Altenuene Derivatives from an Unidentified Freshwater Fungus in the Family Tubeufiaceae

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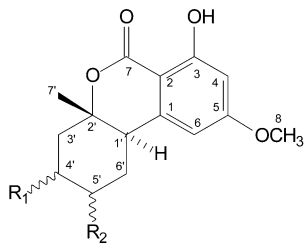
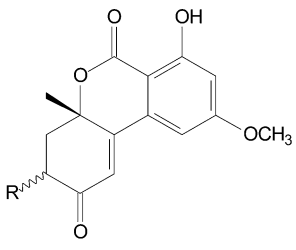
Four new altenuene derivatives called dihydroaltenuenes A (**1**) and B (**2**) and dehydroaltenuenes A (**3**) and B (**4**), along with five known compounds, including isoaltenuene (**5**), altenuene (**6**), and 5′-epialtenuene (**7**), were isolated from cultures of an unidentified freshwater aquatic fungal species in the family Tubeufiaceae. The structures of **1**−**4** were determined by analysis of NMR and MS data. The relative stereochemistry was determined on the basis of ^{1}H NMR *J*-values and NOE data, while the absolute configuration of a representative member of the group (**5**) was assigned by CD spectral analysis of its bis-*N*,*N*-dimethylaminobenzoate derivative. Compounds **1**, **3**, **4**, **5**, and **6** showed antibiotic activity against Gram-positive bacteria.

Fungi are well known as producers of secondary metabolites that display a variety of biological activities. However, several ecological groups of fungi remain underexplored as potential sources of new bioactive natural products. One such group includes fungi that specialize in freshwater aquatic habitats. Our research group has undertaken investigations of freshwater aquatic fungi, and extracts of such isolates have afforded a variety of bioactive metabolites.[1−4] In the course of this work, an unusual isolate identified only as a member of the family Tubeufiacieae was collected in the Cheoah River in the Great Smoky Mountains, Tapoco, North Carolina. Chemical studies of this isolate (A-00471) afforded four new altenuene derivatives (**1**−**4**) and five known compounds. Details of the isolation, structure elucidation, and stereochemical assignment of compounds **1**−**4** are presented here.



1 R_1 = β-OH R_2 = β-OH
2 R_1 = β-OH R_2 = α-OH



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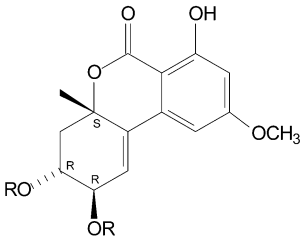
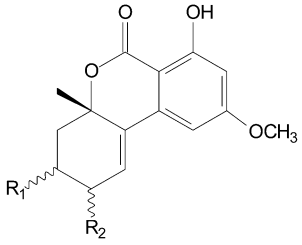
4 R = β-OH



5 R = H
5a R = *p*-bromobenzoyl
5b R = *p*-N,N-dimethylaminobenzoyl



6 R_1 = β-OH R_2 = α-OH
7 R_1 = β-OH R_2 = β-OH

Results and Discussion

The isolate was cultured by solid-substrate fermentation on rice. The EtOAc extract of the resulting cultures exhibited moderate antifungal activity against *Candida albicans* in disk assays and was selected for further investigation. In addition to **1**−**4**, studies of this extract afforded five known compounds: isoaltenuene (**5**), altenuene (**6**), 5′-epialtenuene (**7**), talaroflavone, and 7-hydroxy-2,5-dimethylchromone.[7−10] Altenuene and isomers thereof have been previously reported from isolates of the common fungal genus *Alternaria*. Altenuene, altertoxin, and alternariol are dibenzopyrone derivatives that have been reported to show antimicrobial activity and phytotoxicity.[5,6] The structures of the known compounds were confirmed by comparison of their NMR and MS data with literature values.[7−10] In addition, the relative stereochemical differences that distinguish diastereomers **5**−**7** were independently confirmed by analysis of ^{1}H NMR *J*-values and NOE data for each compound.

