UNIVERSITY
OF WOLLONGONG
AUSTRALIA

University of Wollongong
Research Online

Faculty of Science, Medicine and Health - Papers:
part A

Faculty of Science, Medicine and Health

2017

Antarctic moss biflavonoids show high antioxidant and ultraviolet-screening activity

Melinda J. Waterman

University of Wollongong, melindaw@uow.edu.au

Ari Satia Nugraha

University of Wollongong, asn559@uowmail.edu.au

Rudi Hendra

University of Wollongong, rh965@uowmail.edu.au

Graham E Ball

University of New South Wales, g.ball@unsw.edu.au

Sharon A. Robinson

University of Wollongong, sharonr@uow.edu.au

See next page for additional authors

Publication Details

Waterman, M. J., Nugraha, A. S., Hendra, R., Ball, G. E., Robinson, S. A. & Keller, P. A. (2017). Antarctic moss biflavonoids show high antioxidant and ultraviolet-screening activity. *Journal of Natural Products*, 80 (8), 2224-2231.

Research Online is the open access institutional repository for the University of Wollongong. For further information contact the UOW Library:
research-pubs@uow.edu.au

Antarctic moss biflavonoids show high antioxidant and ultraviolet-screening activity

Abstract

Ceratodon purpureus is a cosmopolitan moss that survives some of the harshest places on Earth: from frozen Antarctica to hot South Australian deserts. In a study on the survival mechanisms of the species, nine compounds were isolated from Australian and Antarctic *C. purpureus*. This included five biflavonoids, with complete structural elucidation of 1 and 2 reported here for the first time, as well as an additional four known phenolic compounds. Dispersion-corrected DFT calculations suggested a rotational barrier, leading to atropisomerism, resulting in the presence of diastereomers for compound 2. All isolates absorbed strongly in the ultraviolet (UV) spectrum, e.g., biflavone 1 (UV-A, 315-400 nm), which displayed the strongest radical-scavenging activity, 13% more efficient than the standard rutin; p-coumaric acid and trans-ferulic acid showed the highest UV-B (280-315 nm) absorption. The more complex and abundant 1 and 2 presumably have dual roles as both UV-screening and antioxidant compounds. They are strongly bound to Antarctic moss cell walls as well as located inside the cells of moss from both locations. The combined high stability and photoprotective abilities of these isolates may account for the known resilience of this species to UV-B radiation and its survival in some of the toughest locations in the world.

Keywords

activity, ultraviolet-screening, antioxidant, show, high, antarctic, moss, biflavonoids

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Waterman, M. J., Nugraha, A. S., Hendra, R., Ball, G. E., Robinson, S. A. & Keller, P. A. (2017). Antarctic moss biflavonoids show high antioxidant and ultraviolet-screening activity. *Journal of Natural Products*, 80 (8), 2224-2231.

Authors

Melinda J. Waterman, Ari Satia Nugraha, Rudi Hendra, Graham E Ball, Sharon A. Robinson, and Paul A. Keller

Antarctic Moss Biflavonoids Show High Antioxidant and Ultraviolet Screening Activity

Melinda J. Waterman,[†] Ari S. Nugraha,[‡] Rudi Hendra,[‡] Graham E. Ball,[§] Sharon A. Robinson,[†]
and Paul A. Keller^{‡,*}

[†]School of Biological Sciences and [‡]School of Chemistry, University of Wollongong,

Wollongong, NSW, 2522, Australia

[§]School of Chemistry, UNSW, Sydney, NSW, 2052, Australia

ABSTRACT: *Ceratodon purpureus* is a cosmopolitan moss that survives some of the harshest places on Earth: from frozen Antarctica to hot South Australian deserts. In a study into survival mechanisms of the species, nine compounds were isolated from Australian and Antarctic *C. purpureus*. This included five biflavonoids with complete structural elucidation of **1** and **2** reported here for the first time, as well as an additional four known phenolic compounds. Dispersion corrected DFT calculations suggested a rotational barrier, leading to atropisomerism, resulting in the presence of diastereomers for compound **2**. All isolates absorbed strongly in the ultraviolet (UV) spectrum, e.g. biflavone **1** (UV-A, 315–400 nm) which displayed the strongest radical-scavenging activity, 13% more efficient than the standard rutin; *p*-coumaric acid and *trans*-ferulic acid showed the highest UV-B (280–315 nm) absorption. The more complex and abundant **1** and **2** presumably have dual roles as both UV screening and antioxidant compounds. They are strongly bound to Antarctic moss cell walls as well as located inside the cells of moss from both locations. The combined high stability and photoprotective abilities of these isolates may account for the known resilience of this species to UV-B radiation and its survival in some of the toughest locations in the world.

Antarctic terrestrial flora (algae, mosses, liverworts, lichens, and two higher plants) endure physiologically extreme conditions such as sub-zero temperatures, freeze-thaw events, desiccation-rehydration cycles, irregular water availability,¹ drying winds, and enhanced ultraviolet (UV) radiation due to stratospheric ozone depletion.² One moss species found in East Antarctica, the cosmopolitan *Ceratodon purpureus* (Hedw.) Brid., is known to be highly tolerant to these many stresses especially UV-B radiation (280–315 nm) and desiccation.³⁻⁶

Ultraviolet radiation (UVR) is harmful to many biologically essential molecules including nucleic acids (e.g. DNA), proteins, lipids, and photosynthetic pigments. Particularly for Antarctic flora, this UVR damage exacerbates an already stressful climate. Plants can typically protect against direct UVR damage by employing intracellular and/or cell wall UV absorbing (or screening) compounds (UVAC) as a first defense strategy, and indirectly via the use of antioxidants to scavenge reactive oxygen species (ROS).⁷⁻¹⁰

Like vascular plants, bryophytes (mosses and liverworts) commonly produce numerous intracellular secondary metabolites with antioxidant and UV photoprotective properties, including flavonoids⁸ and hydroxycinnamic acids, to reduce the direct and indirect effects of damaging UVR penetrating their tissues.¹⁰ High concentrations of UVAC within the cell walls of *C. purpureus* have been suggested to be a major factor in the UV-B radiation tolerance of this Antarctic moss species.⁶ Producing compounds with both UV-screening and antioxidant properties would prove highly valuable for Antarctic plants like *C. purpureus*, since they are frequently exposed to periods of desiccation or freezing. During these events, they are physiologically inactive and thus their ability to repair direct damage is reduced, possibly rendering them susceptible to harmful UV effects.⁵

We aimed to determine the vital chemical ecology of *C. purpureus* that gives rise to its highly resilient nature both in Antarctica where it endures extreme cold climates and in other places around the globe, e.g. in Australia where it lives in hot deserts. Here we report the extraction, separation, isolation, and structural elucidation of intracellular and cell wall compounds from *C. purpureus* and analysis of their UV and antioxidant capacities. In order to conserve the ecologically valuable Antarctic samples of *C. purpureus*, samples of the same species collected from more populous sites in Australia were examined prior to comparing their HPLC profiles with extracts from the Antarctic moss. This is the first elucidation of natural products from *C. purpureus* and the cell walls of any Antarctic moss species as well as the first study to correlate the natural products to their function in Antarctic moss.

RESULTS AND DISCUSSION

Isolation and Structure Elucidation of Intracellular Constituents. In order to conserve Antarctic material, bulk extraction and isolation were undertaken using Australian moss. Therefore, methanol extracts of Australian *C. purpureus* containing intracellular constituents were subjected to HPLC separation to afford the biflavonoids **1-5** (Figure 1) as pale yellow solids. All biflavonoids were stable at room temperature.

NMR spectroscopic analysis of the constituents was performed both in DMSO-*d*₆ and acetone-*d*₆, the former being employed as most biflavonoids have been reported using this solvent. However, recent evidence¹¹ suggests that numerous errors in the structural assignment of natural products, including flavones, have occurred, specifically in the assignment of 3,5-dioxygenated aromatic moieties, when DMSO-*d*₆ was used. Therefore, this study additionally analyzed isolated compounds using acetone-*d*₆.

a) Intracellular isolates from Australian *C. purpureus*

b) Cell wall isolates from Australian *C. purpureus*

Figure 1. a) Intracellular constituent biflavonoids (**1-3** and **5**); b) cell wall isolates; *p*-coumaric acid (**6**), vanillin (**7**), *p*-hydroxybenzaldehyde (**8**) and *trans*-ferulic acid (**9**) from Australian *Ceratodon purpureus*. Isolate **4** is likely also a biflavonoid but its precise structure is unknown. The atropisomeric chirality of **1** was determined by comparative ECD spectroscopy analysis.

Compound **1** was isolated as a yellow powder which decomposed at 295 °C. The IR spectrum indicated stretches at 1,653 and 3,213 cm^{-1} , assigned to carbonyl and hydroxy stretches, respectively. The molecular formula of $\text{C}_{30}\text{H}_{18}\text{O}_{12}$ of compound **1** was confirmed by HRESIMS with an assigned $[\text{M}-\text{H}]^-$ peak at m/z 569.0732. Compound **1** was identified as the known biflavone 8-[5-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)-2,3-dihydroxyphenyl]-2-(3,4-

dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one (5',8''-biluteolin or 5',3'''-dihydroxyamentaflavone), previously identified in other moss species.^{12,13,14} According to Seeger et al. 1990,¹⁴ the most common methanol-soluble moss biflavonoids are dimers of luteolin and their 2,3- or 2'',3''-dihydro derivatives.

