Hydroxylations and Methylations of Quercetin, Fisetin, and Catechin by *Streptomyces griseus*

Mohammed Hosny,† Kajari Dhar, and John P. N. Rosazza*

Division of Medicinal and Natural Products Chemistry, Center for Biocatalysis and Bioprocessing, College of Pharmacy, The University of Iowa, Iowa City, Iowa 52242

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Preparative-scale biotransformation of quercetin (**1**), fisetin (**7**), and (+)-catechin (**12**) with *Streptomyces griseus* (ATCC 13273) resulted in the isolation and characterization of nine known hydroxylated and/or methylated (**2**−**6**, **8**, **9**, **11**, **13a**) metabolites and two previously unknown (**10** and **14**) metabolites. *S. griseus* catalyzed aromatic hydroxylations of rings A and B of quercetin and fisetin. Mono- and dimethoxy ring-B metabolites were obtained with all three substrates. Methylation appeared to occur only when catechol functional groups were present. Metabolite structures were established by FABMS, EIMS, and 1D and 2D NMR analysis.

Flavonoids are among the most ubiquitous groups of polyphenolic compounds in foods of plant origin. As integral constituents of the diet, they may exert a wide range of beneficial effects on human health, including protection against cardiovascular disease and certain forms of cancer.[1] Flavonoids likely produce such biological effects through their free radical-scavenging antioxidative activities and metal ion-chelating abilities.[2] Some flavonoids are more potent than ascorbic acid and tocopherols in scavenging reactive oxygen species. Three types of functionalities appear to increase flavonoid reactive oxygen species scavenging potential: a catechol functionality in ring-B; a C_2-C_3 double bond together with a 4-oxo-functional group;[3] and the presence of 3- and 5-hydroxyl groups on C- and A-rings, respectively.[4] Metabolic *O*-methylation of catechols to respective methoxyphenols decreases the potential for flavonoid redox cycling and oxidation to quinone intermediates.[5]

The ingestion, distribution, and subsequent metabolic disposition of some flavonoids by mammals have been characterized. In mammals, following ingestion and absorption, flavonoids such as quercetin,[6] naringin,[7] (−)-epigallocatechin gallate,[8] catechin,[9] genistein,[10] and daidzein[11] are commonly observed in urine or in bile as glucuronide or sulfate conjugates or methyl ethers. (+)-Catechin is converted in the liver to 3′-*O*-methyl-(+)-catechin glucuronide (**13b**), a major metabolite in both the bile[12] and the urine of the rat.[13] 3′-*O*-Methyl-(+)-catechin (**13a**) is also found as a major (+)-catechin metabolite in human urine.[14] (+)-Catechin was degraded by ring fission to hydroxyphenyl-γ-valerolactone in the rat[15] and in man.[16] Moreover, several flavonoids were substrates for cytochrome P450 in rat liver microsomes.[17]

Flavonoids undergo a wide array of microbial oxidation, reduction, conjugation, and deglycosylation reactions.[18] Bacteria and fungi cleave rhamnose from quercetrin and naringin and further degrade the aglycones by C-ring oxidative fission.[19,20] *Eubacterium limosum*, a strict anaerobe from the human intestinal tract, cleaves 4′-methoxyl groups from biochanin A, formononetin, and glycitein to give genistein, daidzein, and 6,7,4′-trihydroxyisoflavone, respectively.[21] Rao et al. showed that *Rhizobium* and *Bradyrhizobium* strains catalyzed C-ring cleavage of quercetin to phloroglucinol and protocatechuic acid.[22] Quercetinase, a dioxygenase from *Aspergillus flavus* grown on rutin, cleaves ring-C in flavones with a 2,3-double bond and a 3-hydroxyl group.[23−26] *Pseudomonas* sp. grown on (+)-catechin catalyzed initial 8-hydroxylation and subsequent A-ring degradation of the resulting 7,8-catechol.[27,28] Hydroxylations of biochanin A and formononetin by *Fusarium oxysporum* at positions 3′ and 4′ gave a variety of isoflavones, including genistein, daidzein, and pratensein.[29] With *Giberella* sp., (±)-flavanone underwent 4-ketone reduction, 4′-hydroxylation, and C-ring cleavage to chalcones.[30] Abul Hajj et al. examined microbial transformations of (±)-flavanone and (±)-isoflavanone[31] and showed several different hydroxylations, dehydrogenation to flavone, and C-ring fission to chalcones. With flavone and isoflavone, reactions were restricted to 3′- and 4′-hydroxylation and C-ring cleavage.[32] This group also examined the influences of ring-A hydroxyl group substitutions on C-4′-hydroxylation of flavones by *Streptomyces fulvissimus*[33] and of oxygen functional groups on the hydroxylation of flavanoids by *Absidia blackesleeana*.[34] With 5-hydroxyflavone as substrate, *S. fulvissimus* gave 3′- and 4′-hydroxylated metabolites and the unusual 4′-sulfation product as well.[15,35] We earlier demonstrated that the isoflavone genistein underwent extensive sequential hydroxylation and methylation by *Streptomyces griseus*.[36]

