Antioxidant Hydroquinones Substituted by β-1,6-Linked Oligosaccharides in Wheat Germ

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Seven new compounds that demonstrate antioxidant properties, 4-hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**1**), 4-hydroxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**2**), 4-hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)β-D-glucopyranoside (**3**), 4-hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**4**), 4-hydroxy-3,5-dimethoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**5**), 4-hydroxy-3,5-dimethoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**6**), and 4-hydroxy-2-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**7**), were isolated from wheat germ. The structures were determined by spectroscopic and chemical methods. Compound **1** was the most abundant, ~2 mg isolated from each gram of wheat germ. The antioxidant activity of compounds **1**−**7** was determined by the Trolox equivalent antioxidant capacity assay, and **2** and **7** showed higher values than the other compounds. Compounds **1** and **3**−**6** reacted with the radical cation reagent within a few seconds, whereas **2** and **7** required several minutes for complete reaction. Compound **1** was shown to protect plasmid DNA from oxidative stress damage caused by hydrogen peroxide; this effect was concentration-dependent.

Reactive oxygen species are continuously produced in many free radical reactions *in vivo* and can cause oxidative damage of several biological molecules such as lipids, proteins, and nucleic acids, which leads to injury of cells and tissues associated with degenerative diseases. Thus, antioxidants have considerable importance as prophylactic and therapeutic agents against diseases in which oxidants or free radicals are involved. During the last decades, there has been an emerging interest in the development of new types of food products with higher antioxidant activity than traditional foods. The concept of using naturally occurring compounds as antioxidants is also strengthened by the restrictions over the use of synthetic antioxidants in food.[1]

Wheat is an important agricultural commodity and food ingredient across the world. The main constituents of wheat kernel are bran, germ, and endosperm.[2] More than 80% of whole grain mass is represented by endosperm, which consists mostly of starch. Nowadays, wheat flour, which is prepared from endosperm, is more commonly used in food products compared to wheat bran and germ, in spite of the higher content of antioxidants and other important phytochemicals in bran and germ.[3] The antioxidant activity of extracts from cereal products is commonly correlated with the content of phenolic compounds occurring in these cereals.[4,5] These compounds are mostly present in cereals in bound or glycosylated forms and may display some important biological effects.[6,7] The antioxidant fraction of wheat sprouts, for example, was shown to protect DNA from oxidative stress damage caused by hydrogen peroxide.[8]

In the present work, seven new hydrophilic compounds (**1**−**7**), showing antioxidant activity, have been isolated and characterized from wheat germ, among which 4-hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**1**) was the most abundant.

Results and Discussion

Wheat germ was extracted with aqueous 50% EtOH, and the resulting extract was dried and redissolved in aqueous 5% MeCN.

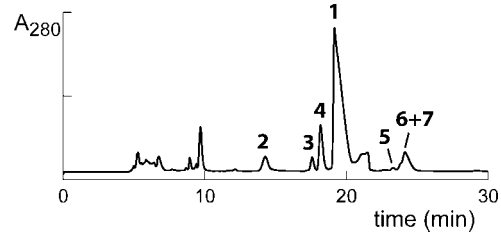
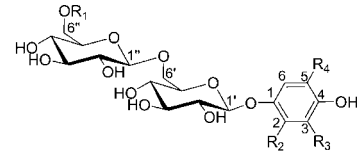


Figure 1. Preparative HPLC chromatogram of wheat germ extracted by aqueous 50% EtOH. The peaks corresponding to the fractions containing antioxidants **1**−**7** are annotated.

The extract was then fractionated by reversed-phase preparative HPLC using isocratic elution with aqueous 5% MeCN containing 10 mM HOAc (Figure 1). The fractions obtained were analyzed with respect to antioxidant activity using the Trolox equivalent antioxidant capacity (TEAC) method, which is based on the ability of antioxidants to scavenge the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$).[9]



1: R_1=β-D-Glc*p*, R_2=H, R_3=OCH_3, R_4=H
2: R_1=β-D-Glc*p*, R_2=H, R_3=H, R_4=H
3: R_1=β-D-Glc*p*-(1→6)-β-D-Glc*p*, R_2=H, R_3=OCH_3, R_4=H
4: R_1=H, R_2=H, R_3=OCH_3, R_4=H
5: R_1=H, R_2=H, R_3=OCH_3, R_4=OCH_3
6: R_1=β-D-Glc*p*, R_2=H, R_3=OCH_3, R_4=OCH_3
7: R_1=β-D-Glc*p*, R_2=OCH_3, R_3=H, R_4=H

From the HPLC, fractions corresponding to six peaks were found to display antioxidant activity. The fractions corresponding to the largest peak were pooled and further fractionated, leading to the isolation of compound **1**, which subsequently was characterized by spectroscopic and chemical methods. Similarly, compounds **2**−**7** were isolated from the other fractions displaying antioxidant activity.

