Gymnoascolides A−C: Aromatic Butenolides from an Australian Isolate of the Soil Ascomycete *Gymnoascus reessii*

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Three new aromatic butenolides, gymnoascolides A−C (**1**−**3**), have been isolated from the Australian soil ascomycete *Gymnoascus reessii* and assigned structures on the basis of detailed spectroscopic analysis. The absolute configurations of gymnoascolides B (**2**) and C (**3**) at C-5 were solved using a combination of chemical derivatization and quantum chemical simulations.

Microbes have long been recognized for their capacity to yield a wide range of structurally novel and biologically potent metabolites. Many of these substances have either found direct application as medicines or provided the inspiration for successful drugs in the fields of human and animal health and crop protection. The pre-eminent role that microbes have played in drug discovery encourages the view that Australia's largely unexplored microbial biodiversity remains an attractive resource worthy of study. This microbial resource encompasses an extraordinary array of varied ecosystems—from the tropics through temperate climes to sub-Antarctic and Antarctic regions, from deserts and tropical rainforests through tropical reefs, estuaries, and all manner of intertidal and marine habitats—all of which can be expected to host diverse microbial communities.

In recent years, we have sought to draw Australian microbial biodiversity into the arena of drug discovery through a concerted and systematic exploration of an Australian microbial isolate library (>250 000 cultures). One dimension of this research targets isolates whose extracts display potent antibiotic properties not readily attributed to known microbial metabolites. Of the hundreds of microbial isolates encountered in our study that meet these criteria, this report focuses on a single strain of *Gymnoascus reessii* (MST-F9977) which in our hands yielded a series of novel aromatic butenolides, the gymnoascolides (**1**−**3**). This discovery is perhaps all the more noteworthy given that literature accounts of chemical investigations into *Gymnoascus* are rare and appear to be limited to reports of such common metabolites as penicillic acid,[1] G-type penicillins,[2] and patulin.[3]

Results and Discussion

The MeOH extract of a liquid culture of *G. reessii* displayed significant growth inhibitory activity against the bacterium, *Bacillus subtilis*, the nematode, *Haemonchus contortus*, and a tumor cell line (murine NS-1). The MeOH extract was concentrated in vacuo and the resulting residue fractionated by C_{18} solid-phase extraction and HPLC, then subjected to chemical (HPLC), spectroscopic (Photodiode array, ESI(±)MS, ^{1}H NMR), and biological profiling to disclose the presence of interesting metabolites. During this fractionation process, a weak but selective antifungal activity was noted in a number of the otherwise inactive fractions. This antifungal selectivity was demonstrated by growth inhibitory activity against the plant pathogen *Septoria nodorum* and a corresponding absence of growth inhibitory activity against the human pathogen *Candida albicans*. Further purification of two of these antifungal fractions yielded the novel aromatic butenolides, gymnoascolides A−C (**1**−**3**).

High-resolution ESI(+)MS analysis of gymnoascolide A (**1**) returned a pseudo molecular ion ($[M + Na]^+$) consistent with a molecular formula ($C_{17}H_{14}O_2$) requiring 11 double bond equivalents (DBE). The ^{1}H and ^{13}C NMR data for **1** (Tables 1 and 2) indicated the presence of two monosubstituted aromatic rings, an ester/lactone carbonyl (δ_C 173.3), two isolated methylene units (δ_C 71.1, δ_H 4.69, s and δ_C 33.9, δ_H 3.96, s), and two sp^2 carbons (δ_C 159.7 and 129.6) consistent with a tetrasubstituted double bond conjugated to the carbonyl. These structural moieties account for all elements in the molecular formula and all but one DBE, confirming gymnoascolide A (**1**) as a lactone (IR 1759 cm^{-1}).

Two-dimensional NMR analysis of **1** confirmed the presence of an α,β-unsaturated butyrolactone moiety, substituted by both phenyl and benzyl functionalities, and permitted complete proton and carbon NMR assignments (Tables 1 and 2). The positions of the phenyl and benzyl substituents were confirmed by NOE difference experiments which revealed enhancements from H-5 to H-2″/6″ (2%), and from H-6 to both H-2′/H-6′ (4%) and H-2″/H-6″ (4%). Thus, the structure for gymnoascolide A (**1**) was assigned as shown.