Quercetin (**1**), fisetin (**7**), and flavan-3-ols such as (+)-catechin (**12**) are among the most common flavonoids occurring in plant foods. As an ongoing study concerned with the metabolic transformation of flavonoids by *S. griseus*,[36] we focused on the abilities of this microorganism to catalyze hydroxylation and/or methylations of these abundant prototypical compounds. The purpose of this study was to examine microbial transformations as a means of preparing rare and useful flavonoid derivatives, which may be difficult-to-obtain mammalian metabolites. Herein, we report the production, isolation, and characterization of nine known (**2**−**6**, **8**, **9**, **11**, and **13a**) and two new (**10** and **14**) oxygenated and methylated metabolites of **1**, **7**, and **12**.

Results and Discussion

Microbial transformations of **1**, **7**, and **12** were conducted by our well-established protocol.[36] Preparative-scale incu-

* To whom correspondence should be addressed. Tel: (319)-335-4902. Fax: (31 9)-335-4901. E-mail: john-rosazza@uiowa.edu.
† Present address: Al-Azhar University, Faculty of Pharmacy, Department of Pharmacognosy, Cairo, Egypt.

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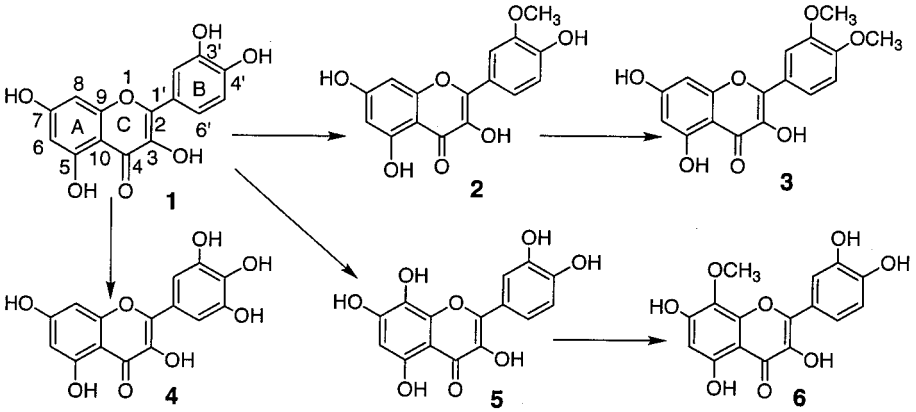
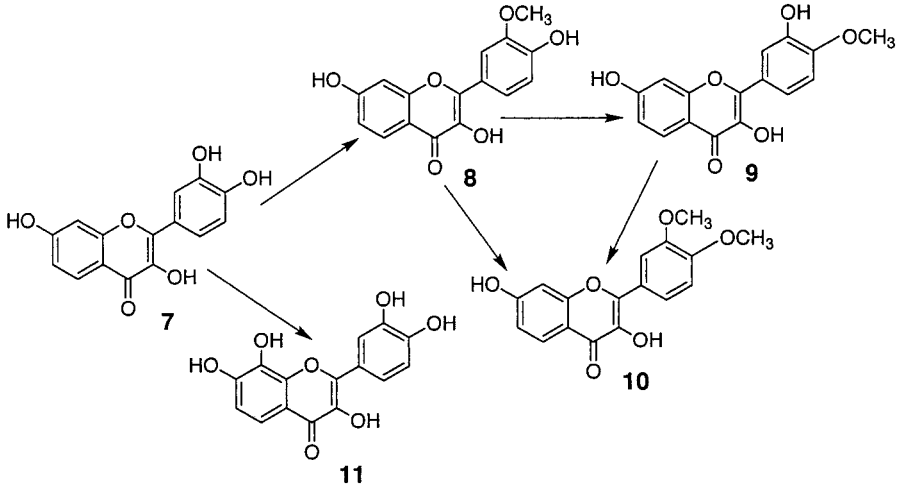
Scheme 1. Quercetin (**1**) Metabolites Produced by *Streptomyces griseus*.



