Bombardolides: New Antifungal and Antibacterial γ-Lactones from the Coprophilous Fungus *Bombardioidea anartia*

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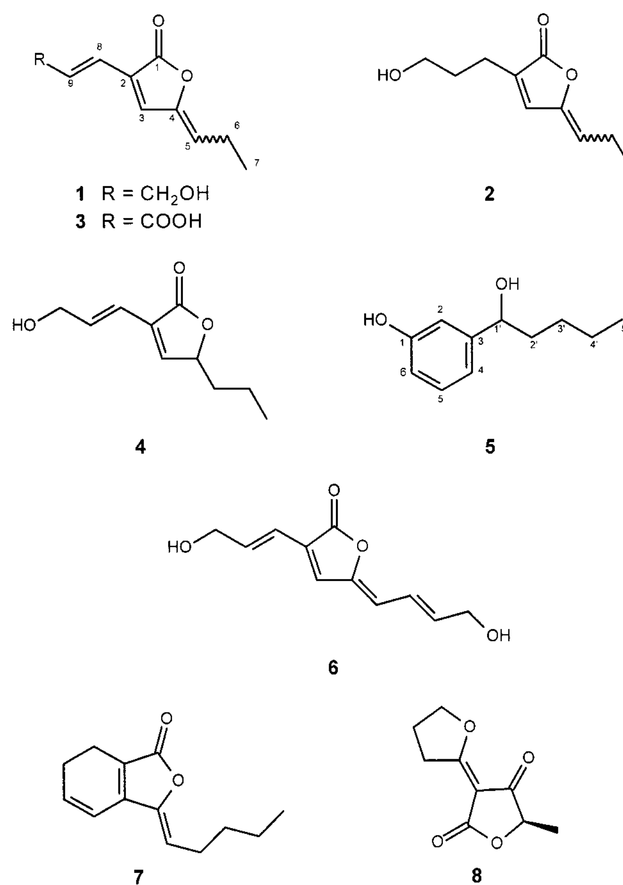
Chemical studies of organic extracts from cultures of the coprophilous fungus *Bombardioidea anartia* have led to the discovery of bombardolides A−D (**1**−**4**), a series of new antifungal and antibacterial metabolites. Three of these metabolites (**1**−**3**) were obtained as inseparable pairs of geometric isomers. A new 3-substituted phenol (**5**) and the known compound asterriquinone B4 were also encountered. The structures of compounds **1**−**5** were determined by analysis of NMR and MS data.

Interference competition among fungi often involves the production of secondary metabolites by one species that deter the growth of competitors. Coprophilous fungi frequently exhibit these types of antagonistic effects,[1] and our chemical studies of such phenomena have led to the isolation of a variety of new antifungal agents.[2−4] During our continuing studies of antagonistic coprophilous species, an extract from cultures of *Bombardioidea anartia* Krug and Scott (JS 272; Lasiosphaeriaceae, originally isolated from deer dung) exhibited activity against *Candida albicans.* Bioassay-guided fractionation of this extract led to the discovery of a group of new γ-lactones, several of which showed anti-*Candida* activity. Some of these compounds also displayed antibacterial effects. Details of the isolation and structure determination of these metabolites are presented here.

The combined EtOAc extracts from several *B. anartia* liquid cultures were subjected to repeated chromatography on silica gel and Sephadex LH-20. Silica gel and/or reversed-phase HPLC of the most active fractions from these separations afforded (*E/Z*)-bombardolide A (**1**), (*E/Z*)-bombardolide B (**2**), and (*E/Z*)-bombardolide C (**3**) as inseparable isomeric mixtures, along with bombardolide D (**4**) as a single entity. These extracts also afforded a new substituted phenol (**5**) and the known compound asterriquinone B4.[5,6] A variety of methods were employed in efforts to separate the isomeric mixtures of bombardolides A−C (**1**−**3**), including argentation chromatography[7] and HPLC using several different combinations of stationary and mobile phases. Unfortunately, none of these approaches were successful in even partially resolving the isomeric pairs. ^{1}H NMR spectra of **1** in different solvents (MeOH-d_4 and acetone-d_6) showed the same integration ratio as that observed when using $CDCl_3$. Despite this complication, it was possible to identify all of these compounds by analysis of NMR and MS data.