Surprisingly, despite previous reports of the structure of biflavone **1**, there has yet to be a full NMR characterization, including the assignment of all protons and carbons in the spectra to their resonances, and importantly, evidence supporting the positioning of the biaryl axis connecting the two flavone moieties. Therefore, we report here for the first time, the full characterization of biflavone **1**, with full NMR assignment and HRMS analysis (Table S1, Figure S16, Supporting Information), including evidence for the precise location of the biaryl axis. This axis is positioned at 5'-8'' as confirmed by analysis of the HMBC spectra (Figure 2), which showed cross-peaks between proton resonances at δ_{H} 7.64 (d, $J = 2.3$ Hz) (H-6') and 6.46 (H-6'') with the carbon resonance at δ_{C} 104.7 (C-8''). Highly useful in confirming the assignments in both biflavonoids **1** and **2** are the correlations from hydroxy protons. Two of the OH resonances in both **1** and **2** are sharp in acetone- d_6 at room temperature, likely due to the formation of strong intramolecular hydrogen bonds. In biflavone **1**, the OH peak at 13.21 ppm shows an HMBC cross-peak to C-4a'' (δ_{C} 105.6) highlighting that it must be the hydroxy group attached to C-5''. The same hydroxy proton shows HMBC correlations to C-5'' at δ_{C} 162.5 and C-6'' at δ_{C} 99.8. Thus, the site of connection must be C-8''. The chirality of the atropisomeric biaryl axis was determined by comparative ECD spectroscopy, with **1** showing a positive Cotton effect at 362 ($\Delta\epsilon +24.6$) nm (Figure S20, Supporting Information), which defines an (*M*)-isomer (*aR* absolute configuration) by comparison with other axially chiral biflavones.^{15,16}

Figure 2. HMBC spectrum of biflavone **1**, showing correlations confirming the location of the biaryl axis as 5'-8''.

Compound **2** was isolated as a yellow powder which decomposed at 295 °C. The IR spectrum showed stretches at 1,653 cm^{-1} and 3,330 cm^{-1} , assigned to carbonyl and hydroxy stretches, respectively. The proposed molecular formula of $\text{C}_{30}\text{H}_{20}\text{O}_{12}$ for compound **2** was confirmed by the assignment of the $[\text{M}+\text{H}]^+$ to the peak observed in the HRESIMS spectrum at m/z 573.1044. The structure of compound **2** was defined through extensive NMR analyses as the known biflavonoid 8-[5-(5,7-dihydroxy-4-oxochroman-2-yl)-2,3-dihydroxyphenyl]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one, or 2,3-dihydro-5',3'''-dihydroxyamentoflavone.¹⁷ Analysis of both the ECD spectrum (Figure S20, Supporting Information) and optical rotation indicated that biflavonoid **2** was present as a racemic mixture.

The ^1H and ^{13}C NMR spectra of compound **2** showed some duplication of resonances. This may result from the presence of diastereomers, arising from the C-2 stereogenic center in combination with the likely C-5'-C-8'' stereogenic axis. Although biflavone atropisomerism was reported in the 1960s, it has been recently noted^{16,18} that the probable presence of biflavone atropisomerism is largely and undeservedly not recognized. As compound **2** possesses three *ortho*-substituents to the C-5'-C-8'' axis, together with the additional C-3'-substituent (*meta* to the axis) providing a buttressing effect, it is highly likely that there is restricted rotation around the biaryl axis leading to the stereogenic element, resulting in diastereomers that could be identified as separate species in the NMR spectra of **2**.

To test if this restricted rotation about the C-5'-C-8'' bond is indeed present, the model compound **10** (Figure 3) which contains the core of compound **2** including the biaryl axis, was subjected to calculations using dispersion corrected DFT methods at two levels of theory, both in vacuum and in continuum model solvent (Supporting Information). One of the DFT methods was recommended in a benchmark study of barriers to biphenyl rotations.¹⁹ A survey of possible low energy conformations and transition structures lead to identification of the lowest energy conformation and transition structures shown in Figure 3.

Figure 3. Lowest energy geometry (left) and lowest energy transition structure for rotation around the biaryl linkage (right) calculated at the TPSS-D3/def2-TZVPP level of theory.

In vacuum, the transition structure was found to be higher in free energy than the minimum energy structure by 21.3 kcal mol⁻¹ using the B97-D3 method or 19.5 kcal mol⁻¹ using the TPSS-D3 method. In acetone, the transition structure is higher in energy by 19.9 kcal mol⁻¹ using the B97-D3 method or 17.8 kcal mol⁻¹ using the TPSS-D3 method. Even using the lowest of these four values as an energy barrier, the rate of exchange due to rotation around the C-5'-C-8" bond is sufficiently slow at room temperature (0.53 s⁻¹ at 298 K for model compound **10** based on $\Delta G^\ddagger = 17.8$ kcal mol⁻¹) that sharp, separate signals should be observable for each diastereomer in derivatives of the model compound **10**, such as compound **2**, which contain a second stereogenic moiety (Table S4, Supporting Information).

In addition to the two major constituents **1** and **2**, three additional compounds were isolated in significantly smaller quantities. It is likely that these are also biflavonoids, based on analysis from mass spectrometric data, UV spectroscopic data, their antioxidative measurements, and partial NMR assignments. The complete structure of compound **4** could not be defined, whereas compound **5** is likely to be a dihydrobiflavone of the type illustrated in Figure 1. Although the moieties defined by rings A-C and D-F-E could be reasonably identified, the connections to ring B could not be established due to both the weak signal to noise ratio in the NMR analysis, and sample contamination. Similarly, the complete structure of compound **3** could not be finalized, but is likely to be a structural isomer of **1**. The moiety defined by rings D-F-E could be determined as could the flavone subunit A-C, the latter connected through C-2, however, the bonds to ring B could not be defined.

Isolation and Characterization of Cell Wall Constituents. After methanol extraction, compounds bound to the cell walls of the remaining plant residue were extracted by alkaline (NaOH) hydrolysis and subjected to successive solid phase extraction and HPLC separation to

afford *p*-coumaric acid [(*E*)-3-(4-hydroxyphenyl)-2-propenoic acid] (**6**), as an off-white solid; vanillin (4-hydroxy-3-methoxybenzaldehyde) (**7**), as a vanilla-scented pale yellow solid; *p*-hydroxybenzaldehyde (**8**); and *trans*-ferulic acid [(*E*)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid] (**9**) (Figure 1). All compounds were stable at room temperature. The MS and UV spectra of these isolates were identical to commercial standards.

Comparisons Between Australian and Antarctic *C. purpureus*. Australian populations of *C. purpureus* were the initial focus for the bulk extraction and isolation of compounds from this species due to the high conservation status of Antarctic flora. Both Australian and Antarctic *C. purpureus* are genetically similar^{20, 21} and are highly tolerant to UV radiation⁶ which suggests that they produce similar UV-active secondary metabolites. Unsurprisingly, compounds **1-5** were present in the Antarctic population. This was confirmed by analytical HPLC (comparison and spiking experiments) and ESIMS analyses, which also showed biflavone **1** and dihydrobiflavone **2** as some of the most abundant UV-active compounds within Antarctic intracellular extracts (Figure S21, Supporting Information). Separate spikings of **1** and **2** to several cell wall extracts from Antarctic samples confirmed that these biflavonoids are also bound to the cell walls of the Antarctic moss (Figure S23, Supporting Information). This adds to the increasing awareness of flavonoid-type compounds being tightly bound to plant cell walls.²² In addition, when Antarctic cell wall extracts were prepared using solid phase extraction, HPLC peaks corresponding to isolates **6-9** were also observed.

The relative abundance of the two biflavonoids varied with cellular location and between the populations (Table 2). The isolated yields of these compounds from Australian *C. purpureus* were 6.0 (**1**) and 9.7 mg.g⁻¹ dry wt. (**2**), which were calculated from the integration of their corresponding peaks in HPLC traces. Applying this calculation to several intracellular and cell

wall extracts showed how variable concentrations of biflavonoids can be between populations and seasons; however, compound **2** was consistently more abundant than **1**. More specifically for the Antarctic samples, biflavonoid **2** was on average 1.3-fold (ranging from one- to three-fold) more abundant than biflavone **1** in the crude intracellular extract but on average 13-fold (ranging from one- to 25-fold) more abundant in the crude cell wall extract. In contrast, biflavonoid **2** was 1.8-fold more abundant than **1** in the Australian populations. Interestingly, these biflavonoids were present as major compounds in 35% of Antarctic *C. purpureus* cell wall extractions but were completely lacking in the cell wall extracts obtained from the Australian samples. The variation in abundance and their presence/absence across different populations may be related to varied stresses and seasonal differences between the two environments.

Table 2. Concentrations (Means $\pm$ s.d.) of Isolates **1-5** Within Australian (n = 7) and Antarctic (n = 8) Samples of *C. purpureus* Based on HPLC Analysis.

	cellular location	concentration (mg.g ⁻¹ dry wt)				
		1	2	3	4	5
Australian	intracellular	5.95 $\pm$ 2.71	9.68 $\pm$ 5.42	0.05 $\pm$ 0.01	0.34 $\pm$ 0.17	0.37 $\pm$ 0.14
	cell wall	-	-	-	-	-
Antarctic	intracellular	3.60 $\pm$ 2.09	4.76 $\pm$ 2.93	0.05 $\pm$ 0.02	0.44 $\pm$ 0.23	1.89 $\pm$ 1.03
	cell wall	0.08 $\pm$ 0.05	0.72 $\pm$ 0.00	-	-	-

These environmental stresses include the different temperature regimes and UVR intensities of the two regions. For example, East Antarctic moss is exposed to frigid, desert conditions (mean monthly range of -12.6 to -5.9 °C)²³ and an extremely short growing season (December-February) leading to very slow growth rates.²⁴ In addition, over the past four decades, this region has seen the largest increase in UV-B radiation due to ozone depletion.²⁵ Antarctic moss may sequester these biflavonoids in its cell walls in order to prevent loss of these complex structures during the frequent freeze/thaw and desiccation/rehydration events that

characterize polar life. On the other hand, south-eastern Australian mosses thrive in more temperate conditions (mean monthly range of 11.5 to 22.5 °C)²³ as well as more constant, high UV radiation levels. The Australian moss species can potentially grow all year round in these more temperate conditions allowing complex biflavonoids **1** and **2** to be maintained in an intracellular environment.