High-resolution ESI(+)MS analysis of gymnoascolide B (**2**) gave a pseudo molecular ion ($[M + Na]^+$) consistent with a molecular formula ($C_{18}H_{16}O_4$) requiring 11 DBE. Comparison of the NMR data for **2** with those of **1** (Tables 1 and 2) confirmed a common phenyl- and benzyl-substituted α,β-unsaturated butyrolactone moiety. NMR differences

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Table 1. ^{1}H NMR ($CDCl_3$, 400 MHz)[a] Data for Gymnoascolides A−C (**1**−**3**)

	1			**2**			**3**		
H	δ_H (m, *J* (Hz))	COSY	HMBC[a]	δ_H (m, *J* (Hz))	COSY	HMBC[a]	δ_H (m, *J* (Hz))	COSY	HMBC[a]
5	4.69 (s)		2, 3, 4, 6, 1′, 1″	5.48 (s)		2, 3, 4, 5-OMe, 1′	5.90 (s)		2, 3, 4, 5-OMe
6	3.96 (s)		2, 4, 5, 1″, 2″/6″	5.88 (d, 8.8)	6-OH	3, 4, 5, 1″, 2″/6″	5.85 (d, 6.2)	6-OH	3, 4, 5, 1″, 2″/6″
6-OH				3.40 (d, 8.8)	6	4, 6	2.25 (d, 6.2)	6	4, 6, 1"
5-OMe				3.61 (s)		5	3.61 (s)		5
2′/6′	7.53 (d, 7.0)	3′	3′/5′, 3	7.62 (d, 7.6)	3′/5′	3	7.4 (m)		
3′/5′	7.47 (dd, 7.0, 7.3)	2′/6′, 4′	2′/6′, 1′	7.5 (m)	2′/6′		7.4 (m)		
4′	7.42 (t, 7.3)	3′/5′	2′/6′	7.5 (m)	2′/6′		7.4 (m)		
2″/6″	7.15 (d, 6.9)	3″/5″	6, 2″/6″, 4″	7.30 (d, 8.0)	3″/5″	6, 2″/6″, 4″	7.4 (m)		
3″/5″	7.34 (dd, 6.9, 7.1)	2″/6″,4″	1″, 3″/5″	7.39 (dd, 7.6, 8.0)	2″/6″,4″	1″, 3″/5″	7.4 (m)		
4″	7.29 (t, 7.1)	3″/5″	2″/6″, 3″/5″	7.33 (t, 7.6)	3″/5″	2″/6″	7.4 (m)		

[a] Assignments have been made as accurately as possible; however, due to overlapping signals, there is some uncertainty with correlations to carbons in the range of 127−129 ppm.

Table 2. ^{13}C NMR ($CDCl_3$, 100 MHz)[a] Data (δ) for Gymnoascolides A−C (**1**−**3**)

C	**1**	**2**	**3**
2	173.3	169.9	170.0
3	127.6[b]	131.4[b]	130.5[b]
4	159.7	154.2	157.3
5	71.1	102.0	102.3
6	33.9	68.7	71.5
5-OMe		58.1	57.8
1″	129.6[b]	128.1[b]	128.7[b]
2″/6″	128.9[c]	129.3	129.2[c]
3″/5″	128.7[c]	128.9	128.9[c]
4″	128.8	129.8	129.3[d]
1″	136.1	140.1	140.3
2″/6″	128.4	125.3	127.3
3″/5″	129.1	128.8	128.4[c]
4″	127.4	128.2	128.7[d]

[a] Assignments assisted by HMBC and HMQC correlations. [b−d] Values within a column may be interchanged.