The *E* and *Z* forms of bombardolide A were assigned the molecular formula $C_{10}H_{12}O_3$ (five degrees of unsaturation) on the basis of ^{13}C NMR and HRFABMS data for the mixture of isomers [$(M + H)^+$ at *m/z* 181.0865, Δ 1.2 mmu]. Integration of the signals in the ^{1}H NMR spectrum revealed that the isomers were present in a 6:1 ratio. The ^{1}H NMR data for each isomer (Table 1) indicated the presence of an isolated vinylic proton, an ethyl group vicinal to a second vinylic proton, and a pair of trans-oriented olefinic protons, one of which was coupled to an oxymethylene group. In addition to signals consistent with these units, the ^{13}C NMR spectrum revealed that each isomer contains one ester or acid carbonyl group. These units accounted for all but one of the unsaturations required by the molecular formula, requiring **1** to be monocyclic.

Selective INEPT data (Table 1) enabled connection of these spin systems. The vinylic proton doublet of the major isomer at δ 6.40 (H-8) was correlated to the carbonyl carbon (C-1), to two protonated sp^2 carbons (C-3 and C-9), to a nonprotonated sp^2 carbon (C-2), and to the oxymethylene

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Table 1. ^{1}H and ^{13}C NMR Data for (*E/Z*)-Bombardolide A (**1**) in $CDCl_3$

	E-isomer (minor)		*Z*-isomer (major)		
position	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	selective INEPT correlations for *Z*-isomer (C#)
1		168.8		168.8	
2		124.4		127.3	
3	7.29 (s)	131.0	7.01 (s)	135.3	2, 4, 5
4		146.9		147.9	
5	5.69 (t, 8.7)	116.1	5.23 (t, 8.4)	118.4	3, 4, 7
6	2.27 (dq, 8.7, 7.8)	20.2	2.38 (dq, 8.4, 7.5)	19.9	
7	1.09 (ov. m)	14.5	1.06 (t, 7.5)	13.6	
8	6.43 (dt, 16, 1.8)	117.6	6.40 (dt, 16, 1.7)	118.3	1, 2, 3, 9, 10
9	6.94 (m)	137.4	6.93 (dt, 16, 4.8)	136.7	
10	4.31 (m)	62.4	4.30 (dd, 4.8, 1.7)	63.0	

carbon (C-10). Polarization transfer to the C-3 signal was also observed when the vinylic proton triplet at δ 5.23 (H-5) was irradiated. Irradiation of the isolated vinylic signal (δ 7.01; H-3) afforded correlations to C-2, C-4, and C-5. Taken together, these results required that the C2–C3 double bond be conjugated with the carboxyl carbonyl and with the other two olefinic units.

At this point, construction of a ring and placement of the exchangeable proton remained in order to complete the structure. The chemical shifts of C-4 and C-10 suggested that these carbons were oxygenated. Connection of the carbonyl group to the oxygen at C-4 to form a butenolide provided the only possible structure that was consistent with the evidence for a trans C8–C9 double bond and the presence of an oxygenated vinylic group at C4–C5. These data permitted assignment of the structure of the major bombardolide A (**1**) isomer. The structure of the minor isomer was established by comparison of its NMR data (Table 1) with those corresponding to the major isomer. The only significant difference in these data was observed for the chemical shift for the H-5 signal of the minor isomer, which appeared downfield at δ 5.69.

To assign the C4–C5 double bond stereochemistry for the two isomers, difference NOE experiments were performed on the well-resolved H-5 signals. Irradiation of the signal at δ 5.23 (major) produced a 4% enhancement of the corresponding H-3 olefinic signal at δ 7.01, while irradiation of the signal at δ 5.69 (minor) did not result in any significant signal enhancement of the H-3 signal at δ 7.30. Therefore, the major component of the mixture was assigned as the *Z*-isomer, and the minor component was assigned as the *E*-isomer.

The NMR data for (*E/Z*)-bombardolide B (**2**) were very similar to those of **1**, except for the replacement of the trans olefinic proton signals with resonances characteristic of a CH_2CH_2 unit. ^{13}C NMR and HRFABMS data permitted assignment of the molecular formula $C_{10}H_{14}O_3$ [$(M + H)^+$ at *m/z* 183.1014, Δ 0.7 mmu]. These data, together with analysis of ^{1}H NMR coupling patterns and δ-values, confirmed the structure of (*E/Z*)-bombardolide B as **2**. In this case, an *E:Z* ratio of 2:1 was observed.