Although biflavonoids are secondary metabolites frequently found in bryophytes, they have only been isolated from intracellular locations using methanol-based solvents²⁶⁻³¹ with most studies ignoring the possibility that they are also located within moss cell walls.^{3, 31} Biflavonoids found in mosses are generally more ubiquitous and structurally more polar than those found in other plants,³⁰⁻³³ increasing the likelihood that such compounds would be also found in moss cell walls. Here, by analyzing both intracellular and cell wall extracts, it was shown that biflavonoids do exist in the cell walls of moss – the first definitive account of such a cellular location. Therefore, this is the first isolation of biflavonoids from moss cell walls as well as from an Antarctic bryophyte.

The phenolic *p*-coumaric acid is a common precursor to many secondary metabolites such as flavonoids. It is not unusual to isolate or identify this compound bound to moss or other plant cell walls³⁴⁻³⁷ and it is commonly found in bryophytes,^{33, 38} moss spores, and pollen.³⁸⁻⁴¹ Similarly, ferulic acid is a typical hydroxycinnamic acid located in plant cell walls.⁴² There are several accounts of both ferulic and *p*-coumaric acids extracted and/or identified from plant cell walls. These include 54 species within the Conifer taxa;³⁷ *Graminae* plants;³⁵ spores of *Vicia faba*, *Betula pendula*, and *Helleborus foetidus*;⁴¹ *Pinus sylvestris*;^{36, 41} and the Antarctic species *Deschampsia antarctica* and *Colobanthus quitensis*.⁴³ In addition, either or both of these two *p*-hydroxycinnamic acids have been reported as chemicals that can be used as UVR proxies^{25, 39, 44}

showing correlation with past UVR levels^{41, 45} and/or been used for reconstructing past ozone climates.^{40, 46}

The location of compounds **1**, **2**, and **6-9** within the cell wall suggest they have a predominant role in photoprotection against UVR damage either through screening-out harmful UV radiation or as scavengers of UV induced radicals such as ROS. Such secondary metabolites could also be produced in this moss to enhance its tolerance to desiccation. In order to define the role of these compounds, their UV absorbing and antioxidant capacities were analyzed.

Ultraviolet and Antioxidant Activities of Compounds 1-9. The absorbance spectra and antioxidant activities of compounds **1-9** were compared with the high performing standard, rutin (Table 3). All isolates absorbed UV radiation (200–400 nm) relatively strongly compared to the standard. Both biflavonoids showed relatively high absorbance across a range of UV wavelengths, exhibiting peaks at 353 ($\epsilon = 12\,300\text{ M}^{-1}\text{cm}^{-1}$) and 255 nm for **1** and 349 ($\epsilon = 7\,030\text{ M}^{-1}\text{cm}^{-1}$) and 287 nm for **2**. However, both **6** and **9** displayed higher absorbance over the entire UV-B (280–315 nm) spectrum demonstrating greater effectiveness as UVACs ($\epsilon_{310} = 23\,100\text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{297} = 22\,410\text{ M}^{-1}\text{cm}^{-1}$). Compound **9** was the most effective at absorbing the shorter UV-A wavelengths giving a maximum peak at 323 nm.

Table 3. Ultraviolet and Antioxidant Activities of the Nine Isolates Compared to Rutin, a Known Standard for DPPH Antioxidant Assays. All UV and DPPH (IC₅₀) Measurements Were Performed on Compounds Isolated from Australian *C. purpureus* only. IC₅₀ Values (μM) Represent Means ± s.d. (n = 3).

compound	ε (M ⁻¹ cm ⁻¹)*		AA (IC ₅₀ , μM)	cellular location	
	λ _{max} 1	λ _{max} 2		Australian <i>C. purpureus</i>	Antarctic <i>C. purpureus</i>
1	12 300 (353 nm)	13 110 (255 nm)	33.5 ± 1.6	intracellular	intracellular and cell wall
2	7 030 (349 nm)	8 510 (275 nm)	127.8 ± 3.8	intracellular	intracellular and cell wall
3	350 (350 nm)	3 290 (264 nm)	> 400	intracellular	intracellular
4	380 (332 nm)	1 490 (287 nm)	> 400	intracellular	intracellular
5	700 (332 nm)	2 550 (289 nm)	> 400	intracellular	intracellular
6	23 110 (310 nm)	12 700 (225 nm)	(29.7 ± 2.3) × 10 ³	cell wall	cell wall
7	8 210 (309 nm)	8 450 (278 nm)	(14.4 ± 0.5) × 10 ³	cell wall	cell wall
8	15 670 (285 nm)	10 930 (221 nm)	(475.6 ± 44.5) × 10 ³	cell wall	cell wall
9	29 850 (323 nm)	22 410 (297 nm)	75.0 ± 16.5	cell wall	cell wall
Rutin	6 920 (359 nm)	8 300 (258 nm)	38.6 ± 0.3	-	-

*wavelengths specified in brackets

Not only did compounds **1-9** prove to be photoprotective UV-screening compounds, further analysis using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay proved their potential as antioxidants (Table 3). Compound **1** demonstrated considerably lower IC₅₀ values (33.5 μM) relative to the strongly-antioxidant standard, rutin (38.6 μM). Additionally, compounds **2** and **9** showed good radical-scavenging activity with IC₅₀ values of 127.8 and 75.0 μM, respectively. Compounds **3-8** showed much weaker antioxidant values with IC₅₀ concentrations in the mM range. Thus, radical-scavenging efficiency in terms of IC₅₀ ranked as follows **1** > rutin > **9** > **2** >> **3-8**.

Biflavone **1** was approximately 13% stronger than rutin, a widely-used positive control for antioxidant assays. Rutin displayed greater radical-scavenging efficiencies compared to that of the commercially available butylated hydroxytoluene,^{47,48} another positive control used in DPPH methods and popular in the food industry. We report here for the first time the testing of

isolate **1** as an antioxidant and the findings show that it could be a strong candidate for further antioxidant applications.

Compounds **1-2** thus combine great potential as radical-scavengers with good UV absorbing capacity particularly over the UV-A wavelengths (315–400 nm). This suggests that these biflavonoids have dual functions in photoprotection: firstly by directly reducing UV radiation transmission to susceptible intracellular components and secondly by quenching UV-induced ROS. Generally, it has been reported that biflavonoids have relatively impressive capacities as antioxidants.⁴⁹ Perhaps **1-2** are constitutively produced and accumulate in the cell to function predominantly as antioxidants but when exposed to more stressful environmental conditions such as high light or UV stresses, these highly abundant intracellular compounds are deposited in the cell wall where their UV-absorbing abilities also offer direct photoprotection. Antarctic moss may sequester more of these compounds in its cell walls in order to prevent loss of these bioactive compounds during the frequent freeze/thaw and desiccation/rehydration events that characterize polar life. In more temperate environments where growth can occur year round, e.g. coastal Australia, an intracellular location for such compounds may be more adaptable.

In contrast to **1** and **2**, the UV spectrum of 4-hydroxycinnamic acid (**6**) confirms that it is much more suited as a UV-B-screening compound^{49, 50} compared to **1** and **2** especially in the outermost layer of the cell as a first defense against harsh UV-B radiation. Similarly, the benzaldehyde **8** showed poor antioxidant activity but good absorption over the UV-B wavelengths, supporting reported studies.⁵¹ Overall, UV photoprotective compounds **1-9** are likely to contribute to the resilient nature of *C. purpureus*.

As the Australian population used was plentiful and steady growing, there was sufficient plant material to allow isolation of these valuable compounds. Therefore, we took advantage of

this abundance to identify and then compare the secondary metabolites that contribute to the resilient nature of this species rather than utilizing the rare and highly conserved Antarctic material. This approach enabled minimal destruction of a precious and protected Antarctic species whilst identifying and quantifying its valuable compounds.

In order to gain understanding of the high UV resilience of *C. purpureus*, we have identified the major UV-active compounds present in both Australian and Antarctic populations of *C. purpureus*. These comprised the five biflavonoids (**1-5**); and phenolics, *p*-coumaric acid, vanillin, *p*-hydroxybenzaldehyde, and ferulic acid. All isolates absorbed in the ultraviolet spectrum and biflavonoids **1-2** showed relatively high antioxidant activities in comparison to the rutin positive control. These properties suggest that these biflavonoids are potentially involved in dual photoprotection mechanisms via both direct (UV-screening) and indirect (radical-scavenging) capacities. These were found as the major intracellular UV-active compounds within the cytoplasm of both Australian and Antarctic *C. purpureus* and also in the cell walls of the latter, substantiating claims that cell wall biflavonoids exist. Differing relative abundances of these compounds were observed between and within populations, which are likely a response to variation in the relative growth climates.