Scheme 2. Fisetin (**7**) Metabolites Produced by *Streptomyces griseus*.



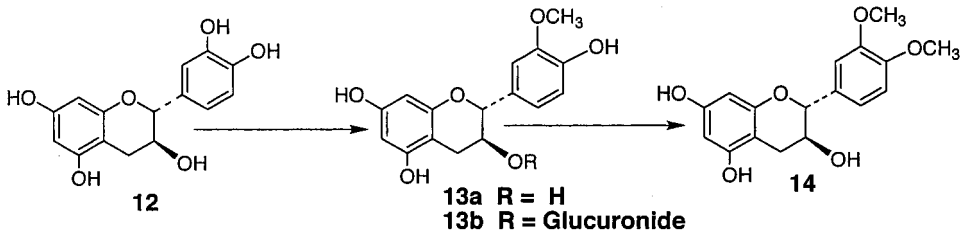
bations using 200 mg of each flavonoid substrate gave several metabolites that were isolated by solvent extraction and subsequent chromatography. In general, the EtOAc-soluble portion containing flavonoids was subjected to flash CC followed by Sephadex LH-20 column chromatography to give five metabolites from quercetin (**2**−**6**), four from fisetin (**8**−**11**), and two from catechin (**13a**, **14**). Metabolite identifications were based on spectral analysis and, where possible, by comparison with literature values for known compounds. The UV spectra of **2**−**6** and **8**−**12** were characterized by major absorptions resembling those of flavonols and flavan-3-ol for metabolites **13a** and **14**.[37,38] Metabolites **2**−**6**, **13a**, and **14** all possessed free hydroxyl groups at the 5- and 7-positions, indicated by bathochromic UV shifts induced by NaOMe, NaOAc, and $AlCl_3$/$AlCl_3$ + HCl. EIMS were diagnostic in that retro Diels−Alder fragmentations gave major A- and B-ring fragment ions that usually showed where hydroxylation and/or methylation occurred.[38] Complete ^1H and ^{13}C NMR spectral analyses using HMBC and HMQC were used in confirming the structures of microbial metabolites that were known compounds and unknown.

Quercetin Metabolites. All of the microbial transformation products of quercetin (**1**) were known flavonoids with UV/visible, ^1H and ^{13}C NMR, and mass spectral analytical results similar to those reported in the literature. Metabolite **2** was obtained by microbial regiospecific methylation of **1** at position 3′ to give isorhamnetin,[37,39] 3,5,7,4′-tetrahydroxy-3′-methoxyflavone. Metabolite **3** was identical to dillenetin,[40] 3,5,7-trihydroxy-3′,4′-dimethoxyflavone, obtained by selective microbial methylation of **1** at positions 3′ and 4′. Metabolite **4** gave *m*/*z* 319 $[M + H]^+$ for $C_{15}H_{11}O_8$ by FABMS for a compound containing one additional hydroxyl group versus **1**. Spectral data showed that **4** was the same as myricetin,[37,38] or 3,5,7,3′,4′,5′-hexahydroxyflavone. Metabolite **5** was identical to the plant flavonoid gossypetin,[39] also known as 3,5,7,8,3′,4′-hexahydroxyflavone, indicating that *S. griseus* had catalyzed 8-hydroxylation of **1**. Quercetin metabolite **6** was characterized as 3,5,7,3′,4′-pentahydroxy-8-methoxyflavone, also known as 8-methoxyquercetin.[37−39,41]