The ^1H and ^{13}C NMR spectra of **1** contained signals at δ_H 5.04/δ_C 102.0, δ_H 4.48/δ_C 103.6, and δ_H 4.42/δ_C 103.6, which are

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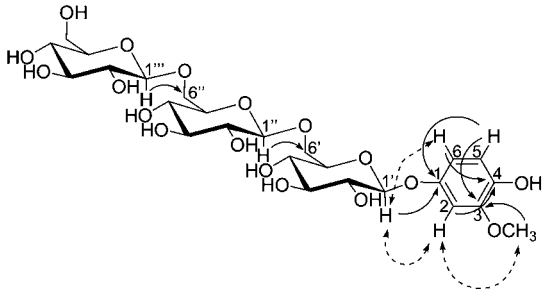
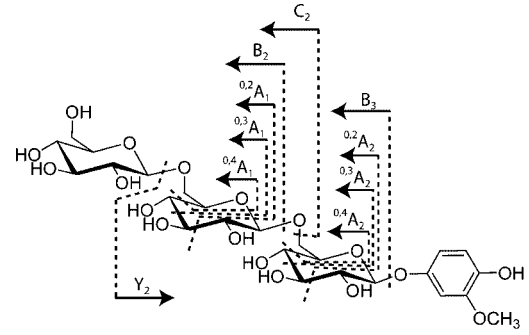


Figure 2. Selected HMBC (solid single-headed arrows) and ROESY (dashed double-headed arrows) correlations for compound **1**.

representative of signals for three anomeric protons and carbons, indicating the presence of three monosaccharide residues. The presence of D-glucose as the only monosaccharide residue in **1** was shown by the method of Gerwig et al.[10] This enantiomeric resolution method is based on the formation of trimethylsilylated (*S* or *R*)-2-butyl glycosides of the monosaccharide units, followed by GC-MS analysis. Subsequently, COSY, TOCSY, and HSQC experiments enabled the identification of the signals for H/C-1 to H/C-6 for all glucose residues. The H-1/H-2 couplings were ~7.9 Hz, whereas the H-2/H-3, H-3/H-4, and H-4/H-5 couplings were in the range 8−10 Hz, all in accordance with a β-glucopyranosidic configuration for all monosaccharide residues in **1**. This was also supported by ROESY data, which showed H-1/H-3 and H-1/H-5 correlations for all monosaccharide components. The chemical shifts of the signals for C-6′ (δ 69.7) and C-6″ (δ 69.3), and for C-6‴ (δ 61.6), indicated C-6′ and C-6″ to be linkage positions in an oligosaccharide.[11] The 1,6-linkage pattern was corroborated by HMBC experiments, which showed correlations between H-1″ and C-6′ and between H-1‴ and C-6″ (Figure 2). The combined data for the carbohydrate part of **1** is thus all in accordance with the presence of a β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl group. The ^{1}H NMR spectrum further contained signals at δ 6.88, 6.85, and 6.71 (one proton each), with a coupling pattern consistent with the presence of a 1,3,4-trisubstituted benzene ring. HMBC experiments demonstrated a cross-peak between the signal for H-1′ (δ_H 5.04) and a carbon signal at δ_C 151.5. This carbon also had an HMBC correlation to a proton at δ_H 6.88, i.e. a signal from a proton in the 1,3,4-trisubstituted benzene ring. Moreover, ROESY experiments showed cross-peaks between the signal for H-1′ and the signals at δ 6.85 (H-6) and 6.71 (H-2, Figure 2). Thus, the NMR data indicated that compound **1** was a β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside with a 1,3,4-trisubstituted phenyl aglycone group. The NMR data also showed the presence of signals at δ_H 3.86 (singlet, three protons) and δ_C 56.9, consistent with a methoxy group. The *O*-methyl protons had an HMBC correlation to a carbon at δ_C 148.9, to which there also was a cross-peak from H-5 at δ 6.88 (H-5, Figure 2). This is consistent with a methoxy group at C-3 of the 1,3,4-trisubstituted phenyl group. The presence of a methoxy group at C-3 was corroborated by a cross-peak to H-2 (δ 6.85) in the ROESY spectrum (Figure 2). The H-2 and H-6 signals at δ 6.85 and 6.71, respectively, showed HMBC cross-peaks to C-4 at δ_C 141.5, a chemical shift indicating a hydroxy group at C-4 of the phenyl group. The HRFAB mass spectrum of the compound showed the $[M + Na]^+$ ion at *m/z* 649.1904, which is consistent with a molecular formula of $C_{25}H_{38}O_{18}$, in accordance with a compound consisting of three hexose residues and a phenyl aglycone substituted by one methoxy and one hydroxy group. ESIMS/MS experiments yielded fragment ions consistent with this structure, including the diagnostic $^{0,4}A_1$ and $^{0,4}A_2$ fragment ions (nomenclature adapted from Domon and Costello[12]), which support the 1,6-linkage between the monosaccharide residues (Figure 3). Thus, the structure



