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## Content

DOI: 10.1021/acs.jnatprod.7b00211

11. **Electrophilic Triterpenoid Enones: A Comparative Thiol-Trapping and Bioactivity Study**
    Danilo Del Prete, Orazio Taglialatela-Scafati, Alberto Minassi, Carmina Sirignano, Cristina Cruz, Maria L. Bellido, Eduardo Muñoz, and Giovanni Appendino
    *Journal of Natural Products* **2017** *80* (8), 2276-2283
    DOI: 10.1021/acs.jnatprod.7b00271
12. **DESIGNER Extracts as Tools to Balance Estrogenic and Chemopreventive Activities of Botanicals for Women's Health**
    Birgit M. Dietz, Shao-Nong Chen, René F. Ramos Alvarenga, Huali Dong, Dejan Nikolić, Martin Biendl, Richard B. van Breemen, Judy L. Bolton, and Guido F. Pauli
    *Journal of Natural Products* **2017** *80* (8), 2284-2294
    DOI: 10.1021/acs.jnatprod.7b00284
13. **Exploring the Sponge Consortium Plakortis symbiotica–Xestospongia deweerdtae as a Potential Source of Antimicrobial Compounds and Probing the Pharmacophore for Antituberculosis Activity of Smenothiazole A by Diverted Total Synthesis**
    Carlos Jiménez-Romero, Joanna E. Rode, Yeiry M. Pérez, Scott G. Franzblau, and Abimael D. Rodríguez
    *Journal of Natural Products* **2017** *80* (8), 2295-2303
    DOI: 10.1021/acs.jnatprod.7b00300
14. **Evaluating Nitrogen-Containing Biosynthetic Products Produced by Saltwater Culturing of Several California Littoral Zone Gram-Negative Bacteria**
    Nicholas Lorig-Roach, Patrick C. Still, David Coppage, Jennifer E. Compton, Mitchell S. Crews, Gabriel Navarro, Karen Tenney, and Phillip Crews
    *Journal of Natural Products* **2017** *80* (8), 2304-2310
    DOI: 10.1021/acs.jnatprod.7b00302
15. **Dioxomorpholines and Derivatives from a Marine-Facultative Aspergillus Species**
    Manuel A. Aparicio-Cuevas, Isabel Rivero-Cruz, Mariano Sánchez-Castellanos, Daniel Menéndez, Huzefa A. Raja, Pedro Joseph-Nathan, María del Carmen González, and Mario Figueroa
    *Journal of Natural Products* **2017** *80* (8), 2311-2318
    DOI: 10.1021/acs.jnatprod.7b00331
16. **Borapetoside E, a Clerodane Diterpenoid Extracted from Tinospora crispa, Improves Hyperglycemia and Hyperlipidemia in High-Fat-Diet-Induced Type 2 Diabetes Mice**
    Yuhui Xu, Yanfen Niu, Yuan Gao, Fang Wang, Wanying Qin, Yanting Lu, Jing Hu, Li Peng, Jikai Liu, and Wenyong Xiong
    *Journal of Natural Products* **2017** *80* (8), 2319-2327
    DOI: 10.1021/acs.jnatprod.7b00365
17. **Discovery and Synthesis of Caracolamide A, an Ion Channel Modulating Dichlorovinylidene Containing Phenethylamide from a Panamanian Marine Cyanobacterium cf. Symploca Species**
    C. Benjamin Naman, Jehad Almaliti, Lorene Armstrong, Eduardo J. Caro-Díaz, Marsha L. Pierce, Evgenia Glukhov, Amanda Fenner, Carmenza Spadafora, Hosana M. Debonsi, Pieter C. Dorrestein, Thomas F. Murray, and William H. Gerwick
    *Journal of Natural Products* **2017** *80* (8), 2328-2334
    DOI: 10.1021/acs.jnatprod.7b00367
18. **Asymmetric Total Synthesis of (−)-Astakolactin and Confirmation of Its Stereostructure**
    Takayuki Tonoi, Yutaka Yoshinaga, Moe Fujishiro, Keisuke Mameda, Takashi Kato, Kentaro Shibamoto, and Isamu Shiina
    *Journal of Natural Products* **2017** *80* (8), 2335-2344
    DOI: 10.1021/acs.jnatprod.7b00368
19. **Chemical Constituents of Propolis from Vietnamese Trigona minor and Their Antiausterity Activity against the PANC-1 Human Pancreatic Cancer Cell Line**
    Hai X. Nguyen, Mai T. T. Nguyen, Nhan T. Nguyen, and Suresh Awale

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Article

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# 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*[,‡]

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Supporting Information

**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.


Antarctic terrestrial flora (algae, mosses, liverworts, lichens, and two higher plants) endure physiologically extreme conditions such as subzero 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 flavonoids[8] 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 such as *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.