between the two were consistent with addition of oxygen substituents to the sp^3 centers at C-5 and C-6 (δ_C 102.0, δ_H 5.48, s, and δ_C 68.7, δ_H 5.88, d) and the appearance of OMe (δ_C 58.1, δ_H 3.61) and OH (δ_H 3.40, d) functionalities. Analysis of the 2D NMR data (Table 1) for **2** unambiguously positioned the OH at C-6 and the OMe at C-5. The phenyl/benzyl positions for **2** were confirmed by comparable NOE enhancements to those observed for **1** (H-5 to H-2″/6″ (3%), and H-6 to both H-2′/H-6′ (10%), and H-2″/H-6″ (5%)), with additional strong enhancements between H-5 and the 5-OMe (12%). The observations outlined above provide evidence for the structure for gymnoascolide B (**2**) as shown, except for its configuration at C-5 and C-6.

Gymnoascolide C (**3**) was found to be closely related to and isomeric with gymnoascolide B (**2**). Unfortunately, the ^{1}H NMR data for **3** (Table 1) failed to disperse the aromatic proton resonances, preventing interpretations comparable to those for **1** and **2**. Significant ^{1}H NMR shift differences between **2** and **3** were observed for H-5 ($\Delta\delta$ 0.42) and 6-OH ($\Delta\delta$ −1.15), with additional small differences observed for C-4, C-6, and C-2″/6″ in the ^{13}C NMR spectrum (Table 2). As both gymnoascolides B (**2**) and C (**3**) were resolved by achiral HPLC, possessed unique ^{1}H NMR spectra, and displayed significant specific rotations, they were deemed to be diastereomers.

To establish whether gymnoascolides B (**2**) and C (**3**) were C-5 or C-6 epimers, a sample of each was oxidized with pyridinium dichromate to yield the keto derivative **4**. The ketones derived individually from **2** and **3** would be expected to possess the same optical rotation if **2** and **3** were C-6 epimers, and opposite optical rotations if **2** and **3** were C-5 epimers. In the event, gymnoascolide B (**2**) yielded (+)-**4**, while gymnoascolide C (**3**) gave (−)-**4**, confirming that **2** and **3** were indeed epimers at C-5.

The absence of structurally related natural or synthetic compounds of known absolute stereostructure in the scientific literature precluded an elucidation of the absolute configuration of **4** by comparison of circular dichroism (CD) spectra. Confronted by this challenge, we turned to quantum chemical CD calculations.[4−6] Arbitrarily starting with the *R*-enantiomer of **4**, the conformational analysis resulted in 112 minimum structures within the range of 3 kcal/mol above the global minimum. For each geometry optimized by means of the PM3[7] Hamiltonian, the respective CD curve was computed using the semiempirical CNDO/S method.[8] The single CD spectra thus received were summed up and weighted following the Boltzmann statistics, that is, according to the heats of formation of the conformers. The resulting overall theoretical CD spectrum was submitted to a UV correction[9] and compared with experimental data (Figure 1, top), indicating that the oxidation product (+)-**4** of gymnoascolide B (**2**) is *R*-configured, and consequently, (−)-**4** has the *S*-configuration. Although the two main peaks of the experimental CD curve are, in principle, reproduced in the calculated spectrum, the agreement is not very good and the comparison can thus not be used as a sole proof. For this reason, a second theoretical approach to simulate the CD curve of **4** was chosen, this time based on a molecular dynamics (MD) simulation.[10] The MD run was carried out using the TRIPOS force field[11] at a virtual temperature of 500 K for a total time period of 500 ps, recording the structure every 0.5 ps. For the 1000 structures thus obtained, the single CD spectra were calculated again by means of the CNDO/S[8] method and averaged arithmetically to give the overall theoretical spectrum of **4**. As shown in Figure 1 (bottom), the CD spectrum calculated for (5*R*)-**4** matches now almost perfectly with the experimental data for (+)-**4**, whereas the curve simulated for (5*S*)-**4** is virtually opposite. Thus, the absolute configurations at C-5 for (+)-**4** and **2** were assigned as *R*, and those of (−)-**4**, and hence **3**, as *S*.

