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Chemical constituents of the lichen, *Candelaria concolor*: A complete NMR and chemical degradative investigation

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A detailed chemical and spectroscopic investigation of the terrestrial lichen *Candelaria concolor* has yielded several lichenic metabolites belonging to the pulvinic acid series, as well as several depside derivatives including pulvinic dilactone (**1**), vulpinic acid (**4**) and calycin (**5**). The chemical transformation of **1** to pulvinic acid (**3**) is reported for the first time, as is the conversion of atranorin (**6**) to 5-chloroatranorin (**7**) and then finally to 5,5′-dichloroatranorin (**8**) under very mild conditions. Also presented is the complete 1D and 2D NMR assignment for compounds **1**, **3**, **4**, **5** and **8**, including partial NMR chemical shift assignments for the unstable depside (**7**). Previously, these metabolites had only been partially assigned by NMR spectroscopy.

Keywords: lichen; natural product; pulvinic acid derivatives; depside; biological activity

1. Introduction

The remarkable symbiotic relationship of lichens is one of the most successful examples of two or more organisms (a fungus – mycobiont and an algal partner – photobiont) living in unison. Lichens occur in the most extreme environments on Earth, such as the cold arctic tundra, rain forests, hot deserts, rocky coasts, as well as toxic spoil heaps, and have evolved to battle extreme humidity, temperature and light (Purvis, 2000). With greater than 18,500 species known worldwide, lichens produce unique classes of secondary metabolites that are rarely encountered in other organisms and, to date, there have been in excess of 800 new chemical entities reported from lichens (Boustie & Grube, 2005; Huneck & Yoshimura, 1996; Rundel, 1978). Structural classes range from the more common phenolic compounds to the usnic acids, depsides, depsidones, depsones, lactones and quinones, which are generally derived by the acetate-malonate pathway, and the pulvinic acid derivatives, derived from the shikimic acid pathway (Boustie & Grube, 2005; Rundel, 1978). There have been numerous reports of the pharmacological properties of lichen substances including antibiotic, antiviral, anti-inflammatory and

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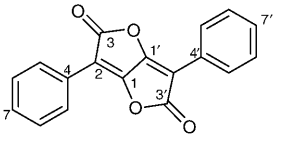
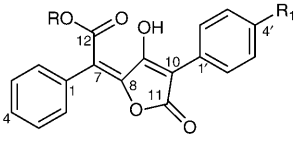
cytotoxic effects (Muller, 2001). One well-known pulvinic acid derivative is vulpinic acid (**4**), which was discovered as early as 1939, and reported to display acute dyspnoea as well as causing an increase in the rate of respiration and salivation in cats (Santesson, 1939). Progressive developments in separation and purification strategies, as well as advancements in the sensitivity of instrumentation, have resulted in the ability to rapidly isolate and identify lichen constituents (Culberson & Culberson, 2001). As early as the 1900s pioneers in lichen natural products chemistry used microcrystal tests, ultraviolet spectroscopy (UV) and paper chromatography to identify lichen metabolites. The 1960s made more use of techniques such as infrared spectroscopy (IR), circular dichroism (CD), and sensitive techniques such as mass spectrometry (MS), for compound identification. It was the late 1980s which saw improvements in chromatographic techniques including high pressure liquid chromatography (HPLC), gas chromatography (GC), together with advancements in spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy, single-crystal X-ray diffraction analysis, as well as improved sensitivity in mass spectrometry techniques, coupled with synthetic techniques to confirm absolute structures (Culberson & Culberson, 2001). These improvements have ultimately enabled far more rapid isolation and structure elucidation. One of the more powerful spectroscopic techniques in the identification of organic compounds is NMR spectroscopy and presently advancements in probe technology, such as cold flow-probes, higher field strength magnets as well as the development of superior 2D NMR experiments have provided new opportunities to fully characterise lichen constituents. This is particularly important for lichen metabolites, which had previously only been partially assigned, since much less compound is now required for complete structural assignment.

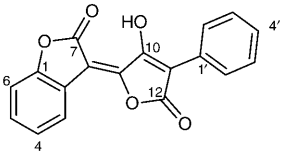
As part of the continuing activities of The Marine And Terrestrial NAtural Product (MATNAP) research group at RMIT University (Melbourne, Victoria, Australia), which includes the study of Australian lichens, we focused our efforts on the chemical profiling of a yellow lichen. Recently we conducted the chemical investigation of a distinctly bright yellow lichen for which we were only able to identify the alga component of the lichen as belonging to a *Treboxia* sp. (Dias, White, & Urban, 2007). We now wish to report the full identification of this lichen as *Chrysothrix xanthina* (Vain.) Kalb. This lichen had afforded pinastric acid (**2**), which presented a significant structural challenge as NMR experiments alone could not unequivocally assign the central lactone ring fragment (Dias et al., 2007). Fortuitously, a single X-ray analysis of **2** could be obtained to further confirm the structure of **2**. As a result of this study we were prompted to investigate another yellow lichen, identified as *Candelaria concolor* (Dickson) B. Stein, collected from Merlynston, Victoria, Australia. To date, reports on the lichen *C. concolor* have been restricted to biomonitoring studies (Loppi & Frati, 2006; Pišút & Pišút, 2006; Purvis, 2000; Ruisi et al., 2005). The crude extract of the lichen *C. concolor* demonstrated significant antibacterial activity, which prompted the isolation of several lichenic compounds, including pulvinic dilactone (**1**), pulvinic acid (**3**) (prepared from **1**), vulpinic acid (**4**), calycin (**5**), atranorin (**6**), and 5-chloroatranorin (**7**), as well as the first reported isolation of 5,5′-dichloroatranorin (**8**). In addition, we report the first complete structural characterisation involving the use of 2D NMR spectroscopy for several previously isolated compounds, including **1** and **3**–**8**. Furthermore, the importance of the selection of the extraction solvent, as well as the chemical degradation of **6**, is also reported.