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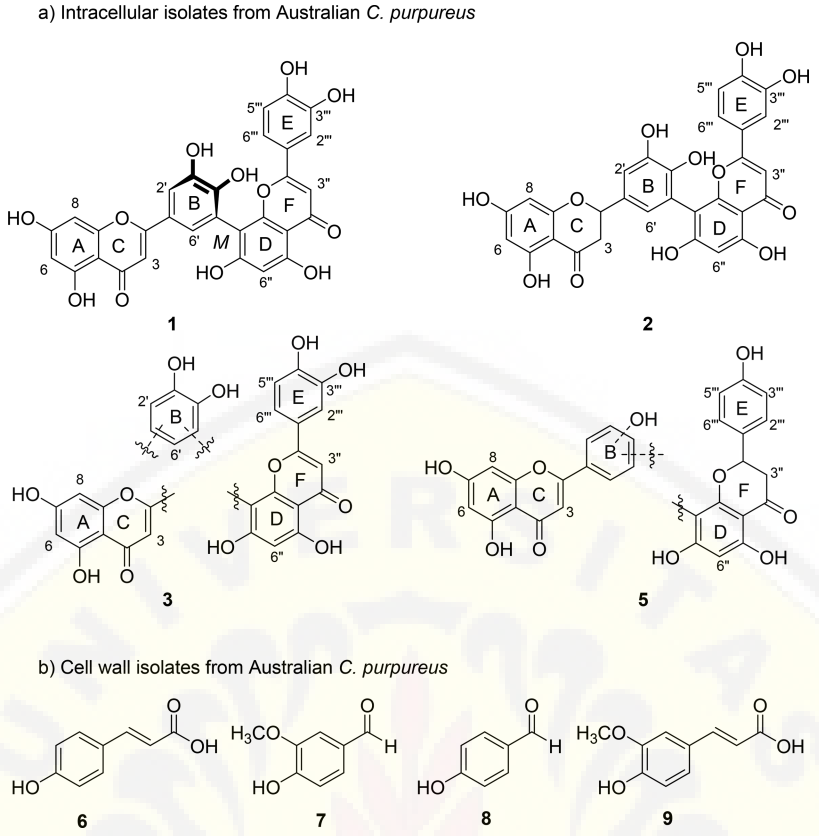


**Figure 1.** (a) Intracellular constituent biflavonoids (**1**–**3** and **5**); (b) cell wall isolates: *p*-coumaric acid (**6**), vanillin (**7**), *p*-hydroxybenzaldehyde (**8**), and *trans*-ferulic acid (**9**) from Australian *Ceratodon purpureus*. Isolate **4** is likely also a biflavonoid, but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