All these compounds from *C. purpureus* were shown to be chemically stable and effective photoprotective compounds. It is suggested that these compounds reduce the amount of UVR damage within this moss species and contribute to its high tolerance to UVR. Whether these compounds are produced in response to, or enhanced by, the exposure of the moss to increasing UVR intensities can now be investigated. If these compounds are UV-induced then they could preserve a record of past UV radiation over the lifetime of this Antarctic moss (e.g. up to 100 years²⁴). This could be achieved by tracing the compounds down the shoots of long living

mosses providing a proxy for past UVR climates in Antarctica and other polar regions. Therefore, these valuable compounds form the basis for larger, more complex future investigations, including their potential for use as paleoproxies for past UV radiation in Antarctica.^{39, 40, 44, 46, 52}

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined using a Buchi M 560 melting point apparatus and are uncorrected. Optical rotations were measured on a Jasco P-2000 polarimeter. UV-visible spectra of samples diluted in MeOH were obtained using a Shimadzu UV-VIS Spectrophotometer UV-1601. ECD Spectra were recorded on a JASCO J-810 spectropolarimeter with pathlength 0.1 cm and concentration between 50-100 μ M in MeOH. IR spectra were recorded with a Shimadzu IR Affinity-1 FT-IR spectrometer fitted with a 1.5 round diamond crystal. ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively on a Varian Inova 500 MHz. Additional 1D and 2D NMR analysis (including gCOSY, gHSQC, gHMBC) was performed using a Bruker Avance III 600 MHz instrument fitted with a 5 mm TCI cryoprobe to confirm the structures of isolates **1-5**. ESIMS spectra were obtained from a Waters LCZ 4000 platform-mounted micromass mass spectrometer. HRESIMS were run on a Waters Q-TOF Ultima mass spectrometer. Low resolution DIEI mass spectrometry was executed on a Shimadzu QP-5050 spectrometer (equipped with a 30 m SGE glass capillary column, temperature range from 0 to 200 °C, helium carrier gas, controlled and analyzed by Shimadzu LabSolutions GC-MS software v1.20). Column chromatography was performed on silica gel 60 (Merck, Germany). Solid phase extraction was undertaken using Waters Oasis® HLB 35 cc extraction cartridges. Analytical HPLC was performed on either a Waters (Waters 1525 pump, Waters 2487 detector, controlled by Breeze software v3.30) with a Symmetry C₁₈ column (5 μ m,

4.9 x 150 mm) or a Shimadzu HPLC system (SOD-M10AVP diode array detector, CTO-20AC column oven, LC-10ATVP pump, SIL-10Ai auto-injector, SC-10AVP system controller, DGU-20ALVP degasser, controlled by Shimadzu Class-VP software v6.12 SP3) with a Wakosil C₁₈ RS column (5 µm, 4.6 x 250 mm). Preparative HPLC was performed on a Waters prep-LC system (LC-600 controller, 2489 detector, LC150 Pump, PD1 degasser) with a Waters reverse-phase OBD SunfireTM C₁₈ column (5 µm, 19 x 150 mm) protected with a Waters SunfireTM C-18 guard column (5 µm, 19 x 10 mm). Absorbances for antioxidant assays were measured on a Molecular Devices SpectraMax 250 microplate reader. All solvents were of HPLC grade (Thermofisher Scientific, Australia). Rutin, vanillin, *p*-coumaric acid and *trans*-ferulic acid standards as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (Sydney, Australia).

Plant Material. Australian populations of *C. purpureus* (Hedw.) Brid. (voucher specimen AustCPMW) were collected from Dapto, New South Wales, Australia (34°29'36.5"S, 150°47'47"E) during May 2015. This fresh moss was thoroughly washed to remove dirt, allowed to air dry and was stored at -20 °C before extraction. Antarctic moss samples were collected in the Windmill Islands region, Antarctica (66°16.9'S, 110°31.5'S) during February 2012 (voucher specimens AntCPMW2012E27-E66) under the Antarctic Treaty (Environment Protection) Act 1980, Permit number ATEP2-12-13-4046 issued by the Commonwealth of Australia, Department of Environment to S. Robinson. Antarctic samples were air dried upon collection and stored at -20 °C prior to transfer to UOW, Australia. Samples were identified either by M. Waterman or J. Williams (School of Biological Sciences, UOW). Compounds from moss gametophytes from the Australian population were extracted and identified before they were compared with those found within Antarctic moss extracts. Voucher specimens of both

populations of *C. purpureus* are stored in the Janet Cosh Herbarium Antarctic Reference Collection at the University of Wollongong.

Extraction and Isolation - Intracellular Sample Preparation of Australian *C. purpureus*. Moss gametophytes (30.5 g dry wt) were ground to a powder under liquid nitrogen in a mortar and pestle. Intracellular compounds were extracted from this powder by stirring in MeOH (0.3 L, in 1.0 L conical flask) for 24 h. The resultant extract was filtered and the residue was extracted ten times. The supernatants were pooled and concentrated *in vacuo* to produce 1.97 g of crude extract. The extract (500 mg) was dissolved in EtOAc-MeOH-MeCN-H₂O (2:2:2:0.5, 6.5 mL). This solution was loaded into a 10 mL syringe filled with silica (to a level of 5 mL) and flushed with EtOAc (10 mL), MeCN (20 mL), MeCN-MeOH-H₂O (9:0.5:0.5, 20 mL) to produce a filtrate (50 mL). This solution was then passed through a HPLC sample filter (0.45 μ m). This procedure was repeated for the remaining extract in applications of 500 mg.

Extraction and Isolation - Isolation of Intracellular Constituents. A gradient elution from 80 to 30% of solvent A (0.1% TFA in H₂O) within 40 min (solvent B, 0.1% TFA in MeCN) was used for the semi-preparative HPLC separation of the prepared intracellular sample. Compounds **1-5** were collected at retention times of 23.50, 24.35, 22.10, 26.80, and 27.30 min, respectively, and were vacuum dried to give pale yellow solids.

Compound 1: (*M*)-8-(5-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)-2,3-dihydroxyphenyl)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one or luteolin-(5'→8'')-luteolin.

A UV-active, pale yellow solid (76.7 mg, 3.1 mg.g⁻¹ dry wt); m.p.: decomposed at 295 °C; $[\alpha]_D^{20}$: -3.8 (c 0.004, MeOH); $\lambda_{\max}$ 255 (14 158) 350 (13 358); ECD (MeOH) λ 220 ($\Delta\epsilon$ +67.8), 261 ($\Delta\epsilon$ +53.9), 331 ($\Delta\epsilon$ -49.9), 362 ($\Delta\epsilon$ +24.6) nm; IR [cm⁻¹]: 3213 (m), 1653 (s), 1430 (s), 1339 (s), 1253 (s), 1165 (s), 836 (s); For ¹H NMR (acetone-*d*₆, 500 MHz) and ¹³C NMR (acetone-*d*₆, 125

MHz) spectroscopic data see Table S1; ESIMS⁺, m/z 571 [M+H]⁺ (100); ESIMS⁻ 569 [M-H]⁻ (50). HRESIMS: calculated for C₃₀H₁₇O₁₂ [M-H]⁻: 569.0720, found 569.0732.

Compound 2: (±)-8-(5-(5,7-dihydroxy-4-oxochroman-2-yl)-2,3-dihydroxyphenyl)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one or eriodictyol-(5'→8'')-luteolin.

A UV-active, pale yellow solid (21.0 mg, 0.8 mg. g⁻¹ dry wt); m.p.: decomposed at 295 °C; [α]_D²⁰: 0 (c 0.150 MeOH); λ_{max} 211 (10 047), 274 (5208), 287 (5000), 348 (4320); IR [cm⁻¹]: 3330 (m), 2956 (s), 1653 (s), 1194 (s), 1189 (s), 1128 (s), 800 (s), 725 (s); For ¹H NMR (acetone-*d*₆, 500 MHz) and ¹³C NMR (acetone-*d*₆, 125 MHz), spectroscopic data see Table S1; ESIMS⁺, m/z 573 [M+H]⁺ (100). HRESIMS: calculated for C₃₀H₂₁O₁₂ [M+H]⁺: 573.1033 found 573.1044.

Extraction and Isolation - Cell Wall Sample Preparation of Australian *C. purpureus*.

Cell wall-bound compounds were extracted using a method adapted from Schnitzler *et al.*³⁶ Separate quantities (24 x ~1.5–1.7 g dry wt) of moss residue after MeOH extraction were sequentially incubated at room temperature (22 °C) twice in NaCl solution (1 M, 15 mL) for 15 min, then in MeOH (10 mL), in MeOH-CHCl₃ (1:1, 15 mL) twice for 1 h, before being washed with MeOH (10 mL). After each incubation, tubes were centrifuged at 3,600 g for 5 min before discarding supernatants. Moss pellets were allowed to air dry before overnight hydrolysis (16 h) was performed at room temperature in the absence of light using NaOH (1 M, 50 mL:1 g dry wt). This alkali extract was filtered and acidified to pH 1 with conc. HCl and then re-filtered. Initial clean-up of the obtained filtrate was performed using solid phase extraction.

Separate loadings (40 mL) of filtered cell wall extract (pH 1) were passed through solid phase extraction cartridges after columns were activated using 100% MeOH (20 mL) and equilibrated with Milli Q water (100 mL). Loaded columns were first washed using Milli Q

water (80 mL) and 5% aq. MeOH (40 mL) before elution using 100% MeOH (5 x 10 mL) with fractions (F1-10; 3 mL each) collected. Fractions 5-9 (F5-9) showed similar analytical HPLC profiles and thus were pooled (147.2 mg).

Extraction and Isolation - Isolation of Cell-Wall Constituents. Cell wall constituents within F5-9 were separated and purified by seven applications (7 x 20.5 mg) of semi-preparative HPLC using a linear gradient from 75 to 55% of solvent A (0.1% formic acid (FA) in H₂O) within 55 min (solvent B, 0.1% FA in MeOH) to obtain *p*-coumaric acid (**6**) (13.7 mg), vanillin (**7**) (1.1 mg), *p*-hydroxybenzaldehyde (**8**) (3.3 mg impure), and ferulic acid (**9**) (0.4 mg impure) collected at retention times of 38, 26, 19, and 40 min, respectively. These isolates were characterized by ¹H NMR, ¹³C NMR, gCOSY, and gHMBC spectroscopic analyses and/or EIDI mass spectrometry then compared with commercial standards.