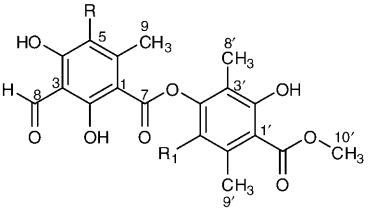
Pulvinic dilactone (**1**)



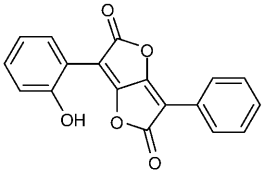
	R	R_1
Pinastric acid (**2**)	CH_3	OCH_3
Pulvinic acid (**3**)	H	H
Vulpinic acid (**4**)	CH_3	H



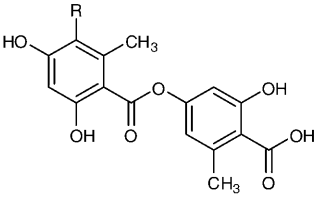
Calycin (**5**) (Correct structure)



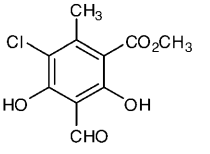
	R	R_1
Atranorin (**6**)	H	H
5-chloroatranorin (**7**)	Cl	H
5,5′-dichloroatranorin (**8**)	Cl	Cl



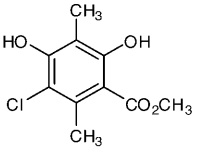
2-hydroxypulvic dilactone (**9**) (Incorrect structure)



Lecanoric acid (**10**) R = H
5-chlorolecanoric acid (**11**) R = Cl



5-chlorohaematommate (**12**)



Methyl-5-chloro-β-orcinol carboxylate (**13**)

2. Results and discussion

2.1. *Isolation of vulpinic acid (4) and calycin (5)*

The yellow lichen was extracted with 3 : 1 MeOH/CH_2Cl_2 and then separated by flash silica chromatography to yield vulpinic acid (**4**) (also known as methylpulvinic acid). Vulpinic acid (**4**) has been known since the 1830s, when early Eskimos used lichens containing the acid to poison wolves, and in Scandinavia the lichen containing this compound was used to kill both wolves and foxes (Knight & Pattenden, 1979; Purvis, 2000). The structure of **4** has been previously confirmed by partial synthesis (Frank, Clark, & Coker, 1950) with the total synthesis achieved in the 1950s (Frank et al., 1950; Moore, Shelden, Deters, & Wikholm, 1969; Pattenden, Pegg, & Kenyon, 1991; Ramage & Griffiths, 1984). There have been several reports of the single crystal X-ray structure of **4**, previously isolated from the lichen *Letharia vulpina* (L.) Hue, with the first crystal structure being reported in 1985 (Brassy, Bachet, Molho, & Molho, 1985). Vulpinic acid (**4**) and its crystal structure has also been reported from the New Zealand liverwort, *T. tenellus*, for which **4** was the major constituent (Toyota, Omatsu, Braggins, & Asakawa, 2004). In 2003 the isolation of **4** and its polymorph from *P. ravenelii* were

described (Duncan et al., 2003). There have been a number of attempts to unambiguously assign the NMR data for **4**, but owing to the number of quaternary carbons present in this molecule, the unequivocal assignment of these resonances has been difficult, as they are often not even observed. To date there have not been any reports of the complete 1D and 2D NMR assignment of a majority of the compounds isolated in this study including **4** (Heurtaux, Lion, Gall, & Mioskowski, 2005; König & Wright, 1999; Patteneden, Pegg, & Kenyon, 1991). This prompted a complete unequivocal NMR assignment of all proton and carbon chemical shifts on the basis of 2D NMR connectivity experiments for all compounds isolated from *C. concolor*.

The complete structure of **4** was determined by detailed spectroscopic analyses involving both 1D and 2D NMR experiments and mass spectrometry. The ESI mass spectrum displayed a m/z 321 $[M-H]^-$, consistent with a molecular formula of $C_{19}H_{14}O_5$ and 13 DBE. The ^{13}C NMR spectrum of **4** showed 15 discrete signals and the DEPT and HSQCAD NMR experiments supported the presence of one methyl, six methines and eight quaternary carbons. The IR spectrum of **4** displayed absorptions at 3426 and 1774 cm^{-1}, indicative of the hydroxy and ester carbonyl moieties. The molecular formula, together with the observation of only 15 distinct carbon signals in the ^{13}C NMR spectrum, immediately suggested an element of symmetry in **4** with four of the methines being equivalent. Analysis of the ^1H NMR and the gCOSY NMR spectra of **4** suggested the presence of two isolated mono-substituted aromatic rings (the first at δ 7.27 dd, $J = 2.0$, 8.0 Hz, (H2/H6); 7.34 dd, $J = 7.0$, 7.5 Hz, (H3/H5) and 7.44, m, (H4) and the second at δ 8.13 dd, $J = 1.5$, 8.5 Hz, (H2′/H6′); 7.41 m, (H3′/H5′) and 7.42 m, (H4′)). The presence of a methyl ester (δ 3.89 s, 54.4 ppm) was supported by ^1H, ^{13}C and HSQCAD NMR experiments and a HMBC correlation (171.7 (C12) ppm) confirmed it to be attached to a carbonyl moiety. Important HMBC correlations were observed from aryl protons on both rings, which ultimately allowed for the placement of the two mono-substituted aromatic rings. In particular, the second mono-substituted aromatic ring showed an important correlation to 105.1 (C10) ppm, thereby confirming the position of this ring, relative to the central lactone ring moiety. Owing to the number of quaternary carbons in the central hydroxyfuranone core and the lack of any HMBC correlations to these carbons, due to their remoteness from the aromatic protons, the remaining quaternary carbons at δ 165.9 (C11), 160.2 (C9) and 154.8 (C8) were assigned on the basis of a direct comparison to the carbon shifts previously assigned for pinastric acid (**2**) (Dias et al., 2007).