^{13}C NMR and HREIMS data for a third related metabolite, (*E/Z*)-bombardolide C (**3**), indicated the molecular formula $C_{10}H_{10}O_4$ (M^+ at *m/z* 194.0589, Δ 1.0 mmu). The NMR data for **3** closely matched those of **1** (including the ratio of signals), except for the absence of the oxymethylene unit, the presence of a second carboxy carbonyl, and simplification of the H-9 signal to a doublet ($J = 16$ Hz). Irradiation of the H-8 signal in a selective INEPT experiment produced correlations to both carbonyl carbons (δ 168.2 and δ 167.2). These data indicated that (*E/Z*)-bombardolide C (**3**) contains a carboxylic acid unit in place of the oxymethylene group present in **1**.

The final member of this group of metabolites, bombardolide D (**4**), was isolated as a single entity. A molecular formula of $C_{10}H_{14}O_3$ (4 degrees of unsaturation) was established by analysis of ^{13}C NMR and HRFABMS data [$(M + H)^+$ at *m/z* 183.1012, Δ 0.4 mmu]. The NMR similarities between **4** and (*E/Z*)-bombardolide A (**1**) enabled straightforward assignment of its structure. The differences in the ^{13}C and ^{1}H NMR spectra were consistent with replacement of the exocyclic ethyl-substituted olefin moiety with an *n*-propyl group coupled to an oxygenated methine. The location of this unit was confirmed through selective INEPT and homonuclear decoupling experiments. Selective INEPT irradiation of the H-8 signal resulted in correlations to C-1, C-3, and C-10, indicating that this portion of the molecule matched that of bombardolide A (**1**). When the oxymethine triplet at δ 4.94 (H-4) was irradiated, a lone correlation to C-3 was observed, supporting the connection of C-4 to C-3. Further evidence for this linkage was provided by a homonuclear decoupling experiment, in which irradiation of H-4 resulted in a sharpening of the H-3 signal. The minimal coupling between the vicinal protons H-3 and H-4 is consistent with a Dreiding model representation, which confirmed that the vicinal angle between these two protons is approximately 90°. These results led to the assignment of structure **4** for bombardolide D.

The molecular formula of compound **5** was assigned as $C_{11}H_{16}O_2$ on the basis of HREIMS and ^{13}C NMR data. The NMR data for **5** were consistent with the presence of a 3-substituted phenol and a 1-hydroxypentyl group. The structures of these units were verified by homonuclear decoupling and selective INEPT experiments, leading to straightforward assignment of structure **5** for this metabolite. Despite its simplicity, compound **5** has not been previously reported as a natural product to our knowledge. Although compounds **4** and **5** show optical rotations, the absolute configurations of these metabolites were not assigned.

The ^{1}H and ^{13}C NMR spectra of a sixth compound contained two sets of very similar signals and were reminiscent of the asterriquinone family of fungal metabolites. Comparison of the spectra to literature data enabled identification of this compound as asterriquinone B4.[5,6] The doubling of NMR signals for this compound is consistent with the presence of a symmetrical structure with restricted rotation caused by steric effects.

The closest known structural analogue to the bombardolides appears to be lissoclinolide (**6**), an antibacterial metabolite isolated from the tunicate *Lissoclinum patella*.[8] Compound **6** has also been reported as tetrenolin, a metabolite of the actinomycete *Micropolyspora venezuelensis*, although the geometry at the exocyclic olefin unit was not assigned in the latter instance.[9,10]

Although there are precedents for the occurrence of natural products as inseparable mixtures of geometric isomers (e.g., gelastatins),[11] our inability to separate the isomeric pairs of **1**−**3** prompted us to survey the literature for other reports of inseparable mixtures of similarly unsaturated butenolides. One example, ligustilide (**7**), has been isolated from plants of the genera *Ligusticum* and *Angelica*. This compound was obtained in one case as the *Z*-isomer,[12] but in another as an inseparable *E*/*Z* mixture.[13] Interestingly, detailed studies described in the latter report revealed that reflux conditions led to a gradual increase in the percentage of the *E*-isomer at the expense of the *Z*-isomer, revealing that the two isomers of **7** can be equilibrated under suitable conditions. An additional precedent for the occurrence of such a mixture in a somewhat similar structural class is provided by the *Penicillium* metabolite carolic acid (**8**). Compound **8** was originally obtained as an inseparable *E*/*Z* mixture. It was later found that the *E*-isomer could be isolated by selective crystallization from EtOH, but was readily reequilibrated to the *E*/*Z* mixture upon exposure to a trace of acid or to light.[14] It is conceivable that such an equilibration process occurred at some stage prior to isolation of **1**−**3**, although isomeric mixtures were present in the initial extract on the basis of the ^{1}H NMR spectrum, and no such equilibration process was reported for lissoclinolide (**6**).