Comparison of Extracts between Australian and Antarctic Populations. Antarctic *C. purpureus* moss samples (10–20 mg dry wt) were extracted using a scaled down method of that used for extracting Australian moss. This involved grinding moss material in microcentrifuge tubes (1.5 mL) each with a 3 mm tungsten carbide bead and using a TissueLyser (Qiagen, Australia) at 30 Hz for 2 min; 1% HCl in MeOH (1.5 mL) was used for intracellular extraction for 3 h on ice (vortexed every 30 min) before being centrifuged and then re-extracted (MeOH; 2 x 1.5 mL). The supernatants (intracellular extracts) were collected and stored at -20 °C before analysis. The remaining moss pellets were sequentially re-suspended using reagents (1.5 mL), air-dried and extracted in NaOH (1.0 mL) as for the Australian moss samples. Samples were centrifuged at 16 000 g for 5 min and the supernatant (cell wall extract) was acidified to pH 1 with conc. HCl. The liquid supernatant was passed through a solid phase extraction cartridge, as previously described.

The presence of compounds **1-9** within Antarctic intracellular (MeOH) and cell wall (NaOH) extracts was determined *via* analytical HPLC. Original and spiked samples of Antarctic MeOH extract were separated using the Waters HPLC system at a flow rate of 1.0 mL.min⁻¹ and linear gradient from 80 to 30% of solvent A (0.1% TFA in H₂O) within 40 min (solvent B, 0.1% TFA in MeCN). Comparison of cell wall constituents was performed using the Shimadzu HPLC system with a 0.7 mL.min⁻¹ flow rate and linear gradient from 80 to 5% solvent A (0.1% TFA in H₂O) within 45 min (solvent B, 0.1% TFA in MeOH).

DPPH Free Radical-Scavenging Assays. The ability of the isolated compounds to scavenge free radicals was determined using a DPPH microplate assay adapted from a range of published methods (reviewed in Brand-Williams et al.⁵³). Sample solutions (50 µL) were loaded into a 96-well plate (in replicates of four) and serially diluted. A separate set of wells were loaded with 50 µL of either MeOH or the model flavonoid, rutin (820 µM) for the negative and positive controls, respectively. To the first three rows of sample solution, DPPH (200 µM, 100 µL) was added and thoroughly mixed, allowing the last row to be used to correct for background absorbance (Abs blank). Plates were incubated for 30 min in the absence of light before the absorbance at 517 nm was measured using a microplate reader. Lower absorbance signifies higher scavenging activities. Free radical-scavenging activities were determined using Equation 1. These percentage activities were graphed against logarithmic sample concentrations and the sample concentrations that sequestered 50% of the DPPH free radicals (i.e. loss of purple color) were interpolated and are presented as IC₅₀ mean ± s.d. values. All antioxidant activity data were tested for significant differences using ANOVA with *post hoc* comparisons performed using Tukey-Kramer HSD tests ($\alpha = 0.05$).

Equation 1: $\text{DPPH}^\bullet \text{ scavenging activity (\%)} = 100 - \left[\frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{neg. control}}} \right] \times 100$

Calculations. Please see Supporting Information for experimental details describing the calculations of the bond rotation restrictions.

ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C 1D and 2D NMR spectra, HRESIMS, FTIR, and HPLC traces for **1-5**. UV extinction coefficients of **1-9** for the 200–500 nm range and details for the DPPH antioxidant assay and computational methods. This material is available free of charge via the internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Email: keller@uow.edu.au Tel: +61 2 4221 4692. Fax: +61 2 4221 4287

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Notes

The authors declare no competing financial interest

ACKNOWLEDGMENT

We thank Joy Williams for formally identifying moss samples, Wilford Lie for his NMR assistance and Andrew Netherwood for graphics production. Funding support was provided by Australian Research Council Discovery Project (DP110101714) and Australian Antarctic

Division (ASG3042 and 4046) grants. Both sources had no involvement in the design of this project. MJ Waterman and AS Nugraha thank the University of Wollongong for an Australian Postgraduate Award and a University Postgraduate Award respectively, and R Hendra thanks the Indonesian Directorate General Higher Education (DGHE) and UOW International Postgraduate Tuition Fee Award (2016) for Scholarships.

REFERENCES

- (1) Robinson, S. A.; Wasley, J.; Tobin, A. K., *Global Change Biol.* **2003**, *9*, 1681-1717.
- (2) Robinson, S. A.; Erickson, D. J., *Global Change Biol.* **2015**, *21*, 515-527.
- (3) Clarke, L. J.; Robinson, S. A., *New Phytol.* **2008**, *179*, 776–783.
- (4) Post, A., *Polar Biol.* **1990**, *10*, 241–245.
- (5) Turnbull, J. D.; Leslie, S. J.; Robinson, S. A., *Funct. Plant Biol.* **2009**, *36*, 214–221.
- (6) Turnbull, J. D.; Robinson, S. A., *Global Change Biol.* **2009**, *15*, 319-329.
- (7) Agati, G.; Azzarello, E.; Pollastri, S.; Tattini, M., *Plant Sci.* **2012**, *196*, 67-76.
- (8) Agati, G.; Tattini, M., *New Phytol.* **2010**, *186*, 786-793.
- (9) Rice-Evans, C.; Miller, N.; Paganga, G., *Trends Plant Sci.* **1997**, *2*, 152-159.
- (10) Robinson, S. A.; Waterman, M. J., Sunsafe bryophytes: Photoprotection from Excess and Damaging Solar Radiation. In *Photosynthesis in Bryophytes and Early Land Plants*, Hanson, D. T.; Rice, S. K., Eds. Springer Netherlands: 2014; Vol. 37, pp 113-130.
- (11) Yang, Y.-N.; Zhu, H.; Chen, Z.; Liu, F.; An, Y.-W.; Feng, Z.-M.; Jiang, J.-S.; Zhang, P.-C., *J. Nat. Prod.* **2015**, *78*, 705-711.
- (12) Anhut, S.; Zinsmeister, H. D.; Mues, R.; Barz, W.; Mackenbrock, K.; Koster, J.; Markham, K. R., *Phytochemistry* **1984**, *23*, 1073–1075.

- (13) Seeger, T.; Geiger, H.; Mues, R.; Zinsmeister, H. D., *Z. Naturforsch., C: Biosci.* **1993**, *48*, 529-30.
- (14) Seeger, T.; Zinsmeister, H. D.; Geiger, H., *Z. Naturforsch., C: Biosci.* **1990**, *45*, 583-6.
- (15) Li, H.-Y.; Nehira, T.; Hagiwara, M.; Harada, N., *J. Org. Chem.* **1997**, *62*, 7222-7227.
- (16) Bringmann, G.; Gunther, C.; Ochse, M.; Schupp, O.; Tasler, S., Stereoselective Total Synthesis of Axially Chiral Products. In *Progress in the Chemistry of Organic Natural Products*, Herz, W.; Falk, H.; Kirby, G. W.; Moore, R. E., Eds. Springer: Vienna, 2001; Vol. 82, pp 78-88.
- (17) Anhut, S.; Seeger, T.; Zinsmeister, H.; Geiger, H., *Z. Naturforsch. Sect. C* **1989**, *44*, 189-192.
- (18) Covington, C. L.; Junior, F. M.; Silva, J. H.; Kuster, R. M.; de Amorim, M. B.; Polavarapu, P. L., *J. Nat. Prod.* **2016**, *79*, 2530-2537.
- (19) Masson, E., *Org. Biomol. Chem.* **2013**, *11*, 2859-2871.
- (20) Clarke, L. J.; Ayre, D. J.; Robinson, S. A., *J. Ecol.* **2008**, *96*, 378-385.
- (21) Wyber, R. A. Phylogeny of Three East Antarctic Mosses. Thesis, University of Wollongong, 2013.
- (22) Markham, K. R.; Ryan, K. G.; Gould, K. S.; Rickards, G. K., *Phytochemistry* **2000**, *54*, 681-687.
- (23) Bureau of Meteorology (BOM) Climate Statistics for Australian Locations.
<http://www.bom.gov.au/climate/data/> (accessed 15th December).
- (24) Clarke, L. J.; Robinson, S. A.; Hua, Q.; Ayre, D. J.; Fink, D., *Global Change Biol.* **2012**, *18*, 301-310.
- (25) Rozema, J.; Boelen, P.; Blokker, P., *Environ. Pollut.* **2005**, *137*, 428-442.
- (26) Brinkmeier, E.; Geiger, H.; Zinsmeister, H. D., *Phytochemistry* **1999**, *52*, 297-302.

- (27) Geiger, H.; Markham, K., *Phytochemistry* **1992**, *31*, 4325–4328.
- (28) Rampendahl, C.; Seeger, T.; Geiger, H.; Zinsmeister, H. D., *Phytochemistry* **1996**, *41*, 1621-1624.
- (29) Taipale, T.; Huttunen, S., *Polar Rec.* **2002**, *38*, 211-218.
- (30) Markham, K. R., Bryophyte Flavonoids, Their Structures, Distribution, and Evolutionary Significance. In *Bryophytes: Their Chemistry and Chemical Taxonomy*, Zinsmeister, H. D.; Mues, R., Eds. Oxford University Press: Oxford, 1990; pp 143-159.
- (31) Geiger, H., Biflavonoids in Bryophytes. In *Bryophytes: Their Chemistry and Chemical Taxonomy*, Zinsmeister, H. D.; Mues, R., Eds. Oxford University Press: Oxford, 1990; pp 161-170.
- (32) Zinsmeister, H. D.; Becker, H.; Eicher, T., *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 130-147.
- (33) Asakawa, Y., Chemical Constituents of the Bryophytes. In *Progress in the Chemistry of Organic Natural Products*, Springer Vienna: 1995; Vol. 65, pp 1-562.
- (34) Davidson, A. J.; Harborne, J. B.; Longton, R. E., *J. Hattori Bot. Lab.* **1989**, *67*, 413–422.
- (35) Harris, P. J.; Hartley, R. D., *Nature* **1976**, *259*, 508-510.
- (36) Schnitzler, J.-P.; Jungblut, T. P.; Heller, W.; Kofferlein, M.; Hutzler, P.; Heinzmann, U.; Schmelzer, E.; Ernst, D.; Langebartels, C.; Sandermann, H., *New Phytol.* **1996**, *132*, 247-258.
- (37) Strack, D.; Heilemann, J.; Mömken, M.; Wray, V., *Phytochemistry* **1988**, *27*, 3517-3521.
- (38) Gorgham, J., Phenolic Compounds, other than Flavonoids, from Bryophytes. In *Bryophytes: Their Chemistry and Chemical Taxonomy*, Zinsmeister, H. D.; Mues, R., Eds. Oxford University Press: Oxford, 1990; pp 171-200.
- (39) Blokker, P.; Boelen, P.; Broekman, R.; Rozema, J., *Plant Ecol.* **2006**, *182*, 197–207.