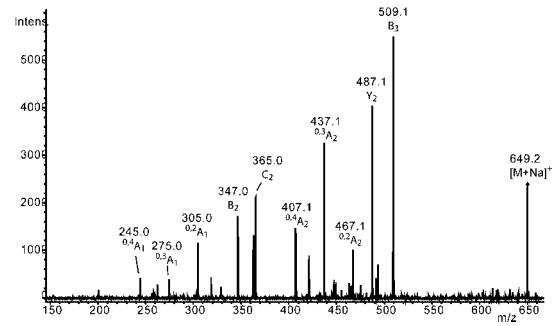


Figure 3. ESIMS/MS spectrum of compound **1** with tentative assignment of fragment ions (Na^+ adduct ions, the nomenclature for fragment ions according to Domon and Costello[12]).

of compound **1** was defined as 4-hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

The NMR data for compound **2** indicated the presence of a β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl group, as for **1**, but the aglycone group did not give any methoxy signal. Instead, there were two two-proton signals for aromatic protons at δ_H 6.88 and 7.08. The ROESY spectrum showed a cross-peak between H-1′ and the signal at δ_H 7.08, indicating the latter signal as H-2 and H-6, and, consequently, the signal at δ_H 6.88 is derived from H-3 and H-5. In the ESI mass spectrum, the $[M + Na]^+$ ion was found at *m/z* 619.2, which is 30 amu less than that of compound **1**, supporting the absence of a methoxy group. The MS/MS data for **2** were similar to the data for **1**, but the Y_2 ion was shifted to *m/z* 457 (*m/z* 487 for **1**), in accord with an aglycone without a methoxy group. This indicated the structure of **2** to be 4-hydroxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside. This structure was corroborated by ROESY, HMBC, and HRFABMS data.

For compound **3** the $[M + Na]^+$ ion was found at *m/z* 811.2, and the NMR data were similar to the data for **1**, except for the presence of signals from one extra glucose residue. This indicated that **3** contained four β-1,6-linked D-glucopyranosyl residues and that the structure was 4-hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside. The ESIMS/MS spectrum of **2** showed a similar fragment ion pattern to the ESIMS/MS spectrum of **1**, including ^{0,4}A ions at *m/z* 245, 407, and 569, diagnostic for the 1,6-linkages. The structure was supported by the combined NMR data, including inter-residual HMBC and ROESY correlations, as well as by the HRFABMS data.

The NMR data for **4**, including ROESY and HMBC data, were in all aspects similar to the data for **1**, except for the absence of signals from the third glucose residue, thus indicating it to be 4-hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside. This was supported by results of the ESIMS analysis of this compound, which showed an $[M + Na]^+$ ion at *m/z* 487.1.

Table 1. Antioxidant Activity (expressed as TEAC units ± SD) of Phenolic Glucosides Isolated from Wheat Germ and the Amounts of Compounds (mg) Purified from 1 g of Wheat Germ

	1	2	3	4	5	6	7
TEAC units	0.97 ± 0.03	1.12 ± 0.04[a]	0.88 ± 0.05	0.94 ± 0.04	0.85 ± 0.04	0.80 ± 0.06	1.76 ± 0.03[a]
amount (mg)	1.87	0.06	0.08	0.17	0.02	0.14	0.01

[a] These compounds reacted continuously with $ABTS^{\bullet+}$; values registered after 5 min are shown.

HRFABMS yielded an $[M + H]^+$ ion at *m/z* 465.1629, which is consistent with a molecular formula of $C_{19}H_{28}O_{13}$, hence in accordance with the proposed structure.