The molecular formula of dihydroaltenuene A (**1**) was determined to be $C_{15}H_{18}O_6$ (7 unsaturations) on the basis of an EIMS molecular ion at *m*/*z* 294, together with NMR data. Analysis of the NMR data suggested that **1** was a new altenuene analogue. One hydrogen-bonded OH signal at δ_H 11.30, two *meta*-coupled aromatic proton signals at δ_H 6.37 and 6.28, and one methoxy group (δ_H 3.84) are all characteristic of the aromatic portion of the altenuenes.[7,8] Comparison of the remaining signals with those of **5**−**7** showed the absence of the olefinic resonance and the presence of one additional methine proton signal at δ_H 3.06 and two methylene proton signals at δ_H 2.30 and 1.74, suggesting saturation of the C1′−C6′ double bond. This conclusion was supported by elucidation of a spin-system corresponding to the C-3′−C6′/C-1′ unit in **1** based on analysis of ^{1}H NMR data. HMBC correlations were observed from the methine proton at δ_H 3.06 (H-1′, J = 13 Hz) to C-2′, C-6′, and C-1 of the aromatic ring, and the methyl singlet at δ_H 1.40 displayed correlations to C-2′ and C-3′. Thus, the gross structure was established as shown in **1**.

The relative stereochemistry of **1** was assigned by analysis of relevant ^{1}H NMR *J*-values and NOE data. H-6′β appeared as a quartet with a coupling constant of 13 Hz, consistent with germinal and axial−axial couplings for a six-membered ring, requiring axial orientations for H-6′β, H-1′, and H-5′, and enabling assignment of the relative stereochemistry shown at C-1′ and C-5′. NOE correlations of the methyl signal at H_3-7′ to H-3′β and H-6′β suggested *trans*-fusion of the lactone ring and cyclohexane rings (Figure 1) and an axial orientation for CH_3-7′ and H-1′. The equatorial orientation proposed for H-4′ was consistent with the small vicinal coupling constants (2.3−3.0 Hz) observed between H-4′ and both H-3α and H-3β.

Dihydroaltenuene B (**2**) also displayed an EIMS molecular ion at *m*/*z* 294, suggesting that it is an isomer of compound **1**. The ^{1}H and ^{13}C NMR spectra were very similar to those of **1**, with the only significant difference in the ^{1}H NMR spectrum being a

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Table 1. ^{1}H NMR Data [δ_H (mult, J_{HH})] for Compounds **1**−**4** ($CDCl_3$, 300 MHz)

position	1	2	3	4
3-OH	11.30 (s)	11.36 (s)	11.38 (s)	11.32 (s)
4	6.37 (d, 2.3)	6.35 (d, 2.3)	6.64 (d, 2.3)	6.61 (d, 2.3)
6	6.28 (d, 2.3)	6.27 (d, 2.3)	6.71 (d, 2.3)	6.69 (d, 2.3)
8	3.84 (s)	3.84 (s)	3.87 (s)	3.89 (s)
1′	3.06 (br d, 13)	3.57 (dd, 13, 3.4)		
3′α	2.05 (dd, 14, 2.3)	2.42 (dd, 13, 4.4)	2.47 (t, 13)	2.83 (dd, 14, 6.0)
3′β	2.40 (dd, 14, 3.0)	2.12 (dd, 13, 2.6)	2.85 (dd, 13, 6.0)	2.40 (dd, 14, 7.3)
4′	4.22 (br m)	4.16 (m)	4.30 (dd, 13, 6.0)	4.44 (dd, 7.3, 6.0)
4′-OH[a]			3.55 (br s)	2.93 (br s)
5′	3.95 (br m)	4.10 (m)		
6′α	2.30 (dt, 13, 3.4)	2.25 (br d, 13)	6.53 (s)	6.43 (s)
6′β	1.74 (q, 13)	1.95 (td, 13, 2.6)		
7′	1.40 (s)	1.39 (s)	1.73 (s)	1.72 (s)

[a] Broad OH signals were observed for the 4′-OH in the spectra for **3** and **4**, but 4′-OH and 5′-OH signals were not discernible in the spectra of **1** and **2**.

