴), 133.4 (C, C3), 130.4 (CH, C2′/C6′), 130.0 (CH, C2‴/C6‴), 125.0 (C, C1‴), 122.4 (C, C1′), 115.5 (CH, C3‴/C5‴), 113.4 (CH, C8‴), 113.3 (CH, C3′/C5′), 103.9 (C, C10), 101.8 (CH, C1″), 98.7 (CH, C6), 93.6 (CH, C8),

75.7 (CH, C3″), 73.8 (CH, C2″/C5″), 69.5 (CH, C4″), 62.4 (CH_2, C6″), 54.9 (CH_3, 4′-OCH_3), not detected (C4).
^{1}H NMR (500 MHz, CD_3OD, δ, ppm) present as a mixture with **19**: 8.08 (2H, d, *J* = 9 Hz, H2′/H6′), 7.42 (1H, d, *J* = 16 Hz, H7‴), 7.35 (2H, d, *J* = 9 Hz, H2‴/H6‴), 6.93 (2H, d, *J* = 9 Hz, H3′/H5′), 6.82 (2H, d, *J* = 9 Hz, H3‴/H5‴), 6.35 (1H, d, *J* = 1.5 Hz, H8), 6.16 (1H, d, *J* = 1.5 Hz, H6), 6.10 (1H, d, *J* = 16 Hz, H8‴), 5.26 (1H, d, *J* = 6.5 Hz, H1″), 4.30 (1H, d, *J* = 11.5 Hz, H6b″), 4.19 (1H, m, H6a″), 3.72 (3H, s, 4'-OCH_3), 3.46* (1H, m, H2″), 3.44* (1H, m, H3″), 3.41* (1H, m, H5″), 3.38* (1H, m, H4″) * indicates overlapping signals.
^{13}C NMR (obtained from gHSQCAD and gHMBC NMR experiments, CD_3OD, ppm): 166.3 (C, C9‴), 165.6 (C, C7), 162.6 (C, C4′), 160.6 (C, C4‴), 158.5 (C, C2) , 157.9 (C, C9), 146.1 (CH, C7‴), 131.7 (CH, C2′/C6′), 130.9 (CH, C2‴/C6‴), 126.4 (C, C1‴), 123.4 (C, C1′), 116.5 (CH, C3‴/C5‴), 114.5 (CH, C8‴), 114.3 (CH, C3′/C5′), 103.8 (CH, C1″), 99.7 (CH, C6), 94.6 (CH, C8), 77.5 (CH, C3″), 75.4 (CH, C2″/C5″), 71.2 (CH, C4″), 63.7 (CH_2, C6″), 55.5 (CH_3, 4′-OCH_3), C3, C4, C5 and C10 not detected.
ESIMS (negative): *m/z* 607 $[M-H]^-$.