The $[M + Na]^+$ ion of compound **5** was found at *m/z* 517.2, i.e., 30 amu higher than for **4**, which indicated the presence of an extra methoxy group. The ESIMS/MS data showed basically the same fragment ions as for **4**, but the Y_2 ion was shifted from *m/z* 325 to *m/z* 355, indicating the extra methoxy group to be on the aglycone moiety. The NMR data for **5** supported a β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl group linked to the aglycone. The aromatic part of the ^{1}H NMR spectrum showed only one two-proton signal (δ_H 6.55, δ_C 96.4), indicating a symmetric substitution pattern. In the ROESY spectrum, the δ_C 6.55 signal displayed cross-peaks to H-1′ at δ_H 5.09 and to a singlet at δ_H 3.86 (six protons, δ_C 57.0). This strongly indicated the aglycone to be a 4-hydroxy-3,5-dimethoxyphenyl group. Thus, **5** was concluded to be 4-hydroxy-3,5-dimethoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside, which also was in accordance with the HRFABMS data. This structure was supported by the combined NMR data, including inter-residual HMBC and ROESY correlations.

The NMR data for compound **6** were very similar to the data for **5**, except for the presence of NMR signals for one additional glucose residue. Thus, the structure of **6** was determined to be 4-hydroxy-3,5-dimethoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside, which was corroborated by ROESY, HMBC, MS/MS, and HRFABMS data.

The ESIMS analysis of compound **7** showed an $[M + Na]^+$ ion at *m/z* 649.4, the same mass as that of compound **1**. The NMR data, including ROESY and HMBC data, showed the presence of a β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl group linked to an aglycone containing one hydroxy group and one methoxy group, just as in **1**. However, the chemical shifts of the signals for the three aromatic protons (δ 6.64, 6.48, and 7.13, for H-3, H-5, and H-6, respectively) were substantially different from the corresponding signals of compound **1**, but the coupling pattern was still in accord with a 1,3,4-trisubstituted benzene ring. A ROESY experiment showed cross-peaks between H-1′ and H-6 (doublet $J = 8.7$ Hz) and between H-3 (doublet $J = 2.7$ Hz) and the methoxy signal, suggesting the aglycone to be 4-hydroxy-2-methoxyphenyl, which also was supported by HMBC data. Thus, **7** was determined to be 4-hydroxy-2-methoxy β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside, which also was in accordance with MS/MS and HRFABMS data.

Compounds **1** and **3**−**7** have not been described previously, whereas **2** has been synthesized by chemical methods.[13] A related compound, 4-hydroxyphenyl β-D-glucopyranoside (arbutin), has been found in many different plants, e.g., in the African shrub *Myrothamnus flabellifolia*, where it is present at ∼20% by dry weight,[14] but also at trace levels (∼1 ppm) in wheat.[15] The corresponding methoxy-substituted compound, i.e., 4-hydroxy-3-methoxyphenyl β-D-glucopyranoside, has been isolated from wheat germ,[16] whereas 4-hydroxy-3,5-dimethoxyphenyl β-D-glucopyranoside has been described in the shrub *Canthium berberidifolium*.[17] The latter two glycosides have also been found with a β-1,6- or β-1,2-linked apiofuranosyl residue in different plants.[17−19] The shrub *C. berberidifolium* was also found to contain 4-hydroxy-2-methoxyphenyl β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside, i.e., a compound with the same aglycone structure as compound **7**.[17] A compound similar to **1** was previously reported to be isolated from wheat, but this compound was suggested to contain a cellotriose oligosaccharide, i.e., three 1,4-linked β-glucose residues, with a 4-hydroxy-3-methoxyphenyl aglycone group.[20,21] This compound was isolated based on its ability to cause solubilization of the glutenin I fraction.[20] The NMR data presented for this compound included only data for aromatic and anomeric protons. The data for these protons, however, closely resemble the data for compound **1**. Methoxyhydroquinone, the aglycone of compound **1**, has not been detected in wheat flour or germ in its free form, but only after acidic hydrolysis or treatment with β-glucosidases.[22]

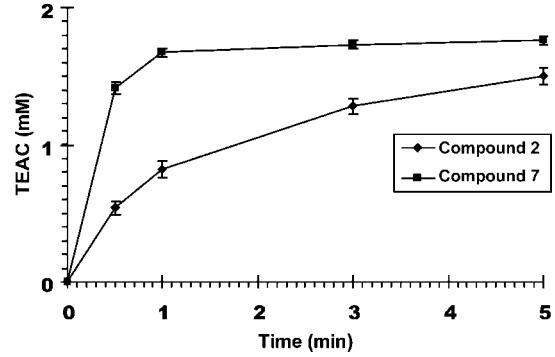


Figure 4. Kinetic curves of the reaction of compounds **2** and **7** with $ABTS^{\bullet+}$. The activity is expressed in TEAC units, i.e., as Trolox concentration (mM) displaying the same activity as 1 mM of the compound.