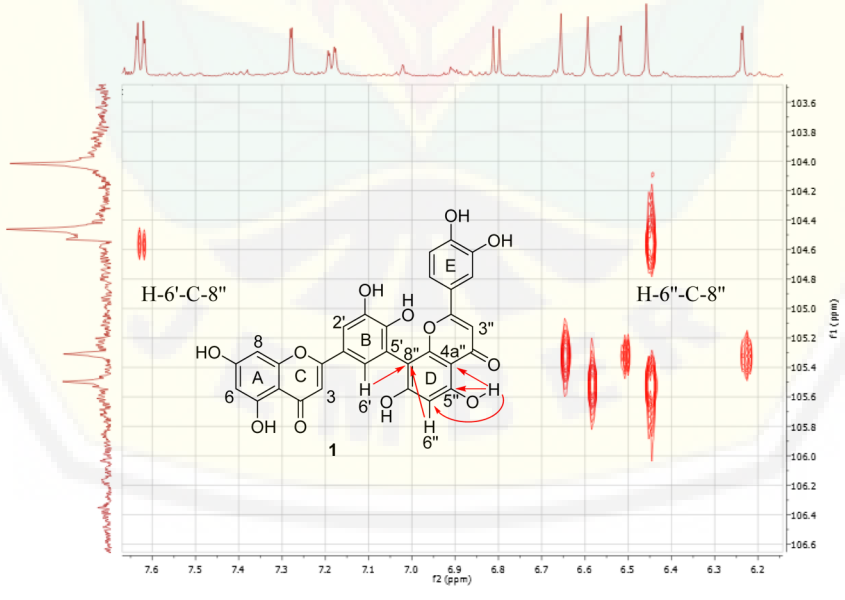


**Figure 2.** HMBC spectrum of biflavone **1**, showing correlations confirming the location of the biaryl axis as 5′–8″.

## 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 in both DMSO-$d_6$ and acetone-$d_6$, the former being employed, as most biflavonoids have been reported using this solvent. However, recent evidence[11] suggests that numerous errors in the structural assignment of natural



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J. Nat. Prod. 2017, 80, 2224–2231

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-$d_6$.

Compound **1** was isolated as a yellow powder that decomposed at 295 °C. The IR spectrum indicated stretches at 1653 and 3213 cm$^{-1}$, assigned to carbonyl and hydroxy stretches, respectively. The molecular formula of $C_{30}H_{18}O_{12}$ of compound **1** was confirmed by HRESIMS with an assigned $[M - H]^-$ 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.[12−14] 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 $\delta_H$ 7.64 (d, $J$ = 2.3 Hz) (H-6′) and 6.46 (H-6″) with the carbon resonance at $\delta_C$ 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″ ($\delta_C$ 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 $\delta_C$ 162.5 and C-6″ at $\delta_C$ 99.8. Thus, the site of connection must be C-8″. The chirality of the atropisomeric biaryl axis was determined by comparative electronic circular dichroism (ECD) spectroscopy, with **1** showing a positive Cotton effect at 362 ($\Delta\varepsilon$ +24.6) nm (Figure S20, Supporting Information), which defines an *M*-isomer (a*R* absolute configuration) by comparison with other axially chiral biflavones.[15,16]

Compound **2** was isolated as a yellow powder that decomposed at 295 °C. The IR spectrum showed stretches at 1653 and 3330 cm$^{-1}$, assigned to carbonyl and hydroxy stretches, respectively. The proposed molecular formula of $C_{30}H_{20}O_{12}$ for compound **2** was confirmed by the assignment of the $[M + H]^+$ 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 $^1$H 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

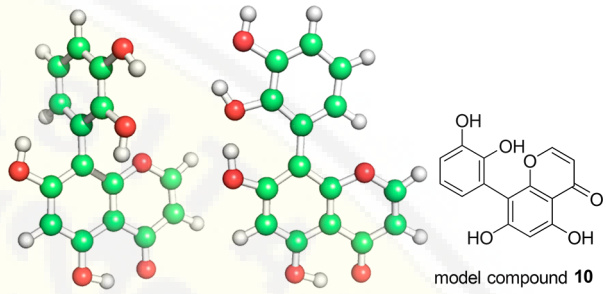


**Figure 3.** Lowest energy geometry (left) and lowest energy transition structure for rotation around the biaryl linkage (right) calculated at the TPSS-D3/def2-TZVPP level of theory.

contains the core of compound **2** including the biaryl axis, was subjected to calculations using dispersion-corrected density functional theory (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 led to identification of the lowest energy conformation and transition structures shown in Figure 3.

Under 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



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Table 1. Concentrations (Means ± SD) of Isolates 1−5 within Australian ($n$ = 7) and Antarctic ($n$ = 8) Samples of *C. purpureus* Based on HPLC Analysis

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

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. Australian and Antarctic *C. purpureus* are genetically similar[20,21] and are highly tolerant to UV radiation,[6] 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 1). 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 1- to 3-fold) more abundant than biflavone **1** in the crude intracellular extract but on average 13-fold (ranging from 1- 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.