Analysis of another fraction resulting from the same flash silica column fractionation step yielded a mixture of vulpinic acid (**4**) and calycin (**5**). This mixture was further purified by additional silica chromatography and resulted in the isolation of **5** as an orange oil. Calycin (**5**) had been previously isolated in 1890 as a deep red compound from the lichen *L. candelaris* Shaer (Åkermark, 1961). Compounds **4** and **5** were also isolated from the lichen *L. clorina* Stein (Grover & S.T.R., 1959). The structure of **5** had initially been reported as 2-hydroxypulvic dilactone (**9**). It was not until the early 1960s that the synthesis of **5** was proposed, for which the correct structure **5** was supported by infrared and ultraviolet spectroscopy (Åkermark, 1961; Kühler, Nilsson, Sandberg, & Wachtmeister, 1984).

The structure of calycin (**5**) was again confirmed using 1D and 2D NMR as well as by mass spectrometry. The ESI mass spectrum displayed a m/z 305 $[M-H]^-$, which suggested a molecular formula of $C_{18}H_{10}O_5$ and 13 DBE. The ^{13}C NMR spectrum of **5** contained 15 signals (nine methines (two of these being equivalent) and eight quaternary carbons (one not being detected)), as supported by a gHSQCAD NMR experiment. The IR spectrum of

5 showed absorptions at 3401 and 1804 cm^{-1}, once again being suggestive of hydroxy and ester carbonyl moieties. The ^1H NMR and gCOSY NMR spectra again identified the presence of two isolated aromatic ring systems. These included a mono-substituted aromatic ring (δ 8.19 d, $J = 7.5$ Hz, (H2′/H6′); 7.47 m, (H3′/H5′); 7.39 dd, $J = 7.5$, 7.5 Hz, (H4′)) and a di-substituted aromatic ring (δ 7.98 d, $J = 7.5$ Hz, (H3); 7.45, m, (H5); 7.32 dd, $J = 7.5$, 8.0 Hz, (H4) and 7.23 d, $J = 8.0$ Hz, (H6)). A key HMBC correlation from δ 7.98 (H3) to the carbon at 107.0 (C8) ppm unequivocally positioned the benzofuranone moiety adjacent to the hydroxyfuranone core, whilst another HMBC correlation positioned the other mono-substituted aromatic ring (δ 8.19 (H2′/H6′) to the carbon at 105.9 (C11) ppm). Positions C10 and C12 were assigned on the basis of comparisons to carbon shifts previously assigned to **2** (Dias et al., 2007).

2.2. *Identification of atranorin (6), 5-chloroatranorin (7) and the isolation of 5,5′-dichloroatranorin (8)*

In an effort to separate **4** and **5** by HPLC, the 3:1 $MeOH/CH_2Cl_2$ crude extract was further sequentially partitioned using CH_2Cl_2 followed by MeOH. The MeOH partition was then subjected to reversed-phase HPLC, which resulted in the isolation of atranorin (**6**). Surprisingly, **4** and **5** were not detected in the MeOH partition. However, the presence of **4** and **5** were detected in the CH_2Cl_2 partition, as confirmed by analytical HPLC. Atranorin (**6**) was first isolated in 1898 and reported to be always accompanied by **4** in the lichen *L. vulpina* (Hesse, 1898; Solberg & Remedios, 1978). In addition, this β-orcinol depside (**6**) has been described as being a significant taxonomic marker for various species of lichens that possess ecological and biological properties (Elix, Whitton, & Jones, 1982; Elix, 1993; Rundel, 1978). It was not until 1999 that the re-isolation together with the complete 1D and 2D NMR for **6** was described (Huneck & Yoshimura, 1996; König & Wright, 1999).

In the present isolation of atranorin (**6**), the structure was confirmed on the basis of 1D and 2D NMR experiments and mass spectrometry. The ESI mass spectrum showed a m/z 373 $[M-H]^-$, consistent with a molecular formula of $C_{19}H_{18}O_8$ and 11 DBE. The ^1H NMR, gHSQCAD and gHMBC NMR spectra supported the assignments of **6** as reported in the literature (König & Wright, 1999). During acquisition of the NMR data for **6** it was observed that changes occurred in the ^1H NMR spectrum (in particular, the disappearance of the ^1H NMR signal at δ 6.41 s, (H5)) after approximately 16 h. Clearly a chemical conversion was occurring and this could be monitored by the acquisition of 1D and 2D NMR experiments as well as ESIMS. The NMR data acquired, together with the supporting ESIMS data, identified the presence of a m/z 407 $[M-H]^-$ (molecular formula of $C_{19}H_{17}ClO_8$ and 11 DBE) and confirmed the presence of the depside 5-chloroatranorin (**7**). Due to the rapid conversion of **6** to **7**, only a gHSQCAD NMR experiment could be acquired for **7** within the time it took for it to further convert to **8**. This permitted the observation of four methyls (δ 2.86 s (H9); 2.09 s (H8′); 2.54 s (H9′); and 3.99 s (H10′)) and one deshielded methine (δ 6.52 s (H5′)). The methine proton at (C5) was absent as it had now been replaced by a chlorine substituent, as confirmed by the isotopic ratio observed in the ESIMS. The identification of 5-chloroatranorin (**6**) dates back to as early as the 1930s (Pfau, 1933). It was postulated, that in the biogenesis of chlorodepsidones, a depside may undergo halogenation and that the halogenated derivative can then undergo ring closure by the elimination of hydrogen halides (Neelakantan, Padmasani, & Seshadri, 1964).