The antioxidant activity of compounds **1**−**7** (Table 1) was determined by the Trolox equivalent antioxidant capacity (TEAC) assay.[9] Compounds **1** and **3**−**6** reacted with $ABTS^{\bullet+}$ very rapidly; the reaction was complete in less than 15 s. All compounds showed similar activities between 0.8 and 1.0 TEAC units. Compounds **2** and **7**, however, showed a continuous reaction with $ABTS^{\bullet+}$ for at least 5 min (Figure 4), and their activities registered after 5 min were significantly higher than those of the other compounds.

The common structural feature of compounds **2** and **7** is the absence of a methoxy group *ortho* to the phenolic hydroxy group; all other compounds have at least one methoxy group in such a position. Thus, the methoxy group adjacent to the phenolic hydroxy group seems to accelerate the process of radical scavenging. This can be directly seen from comparison of the activities of compounds **1** and **2** registered at 15 s time point. Similarly, it was previously observed that ferulic acid, which has a methoxy group *ortho* to a hydroxy group, reacts with $ABTS^{\bullet+}$ faster than *p*-coumaric acid, which has no such group.[9] At the same time, the methoxy group *meta* to the hydroxy group, which is present only in compound **7** and thus makes it different from compound **2**, increases the final value of antioxidant activity and accelerates the reaction to some extent; the reaction of compound **7**, but not **2**, with $ABTS^{\bullet+}$ was almost complete after 1 min (Figure 4).

The antioxidant activities of arbutin and arbutin with one additional α(1→6)-glucopyranosyl group were previously compared using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging method.[23,24] These studies indicated that the glycoside with a disaccharide had a 16−20% lower antioxidant activity than arbutin, suggesting the antioxidant activity to decrease with the size of the carbohydrate part of the glycoside. The antioxidant activities of the 4-hydroxy-3-methoxyphenyl glycosides **1**, **3**, and **4** (trisaccharide, tetrasaccharide, and disaccharide, respectively) in the present

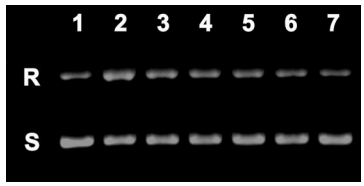


Figure 5. Inhibition of hydrogen peroxide-initiated transition of the plasmid pBR322 from supercoiled to relaxed form by compound **1**. Lanes 1 and 7, control. Lane 2, 0.3% H_2O_2. Lanes 3−6, 0.3% H_2O_2 along with 30, 100, 300 ng or 1 μg of compound **1**, respectively. R, relaxed plasmid form containing one single-strand break. S, supercoiled form (no breaks).

study do not support such a trend. The same is valid for the 4-hydroxy-3,5-dimethoxyphenyl glycosides **5** and **6**. It is known, however, that the DPPH radical has a structure that makes access of large molecules to the reaction site sterically difficult. Thus, steric accessibility is regarded as a major determinant for the reaction of DPPH radicals with radical-scavenging compounds.[25] The ABTS radical-scavenging method was shown to be free of this disadvantage.

The protective effect of compound **1** on plasmid DNA in oxidative stress-like conditions was tested. Hydrogen peroxide is able to generate reactive oxygen forms, which can induce single-strand breaks (SSBs) of plasmid DNA molecules.[26] This causes their transition from the supercoiled to relaxed form, which can be detected by gel electrophoresis. In the control plasmid sample, almost all DNA is present in the native supercoiled form (Figure 5, lanes 1 and 7), but after the reaction with H_2O_2 the number of molecules existing in the relaxed form (due to SSBs) is significantly increased (lane 2). Compound **1**, being introduced into the reaction mixture along with H_2O_2, reduced the number of SSBs in a concentration-dependent manner (lanes 3−6). A similar effect has previously been observed by Falcioni et al.,[8] when EtOH extracts of wheat sprouts prevented induction of SSBs in the same plasmid, induced by H_2O_2/Fe^{2+}; in that case, reactive oxygen forms were formed in a Fenton reaction. The ability to prevent plasmid DNA from SSBs in the presence of ROS has also been shown for some known phenolic-based antioxidants, such as green tea catechins, caffeic acid, and its esters.[27,28]