Most known fungal γ-lactones arise from the polyketide pathway, but the bombardolides do not appear to be regular polyketides. The common fungal γ-lactone penicillic acid is derived from an open-chain form of the corresponding δ-keto acid. Although a keto acid corresponding to an open-chain form of the bombardolides was not detected, these compounds seem most likely to arise through such an intermediate, which, in turn, could arise via oxidative cleavage of an aromatic polyketide precursor.

The antimicrobial activities of compounds **1**−**5** were evaluated in standard disk diffusion assays conducted at 200 μg/disk against *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 29213), and *Candida albicans* (ATCC 10231). Samples of **1**, **2**, **3**, and **5** produced zones of inhibition of 19, 11, 26, and 12 mm in diameter, respectively, against *B. subtilis*. Inhibition zones of 14, 18, and 19 mm were observed for compounds **1**, **3**, and **5**, respectively, in the assay against *S. aureus*. (*E*/*Z*)-Bombardolides A (**1**) and D (**4**) exhibited zones of inhibition of 35 and 20 mm against *C. albicans*, while bombardolides B and C were inactive at this concentration. Compounds **1**−**5** and asterriquinone B4 are the first metabolites to be reported from any member of the genus *Bombardioidea*.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker AC-300 spectrometer operating at 300 and 75 MHz, respectively. Residual protiated solvent signals for $CDCl_3$ and acetone-d_6 (δ 7.24/77.0 and δ 2.04/29.5) were used as internal references. Selective INEPT NMR experiments were performed at 75 MHz with $^nJ_{CH}$ optimized for 7 Hz. FABMS, HRFABMS, and HREIMS data were recorded on a VG ZAB-HF mass spectrometer. EI mass spectra were obtained using a VG Trio 1 instrument operating at 70 eV. HPLC separations of **1**, **2**, **4**, and asterriquinone B4 were accomplished using a Rainin Dynamax silica gel column (5 μm particles, 1.0 × 25 cm) at a flow rate of 3 mL/min with UV detection at 254 nm. Compounds **3** and **5** were isolated using a Beckman Ultrasphere C_{18} column (5 μm particles, 1.0 × 25 cm).

Fungal Material. The isolate of *B. anartia* was obtained from deer dung collected by L. J. Hutchison in the Wasatch National Forest, North Ogden, Utah, in May 1994. The culture was assigned the accession number JS 272 in the D. Malloch culture collection at the University of Toronto. The genus *Bombardioidea* was defined in 1972.[14] Irregularly shaped ascospores and brittle perithecia distinguish this genus from other taxa.

Fermentation, Extraction, and Isolation. Twelve 2 L Erlenmeyer flasks, each containing 400 mL of potato dextrose broth (Difco), were each inoculated with a 1 cm^2 agar plug taken from a stock culture of *B. anartia* maintained on potato dextrose agar. Flask cultures were incubated at 25−28 °C and aerated by agitation on an orbital shaker at 150 rpm for a period of 21 days. The culture filtrate was extracted with EtOAc, and the organic phase was evaporated to give a dark brown oil (960 mg). The extract was subjected to VLC on silica gel (5 × 5 cm) using a hexane/$CHCl_3$/MeOH gradient and collecting 300 mL fractions. The fourth (99:1 $CHCl_3$/MeOH, 328 mg) and fifth (98:2 $CHCl_3$/MeOH, 238 mg) fractions were combined and subjected to Sephadex LH-20 column chromatography, eluting with hexane/toluene/MeOH (3:1:1). Eight fractions (110 mL each) were collected and bioassayed. The fifth and sixth subfractions (280 mg) displayed antifungal activity and were combined and further purified by silica gel column chromatography using 95:5 $CHCl_3$/MeOH (500 mL) as the eluting solvent. Fractions were pooled on the basis of TLC behavior. Approximately 100 mg of the fifth fraction from this column (176 mg) was separated by normal phase HPLC using an EtOAc/hexane gradient solvent system (50−100% EtOAc in hexanes over 20 min) to yield compounds **1** (55 mg), **2** (4.1 mg), **4** (3.7 mg), and asterriquinone B4 (9.1 mg). Fractions six (25 of 61 mg) and nine (29 mg) from the VLC column were subjected to reversed-phased HPLC using a CH_3CN/H_2O gradient (30% CH_3CN in H_2O for 10 min, then 30−100 over 15 min), yielding compounds **3** (9.1 mg) and **5** (3.7 mg). Asterriquinone B4 was identified by comparison of its spectral properties (^{1}H NMR, ^{13}C NMR, and MS) with literature values.[5]