It was not until 1964 that the halogenation of **6** was reported, together with the chlorination of lecanoric acid (**10**), to 5-chlorolecanoric acid (**11**) (Neelakantan et al., 1964; Neelakantan, Seshadri, & Subramanian, 1962).

During the NMR data acquisition of **7** (approximately 14 h post-acquisition), it was observed that the ^{1}H NMR spectrum of **7** showed the disappearance of the signal at (δ 6.52 s, (H5′)) and that all other observed signals, which had been evident for **7** were now slightly shifted further downfield. Analysis of the ^{1}H NMR, gHSQCAD and gHMBC NMR data identified the presence of 17 carbons (one methine, four methyls and twelve quaternary carbons (two not being detected)). The IR spectrum of **8** once again showed absorptions at 3401 and 1735 cm^{-1}, suggestive of hydroxy and ester carbonyl moieties. The ESIMS displayed a m/z 441 $[M-H]^{-}$, consistent with a molecular formula of $C_{19}H_{16}Cl_2O_8$ and 11 DBE. Key HMBC correlations included one from the methyl group at δ 2.65 (H9′) to the carbon bearing the chlorine substituent at 118.4 (C5′) ppm. This enabled the position of the chlorine substituent to be deduced as being adjacent to the bridging ester at (C4′). The methyl group at δ 2.13 (H8′) also showed key HMBC correlations to 148.8 (C4′), 159.6 (C2′) and 119.1 (C3′) ppm. Furthermore, the methyl group δ 2.92 (H9) exhibited a three-bond HMBC correlation to the carbon containing the second chlorine at 115.6 (C5) ppm. The hydroxy group at δ 12.22 (2-OH) displayed a two-bond HMBC correlation to 166.7 (C2) ppm and allowed the carbon (109.1 (C3) ppm) bearing the aldehyde substituent to be positioned. Finally, the methyl ester at δ 4.01 (H10′) showed a three-bond HMBC correlation to the ester carbonyl at 171.2 (C7′) ppm). These key HMBC correlations and similar ^{1}H and ^{13}C chemical shifts observed for **6** and **7** supported the structure of the depside, 5,5′-dichloroatranorin (**8**) (Table 1 and Figure 1).

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of (**8**) in $CDCl_3$.

Position	Δ_H, J (Hz)	δ_C	gHMBC and CIGAR
1	–	103.6, s	–
2	–	166.7, s	–
3	–	109.1, s	–
4	–	ND	–
5	–	115.6, s	–
6	–	149.1, s	–
7	–	ND	–
8	10.38, s	193.9, d	–
9	2.92, s	21.5, q	C1, C5, C6
1′	–	111.9, s	–
2′	–	159.6, s	–
3′	–	119.1, s	–
4′	–	148.8, s	–
5′	–	118.4, s	–
6′	–	136.6, s	–
7′	–	171.2, s	–
8′	2.13, s	10.5, q	C2′, C3′, C4′
9′	2.65, s	19.8, q	C1′, C5′, C6′
10′	4.01, s	53.1, q	C7′
2-OH	12.22, s	–	C1, C2, C3
2′-OH	11.52, s	–	C1′, C2′
4-OH	ND	–	–

Note: ND indicates signal was not detected.

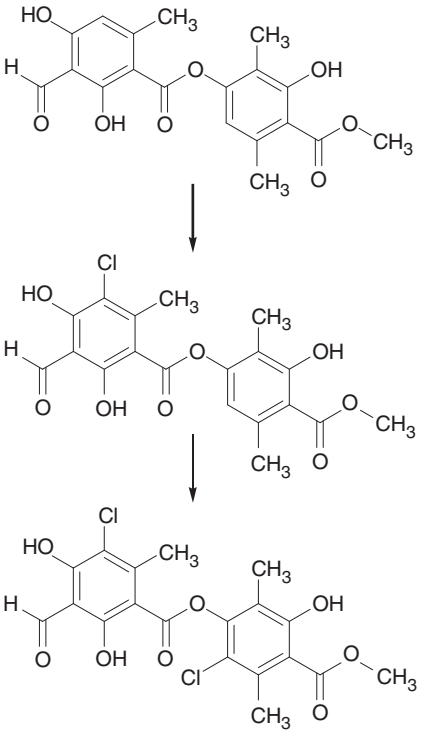


Figure 1. Chemical conversion of **6** to **7** and then finally to **8** in $CDCl_3$ *in situ* (mild conditions).

The first report of **8** appeared in 1964, where the halogenation of depsides was described (Neelakantan et al., 1964). This study reported the halogenation of **6** to **7** and then **7** to **8**, using Cl_2 (2 moles). Methanolysis of **8** produced two fission products, 5-chlorohaematommate (**12**) and methyl-5-chloro-β-orcinol carboxylate (**13**), which were then used to deduce the structure of the chlorinated depside. At the time, 5,5′-dichloroatranorin (**8**) had not been isolated or fully characterised. This represents the first isolation of **8** along with its detailed chemical characterisation.

2.3. *Isolation of pulvinic dilactone (1) and the conversion to pulvinic acid (3)*

The identification of **1** was previously reported in 1883, while the methanolysis of **1** to **3** had been reported in the late 1950s (Frank et al., 1950). HPLC profiling led us to believe that **4** and **5** were in fact the methanolysis products resulting from the initial extraction step. In an effort to confirm this, a separate extraction of *C. concolor* was carried out using CH_2CH_2, resulting in almost 95% pure (on the basis of ^{1}H NMR spectroscopy) pulvinic dilactone (**1**). In terms of the chemical profiling of lichens, selection of the extraction solvent is crucial in correctly determining the 'true' lichen constituents present and should be ideally performed using a selection of solvents.