Experimental Section

General Experimental Procedures. Spectrophotometric measurements were made on a Hitachi U-2001 spectrophotometer. NMR data was acquired on a Bruker DRX600 NMR spectrometer equipped with a 2.5 mm SEI microprobe ($^1H/^{13}C$) and on a Bruker DRX400 NMR spectrometer with a 5 mm QNP probe-head. All NMR experiments were recorded in D_2O at 30 °C, and the chemical shifts determined relative to the methyl signal of internal acetone (δ_C 31.07; δ_H 2.225). For structure elucidation, 1D ^1H NMR, COSY, TOCSY, ROESY (300 ms), DEPT-HSQC, and HMBC (65 ms) were applied, and the pulse sequences were used as provided by the manufacturer. HRFABMS was performed on a Jeol SX/SX102A mass spectrometer, equipped with a FAB ion-source (Xe), using glycerol as matrix and polyethylene glycol as an internal standard. GC-MS was run on a HP5890/5970 instrument. LC-MS was performed on an Agilent 1100 HPLC system connected to a Bruker Esquire-LC ion-trap mass spectrometer using electrospray ionization in the positive ion mode. ESIMS/MS experiments were performed using the same mass spectrometer, with direct injection of samples with a syringe pump. Preparative HPLC was run on a Gilson system. DNA electrophoresis gels were observed and imaged using Bio-Rad Gel Doc XP system.

Plant Material. Wheat germ was manufactured by Kungsörnen (Lantmännen Axa, Järna, Sweden) and bought at a local store.

Extraction and Isolation of Compounds 1−7. Wheat germ (2 g fresh weight) was extracted with aqueous 50% EtOH (20 mL) by vortexing for 5 min at room temperature. Following centrifugation (13 000 rpm, 15 min, Heraeus Sepatech Biofuge 13) and filtration (0.45 μm), the supernatant was dried under reduced pressure. The resulting dry residue (284 mg) was dissolved in 1 mL of aqueous 5% MeCN and fractionated by preparative reversed-phase HPLC (Reprosil-Pur ODS, 250 × 20 mm, 5 μm, with a 30 × 20 mm guard column, 5 μm, Dr. A. Maisch High-Performance LC GmbH) using isocratic elution (aqueous 5% MeCN with 10 mM HOAc, at 10 mL/min). The eluate was monitored at 280 nm by a UV detector. Fractions (1.5 mL) were collected in deep-well plates.

Fractions corresponding to peaks displaying antioxidant activity (Figure 1) were lyophilized (**1**: 6.0 mg; **2**: 0.4 mg; **3**: 0.3 mg; **4**: 1.3 mg; **5**: 0.1 mg; **6** + **7**: 1.3 mg), and each fraction was then dissolved in 1 mL of aqueous 5% MeCN. The fractions were further fractionated by preparative reversed-phase HPLC on the same column using isocratic elution by 7% MeCN with 10 mM HOAc (compounds **1**−**4**) or by 3% MeCN and 10% MeOH with 10 mM HOAc (compounds **5**−**7**), at 10 mL/min, with monitoring of the eluate at 280 nm and collection of 1.0 mL fractions. Fractions containing antioxidant compounds **1**−**7** were lyophilized and weighed using a microbalance.

Antioxidant Activity Measurement. Antioxidant activity was determined by the free radical scavenging ability using the TEAC assay as described by Re et al.[9] A water solution containing ABTS (7 mM) and $K_2S_2O_8$ (2.5 mM) was allowed to stand in the dark at room temperature for 16−18 h for $ABTS^{\bullet+}$ formation and then diluted by aqueous 50% EtOH to a concentration yielding an absorbance of 0.70 ± 0.02 at 734 nm.

For detection of antioxidants in HPLC fractions of wheat germ extract, aliquots (150 μL) were transferred to 96-well microtiter plates, where they were mixed with 150 μL of $ABTS^{\bullet+}$ solution. The decrease in absorbance of the $ABTS^{\bullet+}$ solutions was followed by the naked eye.

For quantification of antioxidant activity of purified compounds **1**−**7**, water solutions (1.00 mM) were added in volumes of 10.0, 20.0, or 30.0 μL to 2.00 mL of $ABTS^{\bullet+}$ solution, and the decrease in absorbance at 734 nm was registered from 15 s to 5 min. The same volumes of 1.00 mM 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) solution were analyzed similarly, and the calibration curves were plotted for compounds **1**−**7** and Trolox and compared. The activities of **1**−**7** were then expressed in TEAC units, i.e., as Trolox concentration (mM) displaying the same activity as 1 mM of the compound. The decrease of absorbance of compounds **1** and **3**−**6** practically did not change during the registration period, as the reaction was complete within the first 15 s. For these compounds, the values obtained after 5 min were used for plotting. For compounds **2** and **7**, a continuous decrease of absorbance was observed during the registration period; therefore, several calibration curves were plotted for the time points 30 s, 1 min, 3 min, and 5 min after reaction start.