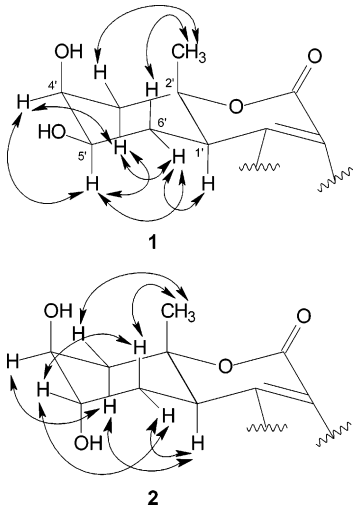

Figure 1. Key NOE correlations of dihydroaltenuenes A (**1**) and B (**2**).

Table 2. ^{13}C NMR Data (δ_C) for Compounds **1**−**4** ($CDCl_3$, 100 MHz)

position	1	2	3	4
1	142.4	143.6	134.6	135.8
2	101.6	102.4	100.5	102.6
3	165.2	164.9	165.1	165.2
4	104.1	104.2	104.3	105.9
5	166.5	166.4	166.6	166.9
6	99.0	98.9	105.3	105.9
7	169.1	169.1	168.0	167.8
8	56.0	55.9	56.3	56.3
1′	42.5	37.7	153.7	153.0
2′	83.3	83.9	81.0	80.1
3′	42.2	40.0	44.4	41.1
4′	69.1	69.6	70.9	68.8
5′	71.2	71.1	197.8	197.4
6′	28.1	27.7	121.2	124.2
7′	21.0	20.9	26.1	29.6

somewhat different splitting pattern ultimately attributed to a different relative configuration at C-5′. Specifically, the H-5′ and H-6′β signals were coupled with an axial−equatorial *J*-value rather than the *trans*-diaxial coupling observed in the spectrum for **1**. NOE correlations (Figure 1) and other relevant *J*-values were consistent with this being the only difference in the two structures.

Dehydroaltenuene A (**3**) was assigned the molecular formula $C_{15}H_{14}O_6$ (9 unsaturations) on the basis of EIMS and NMR data. The ^{1}H and ^{13}C NMR chemical shifts (Tables 1 and 2) matched reasonably well with those of the corresponding signals for the altenuenes and revealed the presence of the same structural features found in the altenuenes,[7,8] except that the oxygenated sp^3 methine carbon C-5′ was replaced by an α,β-unsaturated ketone carbonyl

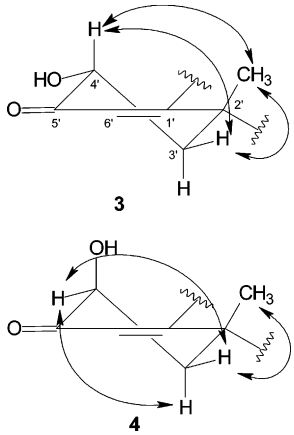

Figure 2. Key NOE correlations of dehydroaltenuenes A (**3**) and B (**4**).

signal at δ_C 197.4. Assignment of the structure as shown in **3** was enabled by analysis of HMBC data, including key correlations from H-6 to C-2, C-5, and C-1′; from H-6′ to C-2′, C-4′, and C-1; and from both H-3′α and H-3′β to the C-5′ carbonyl. The relative stereochemistry of **3** was assigned by analysis of coupling constants and NOE data. The signal for H-4′ showed one vicinal coupling constant (J = 14 Hz) characteristic of axial−axial-type coupling and a smaller one (J = 5.6 Hz) suggestive of axial−equatorial coupling, leading to the assignment of a pseudoaxial orientation for H-4′ as shown in Figure 2. Mutual NOE correlations observed among CH_3-7′, H-4′, and H-3′β indicated orientation of all three of these units on the same face of the molecule.