Fisetin Metabolites. The ^1H NMR, ^{13}C NMR, NOE, and HMBC spectra of fisetin metabolites **8** and **9** all supported the structures of these metabolites as 3,7,4′-trihydroxy-3′-methoxyflavone (**8**), also known as the plant flavonoid geraldol, and 3,7,3′-trihydroxy-4′-methoxyflavone (**9**).[43] Fisetin metabolite **11** was completely comparable to 7,8,3′,4′-tetrahydroxyflavone.[38,39] HRFABMS of fisetin metabolite **10** gave *m*/*z* 315.0873 $[M + H]^+$ for $C_{17}H_{15}O_6$, or a structure containing two more methyl groups than **7**. EIMS fragments at *m*/*z* 163 (B-ring) and 137 (A-ring) showed that the two methyl groups were in ring-B. ^1H NMR and HMBC confirmed that rings-A and -C were essentially the same as those for **7**, **8**, and **9**. Signals for two methoxyl groups were observed at δ 3.93 and 3.84 ppm, and the expected AMX system confirmed a 3′,4′-dimethoxy placement in ring-B. Thus **10** was established unambiguously as 3,7-dihydroxy-3′,4′-dimethoxyflavone, a previously unknown flavonoid.

Catechin Metabolites. Catechin metabolite **13a** gave *m*/*z* 305 $[M + H]^+$ for $C_{16}H_{17}O_6$, indicating that *S. griseus* had introduced a methyl group into the structure of **12**. EIMS, ^1H and ^{13}C NMR, HMBC, and HMQC spectra showed a metabolite structure for **13a** identical to 3′-*O*-

Scheme 3. *Streptomyces griseus* (**13a**) and Mammalian (**13b**) Metabolites of (+)-Catechin (**12**).



methyl-(+)-catechin, a known mammalian metabolite of catechin.[14] In this reaction, *S. griseus* mimics mammalian catechin metabolism. *S. griseus* also gave metabolite **14** with *m/z* 319.1187 $[M + H]^+$ by HRFABMS for $C_{17}H_{19}O_6$, a previously unknown compound containing two more methyl groups than **12**. EIMS fragment ions at *m/z* 180 (B-ring) and 140 (A-ring) showed that the B-ring contained two methoxyl groups. 1D and 2D NMR spectra contained signals for two methoxyl groups, a 5,7-disubstituted A-ring and a 3′,4′-disubstituted B-ring, all indicating that metabolite **14** was 3′,4′-*O*-dimethyl-(+)-catechin.

S. griseus is well known for its abilities to catalyze aromatic hydroxylations of phenols to catechols, and to subsequently methylate the resulting phenolic functional groups.[36,44] With quercetin, fisetin, and catechin, *S. griseus* extended its repertoire for hydroxylation and methylation of flavonoid substrates. Hydroxylation at position-8 occurred with both quercetin and fisetin, but not with catechin. Ring-B regiospecific mono- and dimethylation of catechols occurred with all three substrates.

With *S. griseus*, methylation was observed only in metabolites that contained catechol moieties.[36,44] We recently isolated and characterized an *S*-adenosyl methionine-dependent, catechol-*O*-methyl transferase (COMT) from *S. griseus*.[45] This enzyme is the first COMT found in bacteria. Methylation by the pure enzyme occurred only with catechol substrates. Mammalian *O*-methyltransferases that are capable of methylating flavonoid catechols have been reported.[46] In mammals, *O*-methylation is a well-known detoxication pathway.[5] *S. griseus* appears to mimic mammalian metabolism in producing monomethyl ethers from catechol substrates that are prone to oxidation and redox cycling. Further similarity to mammalian metabolism was seen in the *S. griseus* COMT-mediated methylation of quercetin to form isorhamnetin[27] and catechin to form 3′-methylcatechin.[14] The metabolites dillenetin (**3**) and geraldol (**9**) are rare flavonoid derivatives characterized previously only from the flower heads of *Arnica chamissonis*[47] and from the heartwood of *Auntiza listerana*,[48] respectively. These results show that microorganisms are excellent models that simulate metabolic patterns observed in both plants and mammals.