Pulvinic dilactone (**1**) was isolated as yellow–green oil. The ESI mass spectrum showed the presence of m/z 291 $[M+H]^+$, consistent with a molecular formula of $C_{18}H_{10}O_4$ and 14 DBE. The ^{13}C NMR spectrum of **1** contained seven discrete signals. The highly

symmetrical nature of **1** enabled the presence of 10 methines and 8 quaternary carbons to be concluded, as supported by a gHSQCAD NMR experiment. The ^{1}H NMR and gCOSY NMR experiments identified the presence of two *ortho*-coupled aromatic methines (δ 8.04 d, $J = 8.0$ Hz, (H5/H5′/H9/H9′)) and (δ 7.50 dd, $J = 7.0$, 7.5 Hz (H6/H6′/H8/H8′)) that in turn showed a COSY correlation to an aromatic methine at δ 7.44 dd, $J = 7.0$, 6.5 Hz, (H7/H7′). The only key HMBC correlation was that observed from the protons at δ 8.04, (H5/H5′/H9/H9′) to 101.4 (C2/C2′) ppm, which enabled the positioning of these aromatic protons as being adjacent to the furanone. Positions C1, C1′ and C3, consistent with ester carbons, were assigned on the basis of carbon chemical shift comparisons to those previously assigned in compounds **2**, **4** and **5**.

The conversion of **1** to pulvinic acid (**3**) was carried out, and to the best of our knowledge, no previous allocation of the complete ^{1}H and ^{13}C NMR assignments of **3** has been previously reported. The first report of pulvinic acid (**3**) dates back to 1894 (Volhard, 1894) including a report of the conversion of **1** to **3** by acid hydrolysis (Frank et al., 1950). The synthesis of **3** was carried out in $CDCl_3$, where a solution of permethylated pulvinic acid, treated with iodotrimethylsilane, upon sealing the NMR tube under N_2 was heated to 55°C (Pattenden et al., 1991). After 3 days the reaction mixture was hydrolysed with MeOH to afford **3**, which was characterised by ^{1}H and ^{13}C NMR, but the complete assignment of the NMR data as well as the structure remained unassigned (Pattenden et al., 1991).

In the present study, pulvinic dilactone (**1**) was heated with aqueous acetone for 15 min, resulting in the isolation of **3** (in >95% purity based on ^{1}H NMR spectroscopy). The structure of **3** was confirmed by 1D and 2D NMR experiments, as well as mass spectrometry, and the structure found to be similar to **4**, differing only in the presence of a carboxylic acid at C12. The negative mode ESI mass spectrum showed the presence of a *m/z* 307 $[M-H]^-$ (molecular formula of $C_{18}H_{12}O_5$ and 13 DBE). The IR spectrum of **3** displayed absorptions at 3307 (broad) and 1611 cm^{-1}, suggestive of carboxylic and ester carbonyl moieties. The ^{1}H NMR spectrum, together with the gCOSY NMR identified the presence of two isolated mono-substituted aromatic rings (δ 7.33 m (H4); 7.29, m (H3/H5) and 7.19 d, $J = 7.0$ Hz, (H2/H6) and δ 8.14 d, $J = 7.0$ Hz (H2′/H6′); 7.30, m (H3′/H5′) and 7.12, dd, $J = 7.0$, 7.5 Hz (H4′)). Diagnostic HMBC correlations were observed from aryl protons on both rings (δ 8.14 (H2′/H6′) to 95.7 (C10) ppm) and (δ 7.19 (H2/H6) to 118.3 (C7) ppm), which enabled them to be positioned accordingly. As HMBC correlations were not observed for the exchangeable protons, the remaining quaternary carbon shifts were assigned by comparison to those previously assigned in **2**, **4** and **5**, for which **2** was assigned by a single crystal X-ray analysis (Dias et al., 2007). Figure 2 displays the conversion of **1** to **4** and **5**, as well as the conversion of **1** to **3**.

Compounds **1**, **3**–**5** and **8** were all found to display insignificant activity against the P388 murine leukaemia cell line ($IC_{50} > 12{,}500$ ng mL^{-1} when tested at 1 mg mL^{-1}). Pulvinic dilactone (**1**) moderately inhibited the growth of *Bacillus subtilis*, while compounds **3**–**5** and **8** only showed slight inhibition against *B. subtilis*. Compounds **6** and **7** were unable to be tested due to their instability and ultimate conversion to **8**.

The medicinal properties of lichens dates back to folklore. Hippocrates recommended *Usnea barbata* for uterine trouble, and in the 15th century, *Lobaria pulmonaria* was used for the treatment of catarrhal haemoptysis and pulmonary tuberculosis (Vartia, 1973). In Finnish folklore, lichens have been used to treat athlete's foot and oral doses have been used to treat coughs, usually taken in the form of so-called 'lichen milk' (Vartia, 1973). The antibacterial activity of lichen acids has been reported as early as the 1940s, where it was