Determination of Monosaccharide Composition and Absolute Configuration (adapted from Gerwig et al.[10]). Approx. 0.1 mg of compound **1** was treated with 500 μL of MeOH/AcCl (10:1) at 85 °C for 18 h. The solvent was evaporated under a stream of N_2. Subsequently, the sample was reacted with 500 μL of (*S*)-2-butanol/AcCl (10:1) at 85 °C for 6 h. After evaporation of the solvent with N_2, the residue was treated with 100 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide/chlorotrimethylsilane (99:1, Sylon BFT, Supelco) and 100 μL of pyridine at room temperature for 30 min. The resulting derivative was analyzed directly by GC-MS on an HP-5MS column (80 °C for 5 min, 5 °C/min to 250 °C, injector: 250 °C, transfer line to MS: 250 °C, carrier gas: He at approximately 1 mL/min, splitless injection) and compared with authentic references prepared from (*S*)-2-butanol or racemic 2-butanol. (*S*)-2-Butyl D-Glc*p*: 32.75 and 34.16 min (α/β-forms), (*R*)-2-butyl D-Glc*p* [chromatographic equivalent to (*S*)-2-butyl L-Glc*p*]: 33.01 and 34.18 min (α/β-forms), derivatives of compound **1**: 32.74 and 34.16 min.

4-Hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (1): white powder; $[\alpha]^{24}_D$ −48.7(*c* 0.42, H_2O); UV (11% MeOH and 10 mM HOAc in H_2O) λ_{max} (log ϵ) 196 (4.58), 220 (3.75), 284 (3.48); ^1H NMR (D_2O, 600 MHz) δ 6.88 (1H, d, J = 8.7 Hz, H-5), 6.85 (1H, d, J = 2.8 Hz, H-2), 6.71 (1H, dd, J = 8.7, 2.8 Hz, H-6), 5.04 (1H, d, J = 7.8 Hz, H-1′), 4.48 (1H, d, J = 7.9 Hz, H-1″), 4.42 (1H, d, J = 7.9 Hz, H-1‴), 4.17 (1H, dd, J = 12.1, 1.9 Hz, H-6a′), 4.16 (1H, dd, J = 11.7, 1.6 Hz, H-6a″), 3.90 (1H, dd, J = 12.1, 6.4 Hz, H-6b′), 3.87 (1H, dd, J = 12.2, 2.2 Hz, H-6a‴), 3.86 (3H, s, OCH_3), 3.80 (1H, ddd, J = 9.9, 6.4, 1.9 Hz, H-5′), 3.78 (1H, dd, J = 11.7, 4.8 Hz, H-6b″), 3.66 (1H, dd, J = 12.2, 5.9 Hz, H-6b‴), 3.59 (1H, t, J = 9.2 Hz, H-3′), 3.53 (1H, dd, J = 9.5, 7.8 Hz, H-2′), 3.49 (1H, t, J = 9.5 Hz, H-4′), 3.48 (1H, m, H-5″), 3.45 (2H, t, J = 9 Hz, H-3‴ and H-4″), 3.42 (1H, t, J = 8.3

Hz, H-3″), 3.37 (1H, ddd, J = 9.6, 5.9, 2.2 Hz, H-5‴), 3.32 (1H, t, J = 9.4 Hz, H-4‴), 3.27 (1H, t, J = 8.2 Hz, H-2″), 3.26 (1H, dd, J = 9.4, 7.9 Hz, H-2‴); ^{13}C NMR (D_2O, 100 MHz) δ 151.5 (C, C-1), 148.9 (C, C-3), 141.5 (C, C-4), 116.5 (CH, C-5), 109.8 (CH, C-6), 104.1 (CH, C-2), 103.6 (CH, C-1″ and C-1‴), 102.0 (CH, C-1′), 76.7 (CH, C-5‴), 76.5 (CH, C-3′), 76.4 (CH, C-3″ and C-3‴), 76.1 (CH, C-5′), 75.7 (CH, C-5″), 73.9 (CH, C-2″ and C-2‴), 73.8 (CH, C-2′), 70.4 (CH, C-4‴), 70.3 (CH, C-4′), 70.2 (CH, C-4″), 69.7 (CH_2, C-6′), 69.3 (CH_2, C-6″), 61.6 (CH_2, C-6‴), 56.9 (CH_3, OCH_3); HRFABMS m/z 649.1904 $[M + Na]^+$ (calcd for $C_{25}H_{38}O_{18}Na$ 649.1950).