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, southeastern 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 been isolated only from intracellular locations using methanol-based solvents,[26−31] 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 walls,[34−37] 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 fetidus*;[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



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J. Nat. Prod. 2017, 80, 2224−2231

**Table 2. Ultraviolet and Antioxidant Activities of the Nine Isolates Compared to Rutin, a Known Standard for DPPH Antioxidant Assays[a]**

| | $\varepsilon$ ($M^{-1}$ $cm^{-1}$)[b] | | | cellular location | |
|---|---|---|---|---|---|
| compound | $\lambda_{max\ 1}$ | $\lambda_{max\ 2}$ | AA ($IC_{50}$, $\mu M$) | Australian *C. purpureus* | Antarctic *C. purpureus* |
| **1** | 12 300 (353 nm) | 13 110 (255 nm) | 33.5 ± 1.6 | intracellular | intracellular and cell wall |
| **2** | 7030 (349 nm) | 8510 (275 nm) | 127.8 ± 3.8 | intracellular | intracellular and cell wall |
| **3** | 350 (350 nm) | 3290 (264 nm) | >400 | intracellular | intracellular |
| **4** | 380 (332 nm) | 1490 (287 nm) | >400 | intracellular | intracellular |
| **5** | 700 (332 nm) | 2550 (289 nm) | >400 | intracellular | intracellular |
| **6** | 23 110 (310 nm) | 12 700 (225 nm) | $(29.7 \pm 2.3) \times 10^3$ | cell wall | cell wall |
| **7** | 8210 (309 nm) | 8450 (278 nm) | $(14.4 \pm 0.5) \times 10^3$ | cell wall | cell wall |
| **8** | 15 670 (285 nm) | 10 930 (221 nm) | $(475.6 \pm 44.5) \times 10^3$ | cell wall | cell wall |
| **9** | 29 850 (323 nm) | 22 410 (297 nm) | 75.0 ± 16.5 | cell wall | cell wall |
| rutin | 6920 (359 nm) | 8300 (258 nm) | 38.6 ± 0.3 | | |

[a]All UV and DPPH ($IC_{50}$) measurements were performed on compounds isolated from Australian *C. purpureus* only. $IC_{50}$ values ($\mu m$) represent means ± SD ($n$ = 3). [b]Wavelengths specified in parentheses.

reported as chemicals that can be used as UVR proxies[25,39,44] showing correlation with past UVR levels[41,45] and/or have been used for reconstructing past ozone climates.[40,46]

The location of compounds **1**, **2**, and **6**–**9** within the cell wall suggests 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 2). 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 ($\varepsilon$ = 12 300 $M^{-1}$ $cm^{-1}$) and 255 nm for **1** and 349 ($\varepsilon$ = 7030 $M^{-1}$ $cm^{-1}$) 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 ($\varepsilon_{310}$ = 23 100 $M^{-1}$ $cm^{-1}$ and $\varepsilon_{297}$ = 22 410 $M^{-1}$ $cm^{-1}$). Compound **9** was the most effective at absorbing the shorter UV-A wavelengths, giving a maximum peak at 323 nm.

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 2). Compound **1** demonstrated considerably lower $IC_{50}$ values (33.5 $\mu M$) relative to the strongly antioxidant standard rutin (38.6 $\mu M$). Additionally, compounds **2** and **9** showed good radical-scavenging activity with $IC_{50}$ values of 127.8 and 75.0 $\mu M$, respectively. Compounds **3**–**8** showed much weaker antioxidant values with $IC_{50}$ concentrations in the millimolar range. Thus, radical-scavenging efficiency in terms of $IC_{50}$ 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** and **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: first by directly reducing UV radiation transmission to susceptible intracellular components and second by quenching UV-induced ROS. Generally, it has been reported that biflavonoids have relatively impressive capacities as antioxidants.[49] Perhaps **1** and **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 while identifying and quantifying its valuable compounds.