Assignment of the absolute configuration at C-6 in **2** and **3** proved more elusive. While the secondary alcohol at C-6 was an obvious target for a Mosher analysis, the poorly dispersed aromatic region in the ^{1}H NMR spectra of both **2** and **3** precluded such an approach. We did, however, attempt a modified Horeau analysis, relying on monitoring kinetic resolution during the formation of diastereomeric α-phenylbutyrate esters by chiral HPLC. Unfortunately,

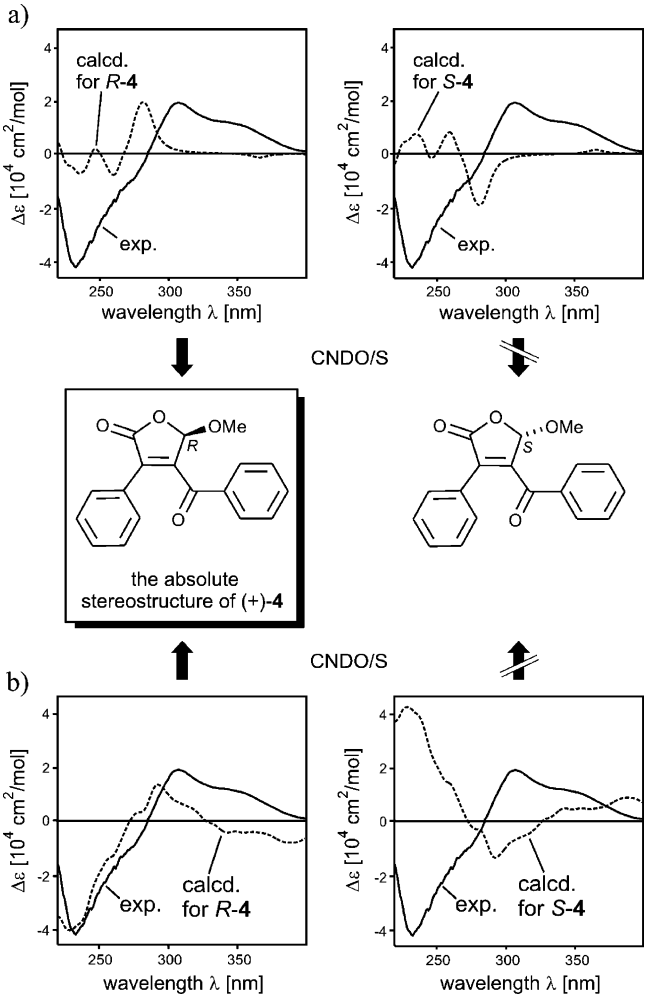


Figure 1. Attribution of the absolute configuration of the oxidized product of gymnoascolide B, (+)-**4** by (a) the PM3-Boltzmann approach; (b) the TRIPOS−MD method. The ellipticities in the calculated CD spectra have been calibrated to fit with the experimental spectra.

despite demonstrating the sensitivity and reliability of this approach on suitable standards, this method proved inconclusive when applied to gymnoascolides, possibly due to a lack of adequate steric discrimination between C-6 substituents. A lack of available material prevented further degradative/chemical approaches to assign the absolute configuration at C-6 in either **2** or **3**.