(*E*/*Z*)-Bombardolide A (1): pale yellow glass; mp 98−99 °C; HPLC t_R 10.4 min under the conditions above; UV (MeOH), λ_{max} 214 (ϵ 3100), 260 nm (ϵ 3800); IR ($CHCl_3$) 3495, 2973, 1769, 1069 cm^{-1}; EIMS *m*/*z* 180 (M$^+$; rel int 32), 151 (46), 136 (31), 125 (32), 110 (56), 81 (73); ^{1}H NMR, ^{13}C NMR, and selective INEPT data, see Table 1; HRFABMS (PEG 200/glycerol/1% TFA) $(M + H)^+$ at *m*/*z* 181.0877, calcd for $C_{10}H_{12}O_3$ + H, 181.0865.

(*E*/*Z*)-Bombardolide B (2): pale yellow glass; mp 94−96 °C; HPLC t_R 13.2 under the conditions above; UV (MeOH), λ_{max} 220 (ϵ 2900), 268 nm (ϵ 2900); IR ($CHCl_3$) 3441, 2935, 1772 cm^{-1}; EIMS *m*/*z* 182 (M$^+$; rel int 14), 164 (16), 138 (29), 112 (100), 111 (83), 98 (68), 84 (96); ^{1}H NMR ($CDCl_3$) *Z*-isomer, δ 6.98 (br s, H-3), 5.15 (t, *J* = 7.8, H-5), 2.38 (dq, 7.8, 7.8, H_2-6), 1.07 (t, 7.8, H_3-7), 1.81 (ov. tt, 7.5, 6.2, H_2-9), 2.46 (br t, 7.5, H_2-8), 3.65 (t, 6.2, H_2-10); *E*-isomer, δ 7.28 (m, H-3), 5.64 (t, *J* = 8.2, H-5), 2.25 (dq, 8.2, 7.8, H_2-6), 1.09 (t, 7.8, H_3-7), 1.83 (ov. tt, 7.5, 6.2, H_2-9), 2.48 (br t, 7.5, H_2-8), 3.66 (t, 6.2, H_2-10); ^{13}C NMR ($CDCl_3$) *Z*-isomer, δ 171.0 (C-1), 132.8 (C-2), 137.6 (C-3), 148.0 (C-4), 116.8 (C-5), 19.7 (C-6), 13.6 (C-7), 30.7 (C-8), 21.4 (C-9), 61.5 (C-10); *E*-isomer, δ 170.5 (C-1), 133.3 (C-2), 135.9 (C-3), 145.8 (C-4), 116.0 (C-5), 20.0 (C-6), 13.6 (C-7), 30.3 (C-8), 21.6 (C-9), 70.6 (C-10); HRFABMS (PEG 200/glycerol/1% TFA) $(M + H)^+$ at *m*/*z* 183.1014, calcd for $C_{10}H_{14}O_3$ + H, 183.1021.

(*E*/*Z*)-Bombardolide C (3): pale yellow solid; mp 174−176 °C; HPLC t_R 27.6 min under the conditions above; UV (MeOH) λ_{max} 212 (ϵ 2900), 246 (ϵ 3000), 314 nm (ϵ 8700); IR (CH_3CN) 3645 (sh), 3544, 3252, 1771, 1721 cm^{-1}; EIMS *m*/*z* 194 (M$^+$; rel int 100), 179 (10), 152 (51), 138 (44), 124 (28), 96 (61); ^{1}H NMR (acetone-d_6) *Z*-isomer, δ 7.86 (br s, H-3), 7.43 (dd, *J* = 16, 0.6, H-8), 6.91 (d, 16, H-9), 5.68 (t, 8.0, H-5), 2.42 (dq, 8.0, 7.8, H_2-6), 1.10 (t, 7.8, H_3-7), *E*-isomer, 8.24 (br s, H-3), 7.44 (dd, 16, 0.6, H-8), 6.94 (d, 16, H-9), 5.95 (d, 8.3, H-5), 2.43 (dq, 8.3, 7.8, H_2-6), 1.12 (t, 7.8, H_3-7); ^{13}C NMR (acetone-d_6) *Z*-isomer, δ 168.2 (C-1), 126.1 (C-2), 142.9 (C-3), 149.1 (C-4), 133.4 (C-8), 20.7 (C-6), 13.6 (C-7), 122.3 (C-5), 124.9 (C-9), 167.1 (C-10); selective INEPT (*Z*-isomer, acetone-d_6, H-# → C-#) H-3