Experimental Section

General Experimental Procedures. TLC was carried out on precoated Si gel 60 F254 (Merck) plates. Chromatograms were visualized by spraying developed plates with freshly prepared 0.5% $NaNO_2$, 0.5% sulfanilic acid in 2% HCl, and 5% NaOH in 50% ethanol. Equal volumes of $NaNO_2$ and sulfanilic acid solutions were mixed immediately prior to use and sprayed into plates, followed by NaOH and warming with a heat gun at 100 °C for 3 min, to give burnt-orange-colored phenolic compounds. Flash column chromatography was performed using J.T. Baker glassware with 40 μm silica gel (Baker) and Sepralyte C_{18}, 40 μm as the stationary adsorbent phase. Optical rotations were measured with a Jasco P-1020 polarimeter, Japan. UV spectra were determined with a Hitachi 340 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker NMR 360 spectrometer (Bruker Instruments, Billerica, MA), operating at 360.13 MHz (^{1}H) and 90.56 MHz (^{13}C), and a Bruker AMX-600 spectrometer operating at 600 MHz (^{1}H). All NMR spectra were obtained in acetone-d_6 using TMS as the internal standard, with chemical shifts (δ) expressed in ppm and coupling constants (J) in Hz. HMBC experiments were carried out using a Bruker AMX-600 spectrometer equipped with an IBM Aspect-2000 processor. NOE experiments were performed using the spectral subtraction technique (NOEDS). The sample for NOE measurements was degassed by bubbling argon through the solution for 40 min. FABMS spectra were performed on a Fisons VG-ZAB-HF reversed geometry (BE configuration, where B is a magnetic sector and E is an electrostatic analyzer) mass spectrometer.

Substrates. Quercetin, fisetin, and (+)-catechin were obtained from Sigma Chemical Company, St. Louis, MO. The purities of substrates were determined by TLC, UV, and ^{1}H NMR spectral analyses.

Microorganism. *S. griseus* (ATCC 13273) was maintained on Sabouraud maltose agar slants and stored in a refrigerator at 4 °C prior to use.

Analytical-Scale Biotransformations. Cultures were grown by a two-stage procedure in 25 mL of soybean meal glucose medium held in stainless steel-capped, 125 mL Delong culture flasks. The soybean meal glucose medium contained (in g/L) 20 dextrose, 5 yeast extract, 5 soybean meal, 5 NaCl, and 5 K_2HPO_4 in distilled water and was adjusted to pH 7.0 with 6 N HCl before being autoclaved at 121 °C for 15 min. Cultures were incubated with shaking at 250 rpm at 28 °C on a New Brunswick Scientific, Innova 5000 Gyrotory three-tier shaker. A 10% inoculum derived from 72 h old stage I cultures was used to initiate stage II cultures, which were incubated for 24 h before receiving 5 mg of substrates in 0.5 mL of *N,N*-dimethylformamide, and incubations were conducted as before. Substrate controls consisted of sterile medium and substrates incubated under the same conditions but without microorganism. Substrate-containing cultures were generally sampled by removing 3 mL of the entire culture at 24, 72, and 144 h after addition of substrate. After determination of pH, they were acidified to pH 2.0 with 6 N HCl and extracted with equal volume of EtOAc/n-BuOH, 9:1. The organic layers were separated by centrifugation for 1 min in a desk-top centrifuge and used for analysis, where 30−40 μL samples were spotted on TLC plates developed with $CHCl_3$/MeOH/H_2O, 80:20:2, and chromatograms were visualized by fluorescence quenching under 254 nm UV light and by spraying developed plates with diazotized sulfanilic acid reagent.

Preparative Biotransformations of Quercetin, Fisetin, and Catechin. Using 24 h old stage II cultures, a total of 200 mg of each substrate was distributed evenly among 20 125-mL DeLong flasks. Substrate-containing cultures were incubated for 144 h for quercetin and fisetin and 72 h for (+)-catechin, at which times the cultures were separately harvested and centrifuged at 7000*g* for 10 min in a Sorvall RC-5 Superspeed refrigerated centrifuge. The supernatants were adjusted to pH 2.0 with 6 N HCl and extracted with EtOAc/n-BuOH, 9:1, 3 × 500 mL. The organic extracts were washed with distilled water, dried over anhydrous sodium sulfate, and vacuum-concentrated to yield viscous residues of 162 mg from quercetin, 133 mg from fisetin, and 88 mg from catechin.