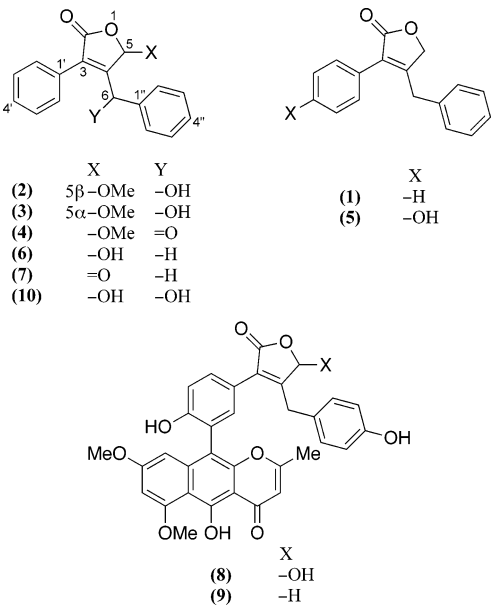
The gymnoascolides possess a rare structure motif. While butenolides and substituted aromatics are common among microbial metabolites,[12] very few natural products, microbial, plant, or other, possess the constellation of phenyl and benzyl substituents about a γ-butenolide as presented in the gymnoascolides A−C (**1**−**3**). The only comparable published compounds are eutypoid A (**5**), isolated in 2002 from a South China Sea marine fungus of the genus *Eutypa*,[13] and microperfuranone (**6**)[14] and the anhydride **7**,[15] isolated in 1998 and 1983 from the terrestrial fungi *Anixiella micropertusa* and *Aspergillus nidulans*, respectively. Similarly, the carbonarins are a more highly substituted suite of related metabolites isolated from the terrestrial fungus *Aspergillus carbonarius* and first recorded in the patent literature in 1996.[16] These include the acetal, carbonarin A (**8**), and the butenolide, carbonarin H (**9**), both of which possess a clear structural overlap with the gymnoascolides. Literature accounts of the carbonarins

Table 3. Antifungal Properties for Gymnoascolide A (**1**) versus Commercially Available Standards[a]

compound name	*C. albicans* LD_{99} mg/mL	*S. nodorum* LD_{99} mg/mL
gymnoascolide A	na	13
cycloheximide	na	6.3
antimycin A3	50	0.20
monorden	12	12
amphotericin B	3.1	3.1
blasticidin S HCl	0.39	1.6

[a] na = not active at 50 mg/mL.

are limited to two patents (1996 and 1997) which document insecticidal properties.[16,17]



Given that methanol was used in the isolation of the gymnoascolides, it is possible that gymnoascolides B (**2**) and C (**3**) are derived from the hypothetical precursor **10**. However, it is worth noting that methanol was also used extensively in the isolation of microperfuranone (**6**), and no methoxylated products were reported in that instance.[14]

Gymnoascolide A (**1**) possessed moderate, selective activity against the pathogenic plant fungus *Septoria nodorum* (MIC = 13 μg/mL), while none of gymnoascolides A−C (**1**−**3**) displayed activity against the human fungal pathogen *C. albicans*, the gram-positive bacteria *B. subtilis*, the livestock nematode *H. contortus*, or a mammalian tumor cell line (murine NS-1). The potency and selectivity of the antifungal properties of gymnoascolide A (**1**) were compared against a range of known antifungal metabolites (as detailed in Table 3), which revealed a selectivity between *C. albicans* and *S. nodorum* comparable with that of cycloheximide, and a potency against *S. nodorum* comparable with that of monorden. Further biological studies on the gymnoascolides are ongoing, as are efforts at total synthesis.

Experimental Section

General Experimental Procedures. As for previous work,[18] except for the following: Initial HPLC work was carried out on a system consisting of two Shimadzu LC-8A preparative liquid chromatographs with a static mixer, Shimadzu SPD-M10AVP diode array detector, and Shimadzu SCL-10AVP system controller. UV−vis absorption spectra were obtained using a Shimadzu UV-1650PC spectrophotom-

eter, while infrared (IR) spectra were acquired using a Shimadzu FTIR-8400 spectrometer.

In addition, for the oxidation products (+)-**4** and (−)-**4**, optical rotations were obtained using a Jasco P-1010 intelligent remote module-type polarimeter, and circular dichroism (CD) spectra were acquired using a JASCO J-810 spectropolarimeter. ^{1}H NMR spectra were acquired using a Bruker Avance 600 spectrometer, and ESI(±)MS data were obtained using a Agilent 1100 Series Separations module equipped with a Agilent 1100 Series LC/MSD mass detector. High-resolution (HR) ESI-MS measurements were obtained on a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III source.

Computational Methods. All calculations were carried out with Linux AMD MP 2400+ workstations. The conformational analysis was performed by means of the semiempirical PM3[7] method as implemented in the program package, Gaussian 98,[19] starting from preoptimized geometries generated by the TRIPOS[11] force field as part of the molecular modeling package, SYBYL 7.0.[11] The molecular dynamics simulations of **4** were run at a virtual temperature of 500 K using the TRIPOS[11] force field with a time step of 2 fs. Bond lengths were constrained using the SHAKE algorithm.[20] The overall simulation time was 500 ps, and every 0.5 ps a single geometry was extracted.