In order to gain an 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 the phenolics *p*-coumaric acid, vanillin,



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*p*-hydroxybenzaldehyde, and ferulic acid. All isolates absorbed in the ultraviolet spectrum and biflavonoids **1** and **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 years[24]). 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-1601 UV−vis spectrophotometer. ECD spectra were recorded on a JASCO J-810 spectropolarimeter with a path length of 0.1 cm and concentration between 50 and 100 $\mu$M in MeOH. IR spectra were recorded with a Shimadzu IR Affinity-1 FT-IR spectrometer fitted with a 1.5 round diamond crystal. $^{1}$H 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 cm$^3$ 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 $C_{18}$ column (5 $\mu$m, 4.9 × 150 mm) or a Shimadzu HPLC system (SOD-M10AVP diode array detector, CTO-20AC column oven, LC-10ATVP pump, SIL-10Ai autoinjector, SC-10AVP system controller, DGU-20ALVP degasser, controlled by Shimadzu Class-VP software v6.12 SP3) with a Wakosil $C_{18}$ RS column (5 $\mu$m, 4.6 × 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 Sunfire $C_{18}$ column (5 $\mu$m, 19 × 150 mm) protected with a Waters Sunfire C-18 guard column (5 $\mu$m, 19 × 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 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 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.A.R. Antarctic samples were air-dried upon collection and stored at −20 °C prior to transfer to UOW, Australia. Samples were identified either by M.J.W. 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 10 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−$H_2O$ (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−$H_2O$ (9:0.5:0.5, 20 mL) to produce a filtrate (50 mL). This solution was then passed through an HPLC sample filter (0.45 $\mu$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 $H_2O$) within 40 min (solvent B, 0.1% TFA in MeCN) was used for the semipreparative 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; UV-active, pale yellow solid (76.7 mg, 3.1 mg g$^{-1}$ dry wt); mp, decomposed at 295 °C; $[\alpha]_D^{20}$ −3.8 (*c* 0.004, MeOH); $\lambda_{max}$ 255 (14 158) 350 (13 358); ECD (MeOH) $\lambda$ 220 ($\Delta\varepsilon$ +67.8), 261 ($\Delta\varepsilon$ +53.9), 331 ($\Delta\varepsilon$ −49.9), 362 ($\Delta\varepsilon$ +24.6) nm; IR [cm$^{-1}$] 3213 (m), 1653 (s), 1430 (s), 1339 (s), 1253 (s), 1165 (s), 836 (s); for $^{1}$H NMR (acetone-$d_6$, 500 MHz) and $^{13}$C NMR (acetone-$d_6$, 125 MHz) spectroscopic data see Table S1; ESIMS$^+$ *m*/*z* 571 [M + H]$^+$ (100); ESIMS$^-$ 569 [M − H]$^-$ (50); HRESIMS calcd for $C_{30}H_{17}O_{12}$ [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; UV-active, pale yellow solid (21.0 mg, 0.8 mg g$^{-1}$ dry wt); mp, decomposed at 295 °C; $[\alpha]_D^{20}$ 0 (c 0.150 MeOH); $\lambda_{max}$ 211 (10047), 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 $^{1}$H NMR (acetone-$d_6$, 500 MHz) and $^{13}$C NMR (acetone-$d_6$, 125 MHz), spectroscopic data see Table S1; ESIMS$^+$ *m*/*z* 573 [M + H]$^+$ (100); HRESIMS calcd for $C_{30}H_{21}O_{12}$ [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 × ∼1.5−1.7 g dry wt) of moss residue after MeOH



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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) and in MeOH−$CHCl_3$ (1:1, 15 mL) twice for 1 h, before being washed with MeOH (10 mL). After each incubation, tubes were centrifuged at 3600*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 concentrated HCl and then refiltered. Initial cleanup 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% aqueous MeOH (40 mL) before elution using 100% MeOH (5 × 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 × 20.5 mg) of semipreparative HPLC using a linear gradient from 75% to 55% of solvent A (0.1% formic acid (FA) in $H_2O$) 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 $^1$H NMR, $^{13}$C 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 × 1.5 mL). The supernatants (intracellular extracts) were collected and stored at −20 °C before analysis. The remaining moss pellets were sequentially resuspended using reagents (1.5 mL), air-dried, and extracted in NaOH (1.0 mL) as for the Australian moss samples. Samples were centrifuged at 16000 *g* for 5 min, and the supernatant (cell wall extract) was acidified to pH 1 with concentrated 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 $H_2O$) 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 $H_2O$) 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 was added DPPH (200 μM, 100 μL), and the solutions were 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 eq 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 $IC_{50}$ mean ± SD 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).

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

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

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00085.

$^1$H 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 on the DPPH antioxidant assay and computational methods (PDF)

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### Notes

The authors declare no competing financial interest.

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