→ C-2, 4, 8; H-5 → C-3, 4, 7; H-8 → C-1, 2, 3, 9, 10; H-9 → C-2, 10; *E*-isomer: δ 170.9 (C-1), 167.1 (C-10), 148.6 (C-4), 142.9 (C-3), 138.5 (C-8), 125.4 (C-2), 118.4 (C-5), 20.9 (C-6), 14.4 (C-7); HREIMS (M^+) at *m/z* 194.0589, calcd for $C_{10}H_{10}O_4$, 194.0579.

Bombardolide D (4): pale yellow glass; mp 93−94 °C; $[\alpha]_D$ −49° (*c* 0.007 g/mL, $CHCl_3$); HPLC t_R 12.0 min under the conditions above; UV (MeOH) λ_{max} 216 (ε 2600), 254 nm (ε 3000); IR ($CHCl_3$) 3500, 2930, 1752 cm^{-1}; EIMS *m/z* 182 (M^+; 2), 153 (15), 135 (29), 121 (39), 95 (38), 71 (100); ^1H NMR ($CDCl_3$) δ 7.13 (br s, H-3), 4.94 (br t, *J* = 4.8, H-4), 1.66 (m, H_2-5), 1.47 (m, H_2-6), 0.95 (t, 7.2, H_3-7), 6.35 (br d, 16, H-8), 6.95 (br dt, 16, 4.9, H-9), 4.29 (ov. m, H_2-10); ^{13}C NMR ($CDCl_3$) δ 171.8 (C-1), 128.8 (C-2), 148.0 (C-3), 80.7 (C-4), 35.5 (C-5), 18.4 (C-6), 13.8 (C-7), 118.2 (C-8), 135.7 (C-9), 63.0 (C-10); Selective INEPT ($CDCl_3$, H-# → C-#): H-4 → C-3; H-8 → C-1, 3, 10; HRFABMS (glycerol/3-NBA) $(M + H)^+$ at *m/z* 183.1012, calcd for $C_{10}H_{14}O_3$ + H, 183.1021.

3-(1′-Hydroxypentyl)phenol (5): pale yellow solid; mp 87−89 °C; $[\alpha]_D$ −20° (*c* 0.004 g/mL, acetone); HPLC t_R 18.4 min under the conditions above; UV (MeOH) λ_{max} 216 (ε 3500), 275 nm (ε 1600); IR (CH_3CN) 3499, 3414, 2938, 1602 cm^{-1}; EIMS *m/z* 180 (M^+, rel int 18), 162 (23), 133 (38), 123 (100), 95 (83); ^1H NMR (acetone-d_6) δ 8.11 (s, Ar-OH), 6.85 (m, H-2), 6.80 (br d, 7.8, H-4), 7.10 (dd, 8.1, 7.8, H-5), 6.67 (ddd, 8.1, 2.6, 1.0, H-6), 4.54 (m, H-1′), 3.96 (d, 4.1, 1′-OH), 1.65 (m, H_2-2′), 1.31 (ov. m, H_2-3′, H_2-4′), 0.86 (dd, 7.5, 6.7, H_3-5′); ^{13}C NMR (acetone-d_6) δ 158.1 (C-1), 149.1 (C-3), 129.7 (C-5), 117.9 (C-4), 114.4 (C-6), 113.6 (C-2), 74.1 (C-1′), 40.2 (C-2′), 28.8 (C-3′), 23.3 (C-4′), 14.3 (C-5′); selective INEPT (acetone-d_6, H-# → C-#): H-6 → C-1, 2, 4; H-1′ → C-2, 3, 4, 2′, 3′; HREIMS (M^+) at *m/z* 180.1140, calcd for $C_{11}H_{16}O_2$, 180.1150.

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