NMR and MS data indicated that dehydroaltenuene B (**4**) is an isomer of **3**, and the limited NMR differences (Tables 1 and 2) again suggested that the difference was in the relative stereochemistry at C-5′. The vicinal *J*-values observed for H-4′ (6.0 and 7.3 Hz) in comparison with those observed for dehydroaltenuene A (**3**) suggested a pseudoequatorial orientation of H-4′. These coupling constants, combined with NOE data (Figure 2), led to assignment of the relative stereochemistry of **4** as shown.

To our knowledge, absolute stereochemistry has not been assigned for altenuene or any of its previously reported stereoisomeric analogues. Interestingly, the original reports indicated that altenuene and 5′-epialtenuene were obtained as racemates,[9,11] and no optical rotation values for the isomers have been reported. In contrast to these reports, nonzero optical rotations were recorded for all three known altenuenes **5**−**7** obtained in this study, as well as the new analogues **1**−**4**. The absolute stereochemistry of the most abundant analogue isolated (isoaltenuene; **5**) was established by preparation of the corresponding bis-*N*,*N*-dimethylaminobenzoate and subsequent analysis of its circular dichroism (CD) spectrum.

Initially, dibromobenzoate **5a** was prepared. However, the CD curve obtained for **5a** (Figure 3) had an irregular shape and did

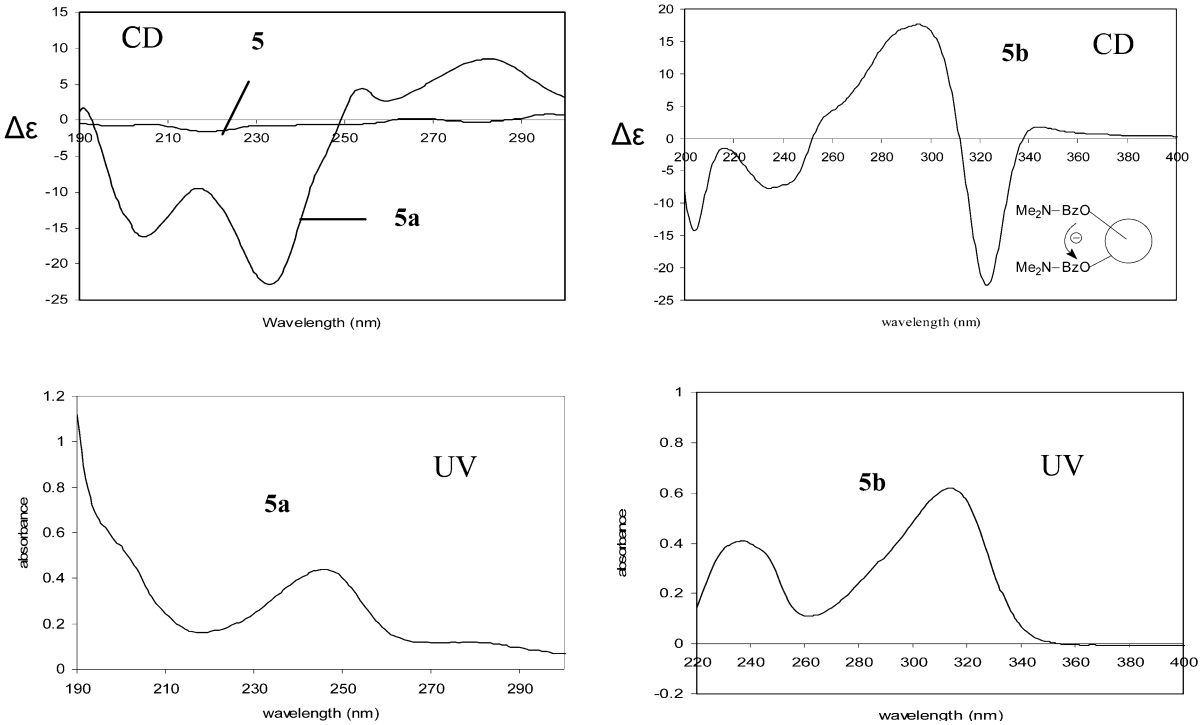

Figure 3. CD and UV spectra of **5a** and **5b** in acetonitrile.