- (40) Lomax, B. H.; Fraser, W. T.; Sephton, M. A.; Callaghan, T. V.; Self, S.; Harfoot, M.; Pyle, J. A.; Wellman, C. H.; Beerling, D. J., *Nat. Geosci.* **2008**, *1*, 592-596.
- (41) Rozema, J.; Broekman, R. A.; Blokker, P.; Meijkamp, B. B.; de Bakker, N.; van de Staaij, J.; van Beem, A.; Arieze, F.; Kars, S. M., *J. Photochem. Photobiol. B: Biol.* **2001**, *62*, 108-117.
- (42) Mathew, S.; Abraham, T. E., *Crit. Rev. Biotechnol.* **2004**, *24*, 59-83.
- (43) Ruhland, C. T.; Day, T. A., *Physiol. Plant.* **2000**, *109*, 244-251.
- (44) Lomax, B. H.; Fraser, W. T.; Harrington, G.; Blackmore, S.; Sephton, M. A.; Harris, N. B. W., *Earth. Planet. Sci. Lett.* **2012**, *353–354*, 22-28.
- (45) Otero, S.; Nunez-Olivera, E.; Martinez-Abaigar, J.; Tomas, R.; Huttunen, S., *Environ. Pollut.* **2009**, *157*, 2335–2344.
- (46) Willis, K. J.; Feurdean, A.; Birks, H. J. B.; Bjune, A. E.; Breman, E.; Broekman, R.; Grytnes, J. A.; New, M.; Singarayer, J. S.; Rozema, J., *New Phytol.* **2011**, *192*, 553-560.
- (47) Lue, B.-M.; Nielsen, N. S.; Jacobsen, C.; Hellgren, L.; Guo, Z.; Xu, X., *Food Chem.* **2010**, *123*, 221-230.
- (48) Yang, J.; Guo, J.; Yuan, J., *LWT-Food Sci. Technol.* **2008**, *41*, 1060-1066.
- (49) Harborne, J.; Williams, C., *Phytochemistry* **2000**, *55*, 481–504.
- (50) Agati, G.; Stefano, G.; Biricolti, S.; Tattini, M., *Ann. Bot.* **2009**, *104*, 853-861.
- (51) Wang, W.; Guo, J.; Zhang, J.; Peng, J.; Liu, T.; Xin, Z., *Food Chem.* **2015**, *171*, 40-49.
- (52) Magri, D., *New Phytol.* **2011**, *192*, 310-312.
- (53) Brand-Williams, W.; Cuvelier, M. E.; Berset, C., *LWT-Food Sci. Technol.* **1995**, *28*, 25-30.

Table of Contents Graphic and Synopsis

Antarctic Moss Biflavonoids Show High Antioxidant and Ultraviolet Screening Activity

Melinda J. Waterman,[†] Ari S. Nugraha,[‡] Rudi Hendra,[‡] Graham E. Ball,[§] Sharon A. Robinson,[†]
and Paul A. Keller^{‡,*}

Five biflavonoids isolated from Antarctic moss (*Ceratodon purpureus*), two of which revealed high ultraviolet absorbance and antioxidant capabilities likely to contribute to its survival in the harsh Antarctic climate (image by Andrew Netherwood).

Supporting Information for the **Antarctic Moss Biflavonoids Show High Antioxidant and Ultraviolet Screening Activity**

Melinda J. Waterman[†], Ari S. Nugraha[‡], Rudi Hendra[‡], Graham E. Ball[§], Sharon A. Robinson[†],
Paul A. Keller^{‡*}

[†]School of Biological Sciences, University of Wollongong, Wollongong, NSW, 2522, Australia

[‡]School of Chemistry, University of Wollongong, Wollongong, NSW, 2522, Australia

[§]School of Chemistry, UNSW, NSW, 2052, Australia

Telephone: +61 2 4221 4692

Fax: +61 2 4221 4287

Email: keller@uow.edu.au

Table of contents	S2
Table S1. ^1H and ^{13}C NMR spectroscopic data for compounds 1 and 2 (in $(\text{CD}_3)_2\text{CO}$)	S3
Fig S1. ^1H -NMR spectrum of biflavone 1	S4
Fig S2. ^{13}C APT-NMR spectrum of biflavone 1	S5
Fig S3. ^{13}C APT-NMR spectrum of biflavone 1 (in absolute mode)	S6
Fig S4. gCOSY spectrum of biflavone 1	S7
Fig S5. gHSQC spectrum of biflavone 1	S8
Fig S6. gHMBC spectrum of biflavone 1	S9
Fig S7. gHMBC, annotated with APT ^{13}C spectrum of biflavone 1 (zoom)	S10
Fig S8. gHMBC spectrum (zoom) for H-C correlation in ring B and C of biflavone 1	S11
Fig S9. ^1H -NMR spectrum of biflavone 1 ($d_6\text{DMSO}$)	S12
Fig S10. ^1H -NMR spectrum of dihydrobiflavone 2	S13
Fig S11. ^{13}C -NMR spectrum of dihydrobiflavone 2	S14
Fig S12. gCOSY spectrum of dihydrobiflavone 2	S15
Fig S13. gHSQC spectrum of dihydrobiflavone 2	S15
Fig S14. gHMBC spectrum of dihydrobiflavone 2	S16
Fig S15. gHMBC spectrum (zoom) for H-C correlation in ring B and D of dihydrobiflavone 2	S17
Table S2. ^1H -NMR spectrum of dihydrobiflavone 3	S18
Table S3. ^1H -NMR spectrum of dihydrobiflavone 5	S19
Fig S16. HRESIMS biflavone 1	S20
Fig S17. HRESIMS dihydrobiflavone 2	S21
Fig S18. & Fig S19. FTIR spectrum of biflavones 1 and dihydrobiflavone 2	S22
Fig S20. CD Spectroscopy of compounds 1 and 2	S23
Fig S21. & Fig S22. HPLC of Antarctic and Australian <i>C. purpureus</i> comparison (intracellular extracts)	S23
Fig S23. & Fig S24. HPLC of Antarctic and Australian <i>C. purpureus</i> comparison (cell wall extracts)	S24
Fig S25. UV extinction coefficients of 1-9	S25
Fig S26. DPPH antioxidant assays of 1-2 and 6-9	S26
Computational methods of 10	S27
Table S4: B97-D3(BJ) DFT calculations summary	S28
Table S5: TPSS-D3 DFT calculations summary	S29
Coordinates of selected optimized Geometries (TPSS-D3/def2-TZVPP)	S30
References	S31

Table S1. ^1H and ^{13}C NMR Spectroscopic Data for Compounds **1** and **2** (in acetone- d_6).

Table S1. ¹ H and ¹³ C NMR Spectroscopic Data for Compounds 1 and 2 (in acetone-d ₆).				
1		2		
	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}
2		165.3	5.46, dd (13.2, 2.9)	80.4
3	6.66, s	104.6	3.23/3.27, dd (17.2, 13.2) 2.78, dd (17.2, 2.9)	43.8, 43.9
4		183.1		197.3/197.4
5	OH – 13.02, s	163.1	OH - 12.17/12.18, s	165.1
6	6.24, d (2.1)	99.7	5.91/5.92, br d (2.2)	96.8
7		164.9		167.6/167.7
8	6.52, d (2.1)	94.9	6.06/6.11, br d (2.2)	96.1
8a		159.0		164.5/164.6
4a		105.4		103.1/103.2
1'		123.3		131.1/131.2
2'	7.62, d (2.3)	113.8	7.15/7.17, d (2.2)	114.7/114.3
3'		147.0		146.5/146.6
4'		148.9		145.1
5'		120.7		119.8/119.9
6'	7.64, d (2.3)	123.7	7.04/7.06, d (2.2)	123.2/122.9
2''		165.5		165.3/165.4
3''	6.59, s	104.1	6.58/6.59, s	103.9/103.8
4''		183.5		183.5
5''	OH – 13.21, s	162.5	OH – 13.17, s	162.3
6''	6.46, s	99.8	6.42, s	99.7
7''		162.5		162.4
8''		104.7		105.3
8a''		156.2		156.1
4a''		105.6	-	105.6
1'''		123.9	-	123.7/123.8
2'''	7.28, d (2.2)	114.5	7.29, d (2.2)	114.4/114.5
3'''		146.4	-	146.4/146.5
4'''		150.1	-	150.2
5'''	6.81, d (8.4)	116.5	6.91/6.92, d (8.4)	116.5
6'''	7.19, dd (2.2, 8.4)	120.2	7.17/7.18, dd (2.2, 8.4)	120.2/120.3

Note: Two diastereomers are apparent in the spectra of compound **2**, presumably due to one chiral centre being present at C2 combined with atropisomerism associated with restricted rotation around the C5'-C8'' axis.^{S1} Where separate resonances have been resolved, both chemical shifts are given in the table.