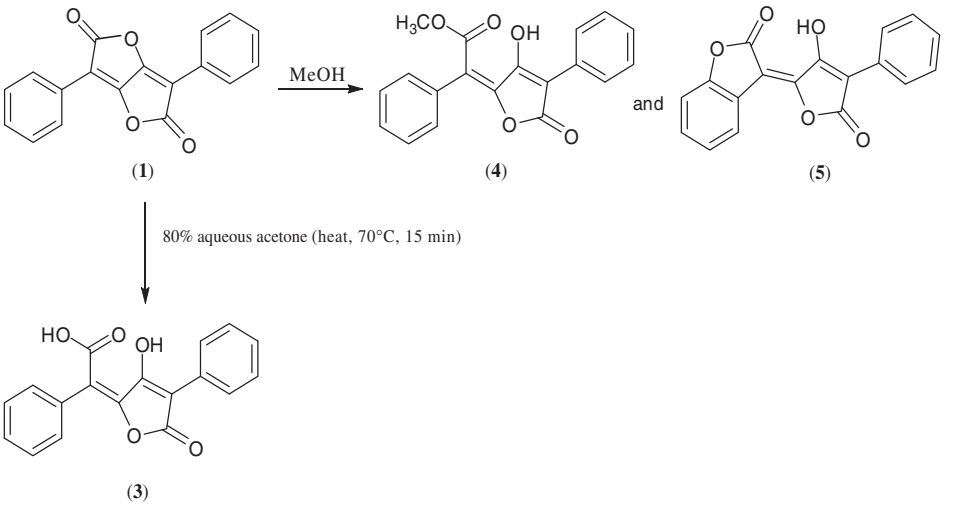


Figure 2. Chemical conversion of **1** to **4** and **5** using MeOH as the extraction solvent and the preparation of **3** from **1**, highlighting the importance of extraction solvent selection.

demonstrated that compounds **4–6** displayed antibiotic activity against Gram-positive bacteria (Krogg, 1954). Around the same time, it was concluded that **1** displayed inotropic effects in the same order of concentration as that necessary for the cardiac glycosides, ouabain and digitoxin (Giarman, 1949). In 1991 it was demonstrated that **4** inhibited the growth of *B. subtilis* (MIC of 15.8 $\mu g\,mL^{-1}$) (Nadir, Rashan, Ayoub, & Awni, 1992). Other reported activities for **4** include anti-inflammatory, inhibition of influenza RNA viruses, analgesic, local anaesthetic activity and toxicity effects (Rao & Prabhakar, 1988; Foden, McCormick, & O'Mant, 1975; Rashan, Ayoub, Al-Omar, & Al-Khayatt, 1990). In another study, **4** displayed activity against the fungus *U. violacea, M. microspora* and *E. repens*, and the bacteria *B. megaterium*, whereas **6** displayed activity only against *E. repens* (König & Wright, 1999).

3. Experimental

3.1. *General experimental procedures*

All organic solvents used were analytical reagent (AR or GR), UV spectroscopic or HPLC grades with milli-Q water also being used. IR spectra were recorded as a film using a NaCl disk on a Perkin-Elmer, Spectrum One FTIR spectrometer. UV/vis spectra were recorded on a Varian CARY 50 Bio spectrophotometer, using ethanol. In addition, a UV profile was obtained from the HPLC (PDA detection) by extraction of the 2D contour plot. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were acquired in $CDCl_3$ or DMSO-d_6 on a 500 MHz Varian INOVA spectrometer with referencing to solvent signals (δ 7.26, 77.0 ppm and δ 2.50, 39.5 ppm, respectively). 1D and 2D NMR experiments included gCOSY, gHSQCAD gHMBC and constant time inverse detection gradient accordian rescaled (CIGAR) experiments. ESI mass spectra were obtained on a Micromass Platform II mass spectrometer equipped with a LC-10AD Shimadzu solvent delivery module (50% CH_3CN/H_2O at a flow rate of 0.1 $mL\,min^{-1}$) in both the positive

and negative ionisation modes, using cone voltages between 20 and 30 V. HRESIMS was carried out on an Agilent 6200 Series TOF system (ESI operation conditions of $8\,L\,min^{-1}$ N_2, 350° drying gas temperature and 4000 V capillary voltage), equipped with an Agilent 1200 Series LC solvent delivery module (100% MeOH at a flow rate of $0.3\,mL\,min^{-1}$) in the negative ionisation mode (in all cases the instruments were calibrated using the 'Agilent Tuning Mix' using purine as the reference compound). TLC was performed on pre-coated aluminium backed silica TLC plates (Merck^{TM} silica gel 60 F_{254}) using the solvent system 65 : 25 : 4 ($CHCl_3$: MeOH : H_2O) or 100% (EtOAc), visualised at 254 and 365 nm and further developed using **A**: iodine vapour and **B**: a ninhydrin dip consisting of 0.3 g ninhydrin in 100 mL of *n*-butanol and 3 mL acetic acid. Silica flash chromatography was carried out with Merck^{TM}; silica gel (60 mesh) using nitrogen and a 50% stepwise solvent elution from 100% hexane to 100% CH_2Cl_2 to 100% EtOAc and finally to 100% MeOH. All analytical HPLC analyses were performed on a Dionex P680 solvent delivery system equipped with a PDA100 UV detector (operated using 'Chromeleon' software). Analytical HPLC analyses were run using either a gradient method (0 min 10% CH_3CN/H_2O; 2 min 10% CH_3CN/H_2O; 14 min 75% CH_3CN/H_2O; 24 min 75% CH_3CN/H_2O; 26 min 100% CH_3CN; 30 min 100% CH_3CN; 32 min 10% CH_3CN/H_2O and 40 min 10% CH_3CN/H_2O) or an isocratic method (100% CH_3CN) on a Phenomenex Prodigy ODS (3) C_{18} 100Å 250×4.6 (5 μ) and on a Phenomenex Luna ODS (3) C_{18} 100Å 250×4.6 (5 μ) column at a flow rate of $1.0\,mL\,min^{-1}$. All semi-preparative HPLC was carried out on a Varian Prostar 210 (Solvent Delivery Module) equipped with a Varian Prostar 335 PDA detector using STAR LC WS Version 6.0 software using an isocratic method (100% CH_3CN) and a Phenomenex Prodigy ODS (3) 100Å C_{18} 250×10 (5 μ) column at a flow rate of $3.5\,mL\,min^{-1}$.