not provide conclusive information, presumably due to interference with the nearby aromatic ring chromophore[8] already present in the starting material. Thus, the bis-*p*-*N*,*N*-dimethylaminobenzoate (**5b**) was prepared. The *N*,*N*-dimethylaminobenzoate chromophore is red-shifted, with absorbance centered at approximately 314 nm,[12] and therefore circumvented the interference problem observed for the dibromobenzoate. The CD spectrum of **5b** (Figure 3) was recorded in MeCN, and a clear negative bisignate Cotton effect was observed centered at the dimethylaminobenzoate chromophore λ_{max}. On the basis of the exciton chirality rule,[12] compound **5** was proposed to have the 2′*S*, 4′*R*, and 5′*R* absolute stereochemistry. Altenuene (**6**), 5′-epialtenuene (**7**), and compounds **1**−**4** are proposed to have the absolute stereochemistry shown based on presumption of the same configuration at C-2′.

Although the original extract showed modest antifungal activity, compounds **1**−**4** showed no activity in disk assays against *Aspergillus flavus* (NRRL 6541), *Fusarium verticillioides* (NRRL 25457), or *Candida albicans* (ATCC 14053) at 200 μg/disk. In antibacterial assays, compounds **1** and **4** were active against *Staphylococcus aureus* (ATCC 29213), each causing a 14-mm zone of inhibition at 100 μg/disk (standard = gentamicin sulfate; 35-mm zone at 25 μg/disk). Compounds **1**, **3**, and **4** also showed activity against *Bacillus subtilis* (ATCC 6051) at the same level, affording zones of 50, 13, and 20 mm, respectively (standard = gentamicin sulfate; 32-mm zone at 25 μg/disk). Interestingly, compound **2** did not show activity in these assays, despite differing from **1** only in the relative stereochemistry at C-5′. None of the compounds showed significant activity against *Escherichia coli* (ATCC 25922) at this level. Isoaltenuene (**5**), altenuene (**6**), and 5′-epialtenuene (**7**) were also tested in these assays (at 100 μg/disk). Altenuene (**6**) showed activity against *S. aureus* and *B. subtilis*, causing zones of inhibition of 18 and 20 mm, respectively. Isoaltenuene (**5**) was active against *B. subtilis*, causing an inhibition zone of 30 mm, while 5′-epialtenuene (**7**) was inactive.

Experimental Section

General Experimental Procedures. Optical rotations were determined with a Rudolph automatic polarimeter, model AP III. UV spectra were recorded with a Varian Cary III UV−visible spectrophotometer, and CD spectra (0.1-cm cell) were collected using an Olis Cary-17 spectrometer. ^{1}H and ^{13}C NMR spectra were measured on Bruker DPX-300 and DRX-400 spectrometers, respectively. HMQC and HMBC data were measured on a Bruker AMX-600. HPLC was carried out using a Beckman system Gold HPLC system with a model 166 detector. Other general procedures and instrumentation have been described previously.[13]

Isolation, Cultivation, and Fermentation of Fungal Material. Species A-00471 was isolated from submerged, decorticated wood collected from the Cheoah River (fast flowing, 26 °C, pH 5), Tapoco, North Carolina, on July 18, 2000. Procedures used in collection and isolation have been described elsewhere.[14] On the basis of the morphological characteristics of this fungus, it belongs in the Dothideomycetes, Pleosporales, and Tubeufiaceae. Due to paucity of material from the field and absence of sexual reproduction in culture, the fungus could not be identified further with confidence. Species A-00471 is characterized by hyaline to tan, superficial, membranous, broadly conical ascomata; cylindrical fissitunicate asci; hamathecium of septate pseudoparaphyses; and 1−3 septate, narrowly fusoid, hyaline ascospores. A voucher specimen has been deposited in the University of Illinois Department of Plant Biology fungal collection with the accession number A-00471.