Figure S1. ^1H -NMR spectrum of biflavone (*M*)-5',3'''-dihydroxyamentoflavone (**1**) (500 MHz, acetone- d_6).

Figure S2. ¹³APT-NMR spectrum of biflavone (*M*)-5',3'''-dihydroxyamentoflavone **1** (500 MHz, acetone-*d*₆).

Figure S3. ¹³APT-NMR spectrum of biflavone (*M*)-5',3'''-dihydroxyamentoflavone **1** (in absolute mode, 500 MHz, acetone-*d*₆).

Figure S4. gDQF-COSY spectrum of biflavone (*M*)-5',3'''-dihydroxyamentoflavone **1** (600 MHz, acetone-*d*₆).

Figure S6. gHMBC, annotated with APT ^{13}C spectrum of biflavone (*M*)-5',3''-dihydroxyamentoflavone **1** (600 MHz, acetone- d_6).

Figure S7. gHMBC, annotated with APT ^{13}C spectrum of biflavone (*M*)-5',3''-dihydroxyamentoflavone 1 (zoom) (600 MHz, acetone- d_6).

Figure S8. gHMBC spectrum (zoom) for H-C correlation in ring B and D of biflavone (*M*)-5',3'''-dihydroxyamentoflavone **1** (600 MHz, acetone-*d*₆).

Figure S9. ^1H -NMR spectrum of biflavone (*M*)-5',3'''-dihydroxyamentoflavone **1** (500 MHz, $\text{DMSO}-d_6$).

Figure S10. ^1H -NMR spectrum of ($\pm$)-2,3-dihydro-5',3'''-dihydroxyamentoflavone **2** (500 MHz, acetone- d_6).

Figure S11. ^{13}C -NMR spectrum of $(\pm)$ -2,3-dihydro-5',3'''-dihydroxyamentoflavone **2** (500 MHz, acetone- d_6).

acetone- d_6). acetone- d_6).

Figure S14. gHMBC, annotated with ^{13}C spectrum of $(\pm)$ -2,3-dihydro-5',3''-dihydroxyamentoflavone **2** (600 MHz, acetone- d_6).

Figure S15. gHMBC spectrum (zoom) for specific H-C correlation in ring B and D of (±)-2,3-dihydro-5',3''-dihydroxyamentoflavone **2** (600 MHz, acetone-*d*₆).

Table S2. ^1H and ^{13}C NMR data for compound **3** (600 MHz, $\text{DMSO}-d_6$).
LRESIMS: 569 $[\text{M}-\text{H}]^+$

Position	DMSO- d_6	
	Proton	Carbon
2		
3	6.63	102.5
4		
5		
6	6.18	98.8
7		
8	6.44	93.9
8a		
4a		
1'		
2'	7.46	122.3
3'		
4'		
5'		
6'	7.49	112.1
2''		
3''	6.67	102.9
4''		
5''		
6''	6.40	98.06
7''		
8''		
8a''		
4a''		
1'''		
2'''	7.07, d (2.0)	113.8
3'''		
4'''		
5'''	6.67, d (8.0)	115.6
6'''	7.02, dd (8.0; 2.0)	118.6

Table S3. ^1H and ^{13}C NMR data of **5** (600 MHz, DMSO- d_6).
LRESIMS: 539 $[\text{M-H}]^+$

Position	Acetone- d_6	
	Proton	Carbon
2		165.1
3	6.63	110.8
4		
5	12.76 (OH)	166.6
6	6.27	100.0
7		
8	6.40	94.9
8a		
4a		105.5
1'		
2'		
3'		
4'		
5'		
6'		
2''	5.51	80.1
3''	2.78	
	3.23	
4''		
5''	12.60 (OH)	163.1
6''		105.5
7''		
8''	6.15	95.9
8a''		
4a''		103.3
1'''		130.8
2'''	7.42	129.2
3'''	6.91	116.3
4'''	8.60 (OH)	158.9
5'''	6.91	116.3
6'''	7.42	129.2

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

869 formula(e) evaluated with 22 results within limits (up to 20 best isotopic matches for each mass)

Elements Used:

C: 0-50 H: 0-60 N: 0-12 O: 0-12

CPMW2-34

PK_Ar_CPMW2_34 66 (1.321) AM2 (Ar,10000.0,0.00,1.00); ABS; Cm (65:67)

1: TOF MS ES+
3.11e+003

Minimum:				-1.5				
Maximum:		5.0	20.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula	
573.1044	573.1006	3.8	6.6	21.5	100.6	0.7	C26 H17 N6 O10	
	573.1033	1.1	1.9	20.5	101.6	1.8	C30 H21 O12	✓
	573.1020	2.4	4.2	26.5	101.8	2.0	C27 H13 N10 O6	
	573.1118	-7.4	-12.9	21.5	102.3	2.4	C25 H17 N8 O9	
	573.1145	-10.1	-17.6	20.5	103.4	3.5	C29 H21 N2 O11	
	573.1046	-0.2	-0.3	25.5	103.4	3.6	C31 H17 N4 O8	
	573.1132	-8.8	-15.4	26.5	103.9	4.0	C26 H13 N12 O5	
	573.0979	6.5	11.3	22.5	104.0	4.2	C22 H13 N12 O8	
	573.1060	-1.6	-2.8	30.5	104.9	5.0	C32 H13 N8 O4	
	573.1078	-3.4	-5.9	17.5	104.9	5.0	C20 H17 N10 O11	
	573.0966	7.8	13.6	17.5	105.6	5.7	C21 H17 N8 O12	
	573.1073	-2.9	-5.1	35.5	106.0	6.2	C33 H9 N12	
	573.0934	11.0	19.2	25.5	106.4	6.5	C32 H17 N2 O9	
	573.1087	-4.3	-7.5	29.5	106.8	6.9	C36 H17 N2 O6	
	573.0947	9.7	16.9	30.5	107.2	7.3	C33 H13 N6 O5	
	573.0974	7.0	12.2	29.5	107.6	7.7	C37 H17 O7	
	573.0961	8.3	14.5	35.5	107.7	7.8	C34 H9 N10 O	
	573.1100	-5.6	-9.8	34.5	107.7	7.9	C37 H13 N6 O2	
	573.0988	5.6	9.8	34.5	107.7	7.9	C38 H13 N4 O3	
	573.1028	1.6	2.8	38.5	108.2	8.3	C43 H13 N2 O	

Figure S16. HRESIMS spectrum of biflavone 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

1370 formula(e) evaluated with 15 results within limits (up to 25 closest results for each mass)

Elements Used:

C: 5-60 H: 0-70 N: 0-18 O: 0-12

CP-mw-40

PK_Ari_CP_mw_40 ESI 52 (1.042) AM2 (Ar,10000.0,0.00,1.00); ABS; Cm (51:56)

1: TOF MS ES-
3.71e+003

Minimum:				-1.5				
Maximum:		5.0	10.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula	
569.0732	569.0733	-0.1	-0.2	27.5	80.4	2.2	C31	H13 N4 O8
	569.0738	-0.6	-1.1	20.5	85.1	7.0	C16	H9 N16 O9
	569.0720	1.2	2.1	22.5	80.7	2.6	C30	H17 O12 ✓
	569.0720	1.2	2.1	33.5	82.2	4.1	C28	H5 N14 O2
	569.0747	-1.5	-2.6	32.5	80.3	2.2	C32	H9 N8 O4
	569.0715	1.7	3.0	40.5	79.7	1.6	C43	H9 N2 O
	569.0707	2.5	4.4	28.5	82.6	4.5	C27	H9 N10 O6
	569.0760	-2.8	-4.9	37.5	80.7	2.6	C33	H5 N12
	569.0765	-3.3	-5.8	19.5	83.9	5.8	C20	H13 N10 O11
	569.0698	3.4	6.0	16.5	87.0	8.9	C11	H9 N18 O11
	569.0693	3.9	6.9	23.5	83.1	5.0	C26	H13 N6 O10
	569.0774	-4.2	-7.4	31.5	79.4	1.3	C36	H13 N2 O6
	569.0779	-4.7	-8.3	24.5	84.1	6.0	C21	H9 N14 O7
	569.0680	5.2	9.1	29.5	84.7	6.6	C23	H5 N16 O4
	569.0787	-5.5	-9.7	36.5	80.3	2.2	C37	H9 N6 O2

Figure S17. HRESIMS spectrum of dihydrobiflavone 2

Figure S18. FT-IR spectrum of biflavone 1

Figure S19. FT-IR spectrum of dihydrobiflavone 2

Figure S20. CD Spectroscopy of compounds **1** (black) and **2** (blue).

Figure S21. HPLC profile of methanol extract of *C. purpureus*. Peaks were recorded at 254 nm.

Figure S22. The comparison of intracellular extracts of Australian and Antarctic populations of *C. purpureus* using HPLC.

Figure S23. HPLC profile of the alkali (cell wall) extract of Australian *C. purpureus*. Extract has undergone clean-up using solid phase extraction and HPLC method has been optimised to improve peak resolution and differs from Figures S21-22. Peaks were recorded at 280 nm.

Figure S24. The comparison of cell wall extracts of Australian and Antarctic populations of *C. purpureus* using HPLC. **A)** top panel represents the extract from Figure S23 and bottom panel represents a cell wall extract of an Antarctic sample after undergoing one clean-up application using solid phase extraction. Identities of the peaks associated with isolates **6-9** were confirmed with spiking experiments. **B)** Crude original and spiked HPLC profiles of a cell wall extract of Antarctic *C. purpureus*. These extracts were separated using a different HPLC method to that used in **A)**. Spiked experiments were conducted separately by adding relative concentrations of isolate **1** or **2** to the original sample.