3.2. *Biological evaluation and details of assays*

Extracts of *C. concolor* (Dickson) B. Stein were evaluated against a P388 Murine Leukaemia cell line (antitumour assay), as well as against a number of bacteria and fungi (antimicrobial assays) at the University of Canterbury, Christchurch, New Zealand. Only moderate antitumour activity was observed for the crude *C. concolor* extract (IC_{50} 31,776 ng mL^{-1} at 10 mg mL^{-1}). The *C. concolor* extract displayed significant antibacterial activity against *B. subtilis*. No activity was observed against *Eschericha coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Trichophyton mentagrophytes* or *Cladosporium resinae*. For further details on the antitumour and antimicrobial assays, please see Dias et al. (2007).

3.3. *Lichen material*

The yellow lichen was collected on 19 August 2007 near Merlynston Railway Station, Victoria, Australia. The lichen and bark were chiselled off a eucalyptus tree and a small ($2 \times 2\,cm^2$) sample sent to Emeritus Prof. John A. Elix (Australian National University, Canberra, ACT, Australia) for taxonomic identification and HPLC profiling. A voucher specimen designated the code 2007-04 is deposited at the School of Applied Sciences (Discipline of Applied Chemistry), RMIT University.

3.4. *Extraction and isolation*

In the first extraction of *C. concolor*, the lichen, together with the bark (170 g) was extracted with 3:1 MeOH/CH_2Cl_2 (750 mL). The crude extract was decanted and concentrated under reduced pressure. Approximately half of the crude extract was then subjected to a flash silica column (50% stepwise elution from petroleum spirits to CH_2Cl_2 and finally to EtOAc) with the third fraction yielding pure vulpinic acid (**4**) (7.2 mg, 0.004%, based on mass of lichen and bark extracted). In addition, calycin (**5**) was identified to be present in a mixture with **4**. This mixture was subjected to further flash silica chromatography using 100% EtOAc as the eluent, affording pure calycin (**5**) (1.5 mg 0.0008%, based on mass of lichen and bark extracted).

In a further effort to separate **4** and **5** by HPLC, the 3:1 MeOH/CH_2Cl_2 crude extract was sequentially partitioned using CH_2Cl_2 followed by MeOH. The MeOH partition was then subjected to reversed-phase HPLC using isocratic conditions (100% CH_3CN with detection at 215 and 254 nm) to yield atranorin (**6**) as a pale yellow oil (1.1 mg, 0.006%, based on mass of lichen and bark extracted). It was immediately noted that atranorin (**6**) rapidly converted to **7** and finally to a stable pale yellow oil, **8**, over a period of 1–2 days in $CDCl_3$ *in situ*.

In a second extraction of the lichen, *C. concolor*, together with the bark (55 g), was extracted with 100% CH_2Cl_2 (50 mL). The crude extract was decanted and concentrated under reduced pressure. The entire extract was then subjected to flash silica chromatography using 100% EtOAc as the eluent, to afford pure **1** (201.3 mg, 0.4%, based on mass of lichen and bark extracted). In this instance, the purity of **1** was assessed on the basis of the ^{1}H NMR analysis.

The chemical conversion of **1** to **3** was demonstrated by heating **1** (75 mg) in 80% aqueous acetone (100 mL) for 15 min, resulting in **3** (>95% purity as assessed by ^{1}H NMR spectroscopy) (65.2 mg, 82.0% yield).

Pulvinic dilactone (**1**) [3,6-diphenylfuro[3,2-*b*]furan-2,5-dione]; isolated as a stable yellow–green oil; IR (film) ν_{max} 3401, 2917, 1816, 1660, 1632 cm^{-1}; UV (EtOH) λ_{max} 205, 231, 285 and 375 nm ($\varepsilon = 20900$, 11100, 12000 and 6000, respectively); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.04, d, $J = 8.0$ Hz (H5/H5′/H9/H9′); 7.50, dd, $J = 7.0$, 7.5 Hz (H6/H6′/H8/H8′); 7.44, dd, $J = 7.0$, 6.5 Hz (H7/H7′); ^{13}C (125 MHz, $CDCl_3$) 165.7 (C3/C3′); 157.1 (C1/C1′); 130.1 (C7/C7′); 129.2 (C6/C6′/C8/C8′); 128.2 (C5/C5′/C9/C9′); 126.4 (C4/C4′); 101.4 (C2/C2′); ESIMS (+ve) (25 V) m/z 291 $[M+H]^+$.

Pulvinic acid (**3**) [(E)-2-(3-hydroxy-5-oxo-4-phenylfuran-2(5H)-ylidene)-2-phenylacetic acid]; isolated as a dark brown oil; IR (film) ν_{max} 3307, 2917, 1739, 1611, 1578, 1563, 1398, 1146 cm^{-1}; UV (EtOH) λ_{max} 200, 260 and 365 nm ($\varepsilon = 16{,}500$, 17,000 and 8600, respectively); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.14, d, $J = 7.0$ Hz (H2′/H6′); 7.33, m (H4); 7.30, m (H3′/H5′); 7.29, m (H3/H5); 7.19, d, $J = 7.0$ Hz (H2/H6); 7.12, dd, $J = 7.0$, 7.5 Hz (H4′), 9-OH and 12-OH not detected; ^{13}C (125 MHz, DMSO-d_6) 172.1 (C12); 169.3 (C11); 167.2 (C9); 153.1 (C8); 136.7 (C1); 133.3 (C1′); 130.8 (C2/C6); 128.5 (C3′/C5′); 127.9 (C3/C5); 127.6 (C4); 125.8 (C2′/C6′); 125.7 (C4′); 118.3 (C7); 95.7 (C10); ESIMS (−ve) (25 V) m/z 307 $[M-H]^-$.