The fungus was subcultured onto 250 g of rice and incubated at 25 °C under 12 h light/12 h dark conditions for 5 weeks. The fermentation mixture was broken up with a spatula and extracted twice with EtOAc (2 × 500 mL). The combined EtOAc solution was filtered and evaporated to afford a crude extract (539 mg).

Extraction and Isolation. The crude extract was partitioned between hexanes and MeCN. The MeCN fraction (290 mg) was fractionated on a Sephadex LH-20 column using a hexanes/CH_2Cl_2/acetone solvent system to obtain 20 fractions. Fraction 10 (28 mg) was further separated by reversed-phase HPLC (30% MeCN/H_2O isocratic over 10 min, 30−70% over 15 min, 70−100% over 5 min) on an Alltech HS BDS 8 μm C_{18} column (10 × 250 mm) at a flow rate of 2 mL/min with UV detection at 215 nm to afford 5′-epialtenuene (**7**; 6.6 mg), talaroflavone[10] (3.6 mg), **1** (2.5 mg), and **3** (1.6 mg). Fraction 12 (33 mg) was separated by gradient elution using MeCN/H_2O under the same HPLC conditions to yield 7-hydroxy-2,5-dimethylchromone[10] (2.4 mg) and **4** (1.3 mg). A subfraction (19 mg) from fraction 12 obtained as a single HPLC peak was further purified by reversed-phase HPLC employing gradient elution (50% MeOH/H_2O isocratic over 30 min, then 50−100% over 10 min) on the same column with UV detection at 230 nm to afford altenuene (**6**; 5.0 mg), isoaltenuene (**5**; 11 mg), and **2** (1.9 mg).

Dihydroaltenuene A (1): colorless glass; $[\alpha]^{25}_D$ +20 (*c* 0.15, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 305 (3.9); 270 (5.3); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HMBC data, H-4 → C-2, 3, 5, 6; H-6 → C2, 4, 5, 1′; H-8 → C-5; OH-3 → C-2, 3; H-1′ → C-1, 2′, 6′; H-3′ → C-1′, 2′, 4′, 5′; H-4′ → C-2′; H-6′ → C-1′, 2′, 5′; H-7′ → C-2′, 3′; EIMS *m*/*z* 294 (M^+; 74), 240 (100), 229 (21), 212 (50); HREIMS *m*/*z* 294.1109 (calcd for $C_{15}H_{18}O_6$, 294.1103).

Dihydroaltenuene B (2): colorless glass; $[\alpha]^{25}_D$ +14 (*c* 0.085, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 304 (4.0), 269 (4.3); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m*/*z* 294 (M^+; 100), 279 (20), 240 (80); HREIMS *m*/*z* 294.1105 (calcd for $C_{15}H_{18}O_6$, 294.1103).

Dehydroaltenuene A (3): colorless glass; $[\alpha]^{25}_D$ +12 (*c* 0.032, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 300 (3.7), 253 (4.1); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HMBC data, H-4 → C-2, 3, 6; H-6 → C-2, 4, 5, 1′; H-8 → C-5; OH-3 → C-2, 3, 4; H-3′→ C-2′, 4′, 5′, 7′; H-4′ → C-3′; H-6′ → C-1, 2′, 4′; H-7′ → C-1′, 2′, 3′; EIMS *m*/*z* 290 (M^+; 26), 245 (80), 217 (100), 202 (13), 174 (29); HREIMS *m*/*z* 290.0793 (calcd for $C_{15}H_{14}O_6$, 290.0790).

Dehydroaltenuene B (4): colorless glass; $[\alpha]^{25}_D$ +65 (*c* 0.026, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 300 (3.9), 253 (4.2); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m*/*z* 290 (M^+; 14), 246 (25), 217 (100), 175 (55); HREIMS *m*/*z* 290.0780 (calcd for $C_{15}H_{14}O_6$, 290.0790).