Isolation of Metabolites. The extracts were separately subjected to Si gel flash column chromatography (1.5 × 50 cm) eluted with CH_2Cl_2/MeOH (95:5→70:30) to yield three fractions, Q1 (47 mg), Q2 (52 mg), and Q3 (34 mg), from quercetin, two fractions, F1 (50 mg) and F2 (48 mg) from fisetin, and two fractions, C1 (35 mg) and C2 (32 mg) from catechin. Subsequently, each fraction was separately resolved by reversed-phase Sepralyte C_{18} Si gel flash column chromatography (1.2 × 50 cm) using a MeOH/H_2O gradient solvent system (40→80%) using column pressures of 0.28 kg/cm^2, at a flow rate of 2 mL/min while 3 mL fractions were collected. Final sample purifications were carried out by Sephadex LH-20 column chromatography eluted with MeOH to afford quercetin metabolites **2** (8 mg), **3** (7 mg), **4** (4 mg), **5** (8 mg), and **6** (7 mg); fisetin metabolites **8** (2 mg), **9** (3 mg), **10** (4 mg), and **11** (9 mg); and catechin metabolites **13a** (4 mg) and **14** (6 mg). All fisetin and quercetin metabolites were obtained as yellow powders. Catechin metabolites were colorless solids.

Metabolite 10: 4 mg; UV (MeOH) λ_{max} 267, 370, + NaOMe 276, 390 (reduced intensity), + $AlCl_3$ 265, 372, + HCl 261, 368, + NaOAc 279, 378, nm; ^{1}H NMR (acetone-d_6, 600 MHz) δ 7.84 (1H, d, J = 8.0, H-5) 6.51 (1H, d, J = 2.1 Hz, H-6), 6.63 (1H, d, J = 2.1 Hz, H-8), 7.50 (1H, d, J = 2.0 Hz, H-2′), 6.89 (1H, d, J = 8.0 Hz, H-5′), 7.62 (1H, dd, J = 8.0/ 2.0 Hz, H-6′), 3.84 (3H, s, 3′-OMe), 3.93 (3H, s, 4′-OMe), 10.65 (1H, s, 7-OH), 9.57 (1H, s, 3-OH); HRFABMS, m/z 315.0873 [M + H]$^+$ (calcd for $C_{17}H_{15}O_6$, 315.0869); ^{13}C NMR (acetone-d_6, 90.56 MHz) δ 146.5 (C-2), 138.2 (C-3), 172.8 (C-4), 126.5 (C-5), 114.6 (C-6), 159.8 (C-7), 103.5 (C-8), 155.5 (C-9), 114.2 (C-10), 124 (C-1′), 112.8 (C-2′), 148.5 (C-3′), 150.9 (C-4′), 116 (C-5′), 121.3 (C-6′), 56.2 (O-CH_3), 56.8 (O-CH_3).

Metabolite 14: 6 mg; $[\alpha]^{25}_D$ + 18.7° (MeOH, *c* 0.72); UV (MeOH) λ_{max} 230, 288, + NaOMe 240, 310 (dec) + $AlCl_3$ 238, 286, + HCl 239, 285, + NaOAc 242, 291, nm; ^{1}H NMR (acetone-d_6, 600 MHz) δ 4.62 (1H, d, J = 8.5, H-2) 3.96 (1H, m, H-3), 2.44 (1H, dd, J = 16.0/8.5 Hz, H-4ax), 2.81 (1H, dd, J = 16.0/5.5 Hz, H-4eq), 5.76 (1H, d, J = 2.0 Hz, H-6), 5.95 (1H, d, J = 2.0 Hz, H-8), 7.08 (1H, d, J = 2.0 Hz, H-2′), 6.95 (1H, d, J = 8.5 Hz, H-5′), 6.88 (1H, dd, J = 8.5/2.0 Hz, H-6′), 3.78 (3H, s, 3′-OMe) 3.75 (3H, s, 4′-OMe); HRFABMS, m/z 319.1187 [M + H]$^+$ (calcd for $C_{17}H_{19}O_6$, 319.1182); ^{13}C NMR (acetone-d_6 90.56 MHz) δ 82 (C-2), 67.9 (C-3), 27.8 (C-4), 156 (C-5), 96.2 (C-6), 156.6 (C-7), 95.5 (C-8), 157.7 (C-9), 100.5 (C-10), 132 (C-1′), 110.5 (C-2′), 149.2 (C-3′), 149.8 (C-4′), 112.4 (C-5′), 120.5 (C-6′), 55.6 O-CH_3), 56.3 (O-CH_3).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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