Vulpinic acid (**4**) [(E)-methyl 2-(3-hydroxy-5-oxo-4-phenylfuran-2(5H)-ylidene)-2-phenylacetate]; isolated as a stable, bright yellow oil; IR (film) ν_{max} 3426, 2919, 2850, 1774, 1677, 1612, 1598, 1440, 1289, 1279, 1065 cm^{-1}; UV (EtOH) λ_{max} 205, 290 and 375 nm ($\varepsilon = 20400$, 5500 and 7000, respectively); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9-OH not detected; 8.13, dd,

$J = 1.5$, 8.5 Hz (H2′/H6′); 7.44, m (H4); 7.42, m (H4′); 7.41, m (H3′/H5′); 7.34, dd, $J = 7.0$, 7.5 Hz (H3/H5); 7.27, dd, $J = 2.0$, 8.0 Hz (H2/H6), 3.89, s (13-OCH_3); ^{13}C (125 MHz, $CDCl_3$) 171.7 (C12); 165.9 (C11); 160.2 (C9); 154.8 (C8); 131.9 (C1); 129.9 (C2/C6); 128.9 (C1′); 128.6 (C4); 128.4 (C3′/C5′); 128.3 (C4′); 128.1 (C3/C5); 127.8 (C2′/C6′); 115.8 (C7); 105.1 (C10); 54.4 (13-OCH_3); ESIMS (−ve) (25 V) m/z 321 $[M-H]^-$.

Calycin (**5**) [(E)-3-(3-hydroxy-5-oxo-4-phenylfuran-2(5H)-ylidene)benzofuran-2(3H)-one]; isolated as a stable orange oil; IR (film) ν_{max} 3401, 2922, 2852, 1804, 1709, 1641, 1473, 1442, 1343, 1040 cm^{-1}; UV (EtOH) λ_{max} 202, 265, 365 and 455 nm ($\varepsilon = 11{,}200$, 10,500, 6100 and 3000, respectively); ^1H NMR (500 MHz, $CDCl_3$) δ 12.60, br s (10-OH); 8.19, d, $J = 7.5$ Hz (H2′/H6′); 7.98, d, $J = 7.5$ Hz (H3); 7.47, m (H3′/H5′); 7.45, m (H5); 7.39, dd, $J = 7.5$, 7.5 Hz (H4′); 7.32, dd, $J = 7.5$, 8.0 Hz (H4); 7.23, d, $J = 8$ Hz (H6); ^{13}C (125 MHz, $CDCl_3$) 173.1 (C7); 165.4 (C12); 160.0 (C10); 153.6 (C1); 152.8 (C9); 131.4 (C5); 129.1 (C4'); 128.6 (C3′/C5′); 128.3 (C1′); 128.0 (C2′/C6′); 125.8 (C3 and C4); 121.6 (C2); 111.1 (C6); 105.9 (C11), 107.0 (C8); ESIMS (−ve) (25 V) m/z 305 $[M-H]^-$.

Atranorin (**6**) [3-hydroxy-4-(methoxycarbonyl)-2,5-dimethylphenyl 3-formyl-2,4-dihydroxy-6-methylbenzoate]; isolated as an unstable colourless oil which rapidly converted to **7** and then finally to the stable compound **8**; ^1H NMR (500 MHz, $CDCl_3$) 12.55, br s (OH); 12.50, br s (OH); 11.95, br s (OH); δ 10.36, s (H8); 6.52, s (H5′); 6.41, s (H5); 3.99, s (10-OCH_3); 2.69, s (9-CH_3); 2.55, s (9′-CH_3); 2.09, s (8′-CH_3); ^{13}C (125 MHz, $CDCl_3$) δ 193.8 (C8); 172.7 (C7′); 163.2 (C2′); 152.6 (C6); 152.0 (C4′); 140.4 (C6′); 117.1 (C3′); 116.1 (C5′); 112.9 (C5); 110.9 (C1′); 103.2 (C1); 52.7 (10′-OCH_3); 25.6 (9-CH_3); 24.2 (9′-CH_3); 9.7 (8′-CH_3); ND (C2); ND (C3); ND (C4); ND (C7); ESIMS (−ve) (25 V) m/z 373 $[M-H]^-$.

5-chloroatranorin (**7**) [3-hydroxy-4-(methoxycarbonyl)-2,5-dimethylphenyl 3-chloro-5-formyl-4,6-dihydroxy-2-methylbenzoate]; isolated as an unstable colourless oil resulting from chemical degradation of **6**; partial identification ^1H NMR (500 MHz, $CDCl_3$) δ 12.33, br s (OH); 11.96, br s (OH); δ10.36, s (H8); 6.52, s (H5′); 3.99, s (10-OCH_3); 2.86, s (9-CH_3); 2.54, s (9′-CH_3); 2.09, s (8′-CH_3) other OH not detected; partial identification based on gHSQCAD; ^{13}C (125 MHz, $CDCl_3$) δ 193.9 (C8); 116.2 (C5′); 52.6 (10′-OCH_3); 24.3 (9′-CH_3); 21.3 (9-CH_3); 9.9 (8′-CH_3); with all remaining quaternary carbons not detected; ESIMS (−ve) (25 V) m/z 407 $[M-H]^-$.

5,5′-dichloroatranorin (**8**) [2-chloro-5-hydroxy-4-(methoxycarbonyl)-3,6-dimethylphenyl 3-chloro-5-formyl-4,6-dihydroxy-2-methylbenzoate]; isolated as a stable colourless oil as the final product from the degradation of (**6**); IR (film) ν_{max} 3401, 2924, 2853, 1735, 1648, 1452, 1302, 1249, 1073 cm^{-1}; UV (MeOH) λ_{max} 285 and 375 nm ($\varepsilon = 4600$ and 1500, respectively); ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data see Table 1; ESIMS (−ve) (25 V) m/z 441 $[M-H]^-$; HRESIMS m/z 441.0143 ($[M-H]^-$; calculated for $C_{19}H_{15}Cl_2O_8$, 441.0222).

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