Isoaltenuene (5): $[\alpha]^{25}_D$ +22 (*c* 0.54, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 243 (4.4); 281 (3.9); 325 (3.7); CD (MeCN) $\Delta\epsilon$ 220 (−1.6), 256 (−0.5). EIMS, ^{1}H NMR, and ^{13}C NMR data matched those previously reported.[8]

Altenuene (6): $[\alpha]^{25}_D$ −7.8 (*c* 0.205, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 240 (4.4); 278 (3.9); 319 (3.7). EIMS, ^{1}H NMR, and ^{13}C NMR data matched those previously reported.[7]

5′-Epialtenuene (7): $[\alpha]^{25}_D$ +18 (*c* 0.185, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 240 (4.5); 278 (3.6); 319 (3.3). EIMS, ^{1}H NMR, and ^{13}C NMR data matched those previously reported.[7]

Preparation of Dibromobenzoate Ester 5a. A solution of **5** (5.7 mg, 0.020 mmol) in CH_2Cl_2 (2 mL) was treated with *p*-bromobenzoyl chloride (9.8 mg, 0.045 mmol) and DMAP (1 crystal). After stirring at ambient temperature for 24 h, saturated $NaHCO_3$ was added. The reaction mixture was extracted with CH_2Cl_2. The combined organic layer was evaporated to dryness and separated by reversed-phase HPLC (MeCN/H_2O 40−100% over 30 min) to afford **5a** (3.7 mg) as a white solid: UV (MeCN) λ_{max} (log ϵ) 244 (4.9); CD (MeCN) $\Delta\epsilon$ 233 (−23), 254 (+4.5), 260 (+2.6), 282 (+8.5); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.34 (s, 3-OH), 6.56 (d, J = 2.3 Hz, H-6), 6.50 (d, J = 2.3 Hz, H-4), 6.20 (d, J = 2.7 Hz, H-6′), 6.09 (dd, J = 8.4, 2.7 Hz, H-5′), 5.56 (ddd, J = 12, 8.4, 4.0 Hz, H-4′), 3.85 (s, H-8), 2.64 (dd, J = 12, 4.0 Hz, H-3′β), 2.54 (t, J = 12 Hz, H-3′α), 1.76 (s, H-7′); EIMS *m*/*z* 660 [(M + 4)$^+$; 0.02], 658 [(M + 2)$^+$; 0.04], 656 (M^+; 0.02), 458 (8), 456 (14), 182 (100).

Preparation of Bis-*N*,*N*-dimethylaminobenzoate Ester 5b. In the same fashion, compound **5** (3.0 mg, 0.010 mmol) was treated with *p*-*N*,*N*-dimethylaminobenzoyl chloride (3.9 mg, 0.021 mmol) in CH_2Cl_2 (1 mL) to give the corresponding **5b** (0.7 mg) as a white solid: UV (MeCN) λ_{max} (log ϵ) 239 (4.1), 314 (4.4); CD (MeCN) $\Delta\epsilon$ 233 (−7.6), 256 (+3.0), 292 (17.3), 333 (−22.6); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.37 (s, 3-OH), 6.55 (d, J = 2.3 Hz, H-6), 6.48 (d, J = 2.3 Hz, H-4), 6.26 (d, J = 2.7 Hz, H-6′), 6.03 (dd, J = 8.4, 2.7 Hz, H-5′), 5.55 (ddd, J = 13, 8.4, 4.1 Hz, H-4′), 3.84 (s, H-8), 2.65 (dd, J = 13, 4.1 Hz, H-3′β), 2.47 (t, J = 13 Hz, H-3′α), 1.76 (s, H-7′); EIMS *m*/*z* 586 (M^+; 0.1), 438 (1), 164 (100), 148 (76).

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Supporting Information Available: ^{1}H NMR spectra for compounds **1**−**4** and ^{13}C NMR spectra for compounds **1** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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