4′-Methoxy-*cis*-tiliroside (**19**), kaempferol 4′-methyl ether 3-*O*-β-D-(6-*O*-*cis*-*p*-coumaroyl) glucopyranoside was isolated as a yellow powder.
IR (film) ν_{max}: 3342, 2919, 2851, 1651, 1605, 1510, 1456,1371, 1358, 1302, 1259, 1181 cm^{-1}.
UV (MeOH) λ_{max} 204, 224 sh, 268, 299 sh, 313, 354 sh nm (ε = 4522, 2899, 2254, 2238, 2389, 1357 respectively).
^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 12.48 (brs, 5OH), 8.03 (2H, d, *J* = 9 Hz, H2′/H6′), 7.55 (2H, d, *J* = 8 Hz, H2‴/H6‴), 6.99 (2H, d, *J* = 8.5 Hz, H3′/H5′), 6.67* (1H, H7‴), 6.67* (2H, d, *J* = 9 Hz, H3‴/H5‴), 6.30 (1H, s, H8), 6.14 (1H, s, H6), 5.49 (1H, d, *J* = 13.5, H8‴), 5.48 (1H, m, 2″OH), 5.38 (1H, d, *J* = 7.5 Hz, H1″), 5.26 (m, 4″OH), 5.20 (m, 3″OH), 4.15 (1H, m, H6b″), 4.06 (1H, dd, *J* = 5.5, 11.5 Hz H6a″), 3.77 (3H, s, 4′-OCH_3), 3.36* (1H, m, H5″), 3.24* (1H, m, H3″), 3.23* (1H, m, H2″), 3.16* (1H, m, H4″) * indicates overlapping signals.
^{13}C NMR (obtained from gHSQCAD and gHMBC NMR experiments, DMSO-d_6, ppm): 165.5 (C, C9‴), 161.2 (C, C4′), 158.8 (C, C4‴), 156.7 (C, C9), 156.0 (C, C2), 143.5 (CH, C7‴), 133.3 (C, C3), 132.5 (CH, C2‴/C6‴), 130.4 (CH, C2′/C6′), 125.3 (C, C1‴), 122.4 (C, C1′), 114.5 (CH, C3‴/C5‴), 114.3 (CH, C8‴), 113.3 (CH, C3′/C5′), 103.5 (C, C10), 101.2 (CH, C1″), 99.0 (CH, C6), 93.8 (CH, C8), 75.9 (CH, C3″), 73.9 (CH, C2″), 73.7 (CH, C5″), 69.5 (CH, C4″), 62.4 (CH_2, C6″), 55.2 (CH_3, 4′-OCH_3), not detected (C4, C5, C7).
^{1}H NMR (500 MHz, CD_3OD, δ, ppm) present as a mixture with **18** in a ratio of 1:2 [compound **19**: compound **18**]: 8.04 (2H, d, *J* = 9 Hz, H2′/H6′), 7.55 (2H, d, *J* = 8.5 Hz, H2‴/H6‴), 6.93 (2H, d, *J* = 9 Hz, H3′/H5′), 6.72 (1H, d, *J* = 13 Hz, H7‴), 6.69 (2H, d, *J* = 8.5 Hz, H3‴/H5‴), 6.35 (1H, d, *J* = 2 Hz, H8), 6.21 (1H, d, *J* = 2 Hz, H6), 5.53 (1H, d, *J* = 13 Hz, H8‴), 5.17 (1H, d, *J* = 7 Hz, H1″), 4.17 (2H, m, H6″), 3.77 (3H, s, 4'-OCH_3), 3.30-3.47* (4H, m, H2″/H3″/H4″/H5″) * indicates overlapping signals.
^{13}C NMR (obtained from gHSQCAD NMR experiment, present as a mixture with **18** in a ratio of 1:2 [compound **19**: compound **18**], CD_3OD, ppm): 145.1 (CH, C7‴), 133.6 (CH, C2‴/C6‴), 131.7 (CH, C2′/C6′), 115.6 (CH, C8‴), 115.4 (CH, C3‴/C5‴), 114.3 (CH, C3′/C5), 104.0 (CH, C1″), 99.7 (CH, C6), 94.6 (CH, C8), 63.7 (CH_2, C6″), 54.3 (CH_3, 4′-OCH_3), all other carbons were not detected.
HR-ESI-MS (negative): *m/z* at 607.1461 $[M-H]^-$ (calcd. for $C_{31}H_{27}O_{13}$: *m/z* 607.1452).
ESIMS (negative): *m/z* 607 $[M-H]^-$, (positive): *m/z* 609 $[M+H]^+$, 630 $[M+Na]^+$.

Acknowledgments - The Marine and Terrestrial Natural Product (MATNAP) research group thanks Dr R. Tinker for his assistance with the collection of the plant specimen; Ms G. Ellis (University of Canterbury, Christchurch, New Zealand) for the biological testing; Ms Sally Duck (Monash University) for the high resolution mass spectrometric analyses; Dr Julie Niere for NMR discussions and the award of an Australian Postgraduate Research Award (APA) scholarship.

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