Figure S25. Extinction coefficients in the UV-Vis spectrum (200–500 nm) of **1-9** in comparison to the rutin standard. All samples were diluted in MeOH.

Figure S26. DPPH antioxidant scavenging activities of isolates 1-2, 6-9 and a standard (rutin). Samples were run in triplicate and absorbances measured at 517 nm after 30 min with DPPH. Error bars represent s.d. Isolates 3-5 had low antioxidant activity (< 10%) in the μM range (results not shown) but assays in the mM range were not undertaken due to their low isolated yields. Note that the substrate concentrations differ between 1-2, 9/rutin and 6-8.

Computational methods of **10**

DFT calculations were performed using the Gaussian 09 software package,^{S2} revision D01 with the B97-D3(BJ)^{S3} and TPSS-D3^{S4} (exchange and correlation) functionals, which both employ empirical dispersion corrections,^{S5} the former with Becke-Johnson damping.^{S6}

Four initial geometries of model compound **10** were originally explored, in which the direction of the hydroxyl groups was varied. The starting geometries were subjected to relaxed potential energy surface scans (B97-D3(BJ)/def2-SV(P)), wherein the two halves of the biaryl link were rotated with respect to each other through 360° degrees in 5° steps in order to identify possible low energy minima and low energy transition states. Four minimum energy geometries (**min1** - **min4**) and four transition structures (**ts1** - **ts4**) were selected then optimized at the B97-D3(BJ)/def2-TZVPP level of theory in vacuum. All minimum energy structures were calculated without any symmetry constraints and were confirmed to be minima by calculating their normal vibrations within the harmonic approximation and observing that there were no imaginary frequencies. Transition structures were likewise confirmed to have one imaginary frequency and the validity of the key transition structures involved in rotation (**ts1** and **ts2**) was confirmed using IRC calculations. Zero point vibrational energies were unscaled. Free energies are calculated at 298.15 K. Free energies of solvation were calculated from the difference in energies of single point B3LYP/6-31G(d) calculations in SMD acetone solvent^{S7} and in vacuum using the B97-D3(BJ)/def2-TZVPP vacuum geometries.

The three lowest energy minimum energy structures and two lowest energy transition structures were also reoptimized at the TPSS-D3/def2-TZVPP level in vacuum. In this case, free energies of solvation were calculated from the difference in energies of single point TPSS-D3/def2-TZVPP calculations in IEFPCM acetone solvent and in vacuum using the TPSS-D3/def2-TZVPP vacuum geometries.

Table S4: B97-D3(BJ) DFT calculations summary

Species	Electronic Energy B97-D3(BJ)/def2- TZVPP (Hartree)	Zero point vibrational energy ZPVE (kcal mol ⁻¹)	Enthalpy correction, H (kcal mol ⁻¹)	Entropy correction, S (cal K ⁻¹ mol ⁻¹)	Single Point Electronic Energy B3LYP/6-31G(d) SMD acetone solvent (Hartree)	Single Point Electronic Energy B3LYP/6-31G(d) vacuum (Hartree)	ΔG solvation (kcal mol ⁻¹)	Relative free energy, vacuum (kcal mol ⁻¹)	Relative free energy, acetone (kcal mol ⁻¹)
min1	-1028.756138	138.74	11.20	132.20	- 1028.974816	-1028.950529	-15.24	0.00	0.00
min2	-1028.750064	138.72	11.08	129.85	- 1028.975273	-1028.948290	-16.93	4.37	2.68
min3	-1028.749877	138.59	11.21	130.49	- 1028.974597	-1028.947834	-16.79	4.30	2.75
min4	-1028.751818	138.50	11.35	133.68	- 1028.974237	-1028.947102	-17.03	2.18	0.39
ts1	-1028.723172	138.25	10.69	126.79	- 1028.948143	-1028.921623	-16.64	21.30	19.90
ts2	-1028.721056	138.61	10.45	124.39	-1028.94662	-1028.918930	-17.38	23.47	21.33
ts3	-1028.717082	138.64	10.53	125.46	- 1028.941414	-1028.914035	-17.18	25.75	23.81
ts4	-1028.718167	138.80	10.39	123.46	- 1028.938316	-1028.913720	-15.43	25.68	25.49

min1

min2

min3

min4

ts1

ts2

ts3

ts4

Table S5: TPSS-D3 DFT calculations summary

Species	Electronic Energy TPSS-D3/def2- TZVPP (Hartree)	Zero point vibrational energy ZPVE (kcal mol ⁻¹)	Enthalpy correction, H (kcal mol ⁻¹)	Entropy correction, S (cal K ⁻¹ mol ⁻¹)	Single Point Electronic Energy TPSS-D3/def2- TZVPP IEFPCM acetone solvent (Hartree)	ΔG solvation (kcal mol ⁻¹)	Relative free energy, vacuum (kcal mol ⁻¹)	Relative free energy, acetone (kcal mol ⁻¹)
min1	-1029.564052	138.86	11.18	131.12	-1029.576059	-7.53	0.00	0.00
min2	-1029.559053	138.99	10.99	128.44	-1029.573858	-9.29	3.88	2.13
min4	-1029.559895	138.74	11.27	131.11	-1029.574597	-9.23	2.58	0.89
ts1	-1029.533727	138.46	10.67	126.49	-1029.54844	-9.23	19.51	17.81
ts2	-1029.531519	138.78	10.43	123.93	-1029.546892	-9.65	21.72	19.61

Coordinates of selected optimized Geometries (TPSS-D3/def2-TZVPP)

Species: **Lowest energy geometry, min1**

Energy (a.u.): -1029.564052

O	-0.468524	-1.595581	-0.567360
C	-1.378625	-2.595042	-0.680463
C	-2.706478	-2.442099	-0.500350
C	-3.254051	-1.140500	-0.164138
C	-2.275647	-0.076533	-0.041704
C	-0.906555	-0.327438	-0.241409
C	-2.673990	1.256527	0.278164
C	-1.720678	2.265255	0.382846
C	-0.372703	1.963875	0.176023
C	0.079458	0.658470	-0.136188
C	2.232404	-0.384522	0.579230
C	3.605551	-0.630107	0.420005
C	4.282159	-0.082698	-0.667075
C	3.593227	0.706116	-1.590188
C	2.228578	0.944145	-1.436602
C	1.526698	0.393332	-0.347554
O	4.282469	-1.392365	1.331641
O	-4.483520	-0.944327	0.008824
O	0.515216	2.984390	0.294486
O	-3.969505	1.532815	0.476029
O	1.654213	-0.944896	1.694769
H	-3.372580	-3.289403	-0.609290
H	-2.019627	3.278277	0.624281
H	5.343815	-0.280367	-0.772456
H	1.683931	1.534294	-2.167459
H	3.648437	-1.662096	2.017888
H	1.406707	2.633251	0.101493
H	-4.455972	0.655498	0.342810
H	0.730525	-0.642845	1.744561
H	4.124554	1.127664	-2.437124
H	-0.888661	-3.527241	-0.935395

Species: **Lowest energy transition**

structure, ts1

Energy (a.u.): -1029.533727

O	0.976103	1.788668	0.321340
C	2.040794	2.621244	0.332262
C	3.310549	2.225475	0.136771
C	3.576533	0.820747	-0.074782
C	2.419407	-0.057899	-0.051220
C	1.102915	0.429896	0.101495
C	2.614749	-1.464826	-0.156295
C	1.518953	-2.282300	0.002298
C	0.224369	-1.770833	0.177132
C	-0.094217	-0.360040	0.092843
C	-2.722481	-0.430684	-0.033704
C	-3.960238	0.233767	-0.076132
C	-4.066663	1.608104	-0.158147
C	-2.869017	2.314250	-0.218154
C	-1.646833	1.663235	-0.145803
C	-1.476132	0.253370	-0.000672
O	-5.050963	-0.627465	-0.054110
O	4.745510	0.387155	-0.256607
O	-0.633635	-2.761295	0.475546
O	3.834887	-1.980470	-0.345167
O	-2.851044	-1.809574	-0.071143
H	4.127236	2.936444	0.150796
H	1.636702	-3.358861	0.028856
H	-5.035458	2.098542	-0.195384
H	-0.780301	2.289012	-0.210308
H	-5.871620	-0.120504	-0.123338
H	-1.576755	-2.476686	0.405475
H	4.458156	-1.180512	-0.368697
H	-3.802550	-2.018715	-0.014435
H	-2.880122	3.394438	-0.321684
H	1.721724	3.639647	0.522503

References

- (1) Bringmann, G.; Gunther, C.; Ochse, M.; Schupp, O.; Tasler, S., Stereoselective total Synthesis of Axially Chiral Products. In *Progress in the Chemistry of Organic Natural Products*, Herz, W.; Falk, H.; Kirby, G. W.; Moore, R. E., Eds. Springer: Vienna, 2001; Vol. 82, pp 78-88.
- (2) Frisch, M. J. T., G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, N. J.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. *Gaussian 09, Revision A.1*, Gaussian, Inc.: Wallingford CT, 2009.
- (3) Becke, A. D., *J. Chem. Phys.* **1997**, *107*, 8554-8560.
- (4) Tao, J.; Perdew, J. P.; Staroverov, V. N.; Scuseria, G. E., *Phys. Rev. Lett.* **2003**, *91*, 146401.
- (5) Grimme, S.; Antony, J.; Ehrlich, S.; Krieg, H., *J. Chem. Phys.* **2010**, *132*, 154104.
- (6) Grimme, S.; Ehrlich, S.; Goerigk, L., *J. Comput. Chem.* **2011**, *32*, 1456-1465.
- (7) Marenich, A. V.; Cramer, C. J.; Truhlar, D. G., *J. Phys. Chem. B* **2009**, *113*, 6378-6396.