The wave functions required for the computation of the rotational strengths for the electronic transitions from the ground state to excited states were obtained by CNDO/S[8] calculations followed by single configuration interaction (SCI) computations, including 784 singly occupied configurations and the ground state determinant. These computations were carried out using the BDZDO/MCDSPD[21] program package. The single CD spectra were summed up and weighted following the Boltzmann statistics, that is, according to the respective heats of formation. The rotational strengths were transformed into $\Delta\epsilon$ values and, for a better visualization, superimposed with a Gaussian band shape function.

Biological Material. The fungal strain MST-F9977 was isolated from a roadside soil sample collected near Sussex Inlet on the southern coast of New South Wales, Australia, in an area regenerating from a recent bushfire. The isolate was identified as an ascomycete, *Gymnoascus reessii*, on morphological grounds. On malt extract agar, it is characterized by orange hyphae and a pinkish reverse.

Assay Details. Procedures for antibacterial,[22] cytotoxicity,[22] and nematocidal[23] assays have been described previously. Antifungal activity was determined in an agar-based, microtiter plate bioassay. Spores of *Candida albicans* or *Septoria nodorum* (20 000 sp/well) were applied to potato dextrose agar in the wells of a microtiter plate, containing serial 2-fold dilutions of the test compound. The plates were incubated at 24 °C before a qualitative assessment of fungal growth was made at 48 h, with the LD_{99} determined as the lowest concentration of the test compound at which no growth of the fungus was observed.

Extraction and Isolation. A solid fermentation (100 g wheat, 21 days, 28 °C) was extracted with MeOH. This extract was concentrated in vacuo to an aqueous residue that was diluted with H_2O to a final volume of 1 L. This was passed through two parallel C_{18} solid-phase extraction (SPE) cartridges (2 × 10 g, Varian HF C_{18}), eluting with MeOH (2 × 40 mL each). On evaporation of the combined MeOH eluants, a residue (~0.5 g) was obtained that was subjected to preparative HPLC (60 mL/min with gradient elution of 70 to 10% H_2O/MeCN over 20 min followed by MeCN for 10 min, through a 5 μm Platinum EPS C_{18} 50 × 100 mm column). One hundred fractions were collected, concentrated in vacuo, and combined on the basis of analytical HPLC analysis.

One of these combined fractions (38 mg) possessed interesting aromatic ^{1}H NMR resonances and was further fractionated by C_{18} SPE (10% stepwise gradient elution from 60% H_2O/MeOH to MeOH, through a 500 mg Alltech Extract-Clean C_{18} cartridge) and C_{18} HPLC (3 injections, 10 mL/min gradient elution from 70% H_2O/MeCN (0.01% TFA) to 30% H_2O/MeCN (0.01% TFA) over 20 min, through a 5 μm Phenomenex LUNA $C_{18(2)}$ 150 × 21.2 mm column) to yield gymnoascolide A (**1**) (9.1 mg, 1.8%).

Subsequently, a more polar fraction (44 mg) that appeared to contain similar metabolites was fractionated using C_{18} SPE (as for the previous fraction), followed by C_8 HPLC (10 injections, 2.5 mL/min gradient elution from 60% H_2O/MeCN (0.01% TFA) to 20% H_2O/MeCN (0.01% TFA) over 20 min, through a 5 μm Phenomenex LUNA $C_{8(2)}$ 10 × 250 mm column) then phenyl−hexyl HPLC (20 injections, 1 mL/min isocratic elution with 50% H_2O/MeCN, through a 5 μm Phenomenex phenyl−hexyl 4.6 × 150 mm column), yielding gymnoascolide B (**2**) (2.2 mg, 0.43%) and gymnoascolide C (**3**) (1.5 mg, 0.29%).

All percent yields for purified metabolites were calculated against the combined residue (0.5 g) recovered after elution of the extract from two parallel C_{18} SPE preparative cartridges.

Gymnoascolide A (1): colorless oil; IR ($CHCl_3$) ν_{max} 3064−3011, 2928, 1759, 1495, 1467, 1126, 1045, 957 cm^{-1}; UV (EtOH) λ_{max} (ϵ) 248 (10500) nm; ^{1}H NMR data ($CDCl_3$, 400 MHz) see Table 1; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 2; ESI(+)MS (30 kV) *m/z* 523 $[2M + Na]^+$, 273 $[M + Na]^+$, 251 $[M + H]^+$; HRESI(+)MS *m/z* 273.0880 ($[M + Na]^+$, $C_{17}H_{14}O_2$Na requires 273.0891).

Gymnoascolide B (2): white solid; $[\alpha]_D$ −105° (*c* 0.044, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3543, 2930, 1774, 1495, 1448, 1369, 1331, 1234, 1109 cm^{-1}; UV (EtOH) λ_{max} (ϵ) 259 (4900) nm; ^{1}H NMR data ($CDCl_3$, 400 MHz) see Table 1; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 2; ESI(+)MS (30 kV) *m/z* 319 $[M + Na]^+$; HRESI(+)MS *m/z* 319.0942 ($[M + Na]^+$, $C_{18}H_{16}O_4$Na requires 319.0946).

Gymnoascolide C (3): white solid; $[\alpha]_D$ −110° (*c* 0.022, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3570, 2928, 2855, 1767, 1494, 1446, 1371, 1334, 1117 cm^{-1}; UV (EtOH) λ_{max} (ϵ) 255 (5500) nm; ^{1}H NMR data ($CDCl_3$, 400 MHz) see Table 1; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 2; ESI(+)MS (30 kV) *m/z* 319 $[M + Na]^+$; HRESI(+)MS *m/z* 319.0941 ($[M + Na]^+$, $C_{18}H_{16}O_4$Na requires 319.0946).

Oxidation of Gymnoascolide B (2). A sample of **2** (1.1 mg) in CH_2Cl_2 (2 mL) was treated with pyridinium dichromate (2.2 mg) and the suspension stirred overnight, after which the mixture was filtered through a small plug of silica to remove chromium salts and washed well with ether. The combined washings were concentrated in vacuo to yield (+)-**4** (0.9 mg, 82%) as a colorless oil: $[\alpha]_D$ +175° (*c* 0.018, $CHCl_3$); UV (EtOH) λ_{max} (ϵ) 256 (6700) nm, 293 (sh) (3400); CD (MeOH) λ_{max} (ϵ) 233 (−41000), 307 (18500) nm; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.78 (2H, m), 7.50 (1H, m), 7.45 (2H, m), 7.33 (2H, m), 7.28 (1H, m), 7.23 (2H, m), 6.20 (1H, s), 3.64 (3H, s); ESI(+)MS *m/z* 317 $[M + Na]^+$; HRESI(+)MS *m/z* 317.0793 ($[M + Na]^+$, $C_{18}H_{14}O_4$Na requires 317.0790).

Oxidation of Gymnoascolide C (3). A sample of **3** (0.7 mg) was oxidized in the same manner as described above, yielding (−)-**4** (0.7 mg, 100%) as a colorless oil: $[\alpha]_D$ −160° (*c* 0.014, $CHCl_3$); UV (EtOH) λ_{max} (ϵ) 254 (6200) nm; 293 (sh) (3000); CD (MeOH) λ_{max} (ϵ) 233 (34000), 307 (−15000) nm; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.78 (2H, m), 7.50 (1H, m), 7.45 (2H, m), 7.33 (2H, m), 7.28 (1H, m), 7.23 (2H, m), 6.20 (1H, s), 3.64 (3H, s); ESI(+)MS *m/z* 317 $[M + Na]^+$; HRESI(+)MS *m/z* 317.0784 ($[M + Na]^+$, $C_{18}H_{14}O_4$Na requires 317.0790).

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