4-Hydroxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (2): white powder; UV (11% MeOH and 10 mM HOAc in H_2O) λ_{max} (log ϵ) 192 (4.68), 222 (3.97), 284 (3.46); ^{1}H NMR (D_2O, 600 MHz) δ 7.08 (2H, d, J = 9.1 Hz, H-2 and H-6), 6.88 (2H, d, J = 9.1 Hz, H-3 and H-5), 5.03 (1H, d, J = 7.5 Hz, H-1′), 4.49 (1H, d, J = 7.9 Hz, H-1″), 4.44 (1H, d, J = 7.9 Hz, H-1‴), 4.18 (2H, d, J = 12 Hz, H-6a′ and H-6a″), 3.90 (1H, dd, J = 12, 3.9 Hz, H-6b′), 3.90 (1H, d, J = 12 Hz, H-6a‴), 3.82 (1H, dd, J = 12, 4.9 Hz, H-6b″), 3.80 (1H, m, H-5′), 3.70 (1H, dd, J = 12, 5.8 Hz, H-6b‴), 3.60 (1H, t, J = 9 Hz, H-3′), 3.54 (1H, t, J = 8 Hz, H-2′), 3.52 (1H, t, J = 8.9 Hz, H-4′), 3.51 (1H, m, H-5″), 3.47 (2H, t, J = 9 Hz, H-3‴ and H-4″), 3.45 (1H, t, J = 9 Hz, H-3″), 3.40 (1H, m, H-5‴), 3.36 (1H, t, J = 9 Hz, H-4‴), 3.30 (1H, t, J = 9 Hz, H-2″), 3.29 (1H, t, J = 9 Hz, H-2‴); ^{13}C NMR (D_2O, 150 MHz) δ 151.7 (C, C-4), 150.8 (C, C-1), 119.1 (CH, C-2 and C-6), 117.0 (CH, C-3 and C-5), 103.5 (CH, C-1‴), 103.4 (CH, C-1″), 101.6 (CH, C-1′), 76.5 (CH, C-5‴), 76.3 (CH, C-3″ and C-3‴), 76.2 (CH, C-3′), 76.0 (CH, C-5′), 75.6 (CH, C-5″), 73.8 (CH, C-2″ and C-2‴), 73.6 (CH, C-2′), 70.3 (CH, C-4‴), 70.1 (CH, C-4′), 70.0 (CH, C-4″), 69.4 (CH_2, C-6′), 69.3 (CH_2, C-6″), 61.5 (CH_2, C-6‴); HRFABMS m/z 597.2051 $[M + H]^+$ (calcd for $C_{24}H_{37}O_{17}$ 597.2031).

4-Hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (3): white powder; UV (11% MeOH and 10 mM HOAc in H_2O) λ_{max} (log ϵ) 196 (4.56), 220 (3.73), 284 (3.45); ^{1}H NMR (D_2O, 600 MHz) δ 6.90 (1H, d, J = 8.7 Hz, H-5), 6.86 (1H, d, J = 2.4 Hz, H-2), 6.72 (1H, dd, J = 8.7, 2.4 Hz, H-6), 5.05 (1H, d, J = 7.8 Hz, H-1′), 4.49 (1H, d, J = 8.0 Hz, H-1″), 4.47 (1H, d, J = 8.0 Hz, H-1⁗), 4.45 (1H, d, J = 8.0 Hz, H-1‴), 4.19 (1H, obsc., H-6a′), 4.18 (2H, obsc., H-6a″ and H-6a‴), 3.91 (2H, obsc., H-6b′ and H-6a⁗), 3.87 (3H, s, OCH_3), 3.81 (1H, obsc., H-6b‴), 3.80 (2H, obsc., H-5′ and H-6b″), 3.72 (1H, dd, J = 12.4, 5.3 Hz, H-6b⁗), 3.61 (1H, t, J = 9.1 Hz, H-3′), 3.55 (1H, t, J = 9 Hz, H-2′), 3.54 (1H, obsc., H-5‴), 3.52 (1H, t, J = 9 Hz, H-4′), 3.50 (1H, obsc., H-5″), 3.49 (1H, obsc., H-3⁗), 3.45−3.43 (4H, obsc., H-3″, H-4″, H-3‴ and H-4‴), 3.42 (1H, obsc., H-5⁗), 3.40 (1H, t, J = 9 Hz, H-4⁗), 3.31 (1H, t, J = 8 Hz, H-2⁗), 3.30 (1H, t, J = 8 Hz, H-2‴), 3.29 (1H, t, J = 8 Hz, H-2″); ^{13}C NMR (D_2O, 150 MHz) δ 151.4 (C, C-1), 148.8 (C, C-3), 141.5 (C, C-4), 116.3 (CH, C-5), 109.6 (CH, C-6), 104.0 (CH, C-2), 103.6 (CH, C-1‴ and C-1⁗), 103.4 (CH, C-1″), 101.8 (CH, C-1′), 76.5 (CH, C-5⁗), 76.3 (CH, C-3⁗), 76.2 (CH, C-3′), 76 (CH, C-3″, C-3‴), 75.9 (CH, C-5′), 75.8 (CH, C-5″), 75.6 (CH, C-5‴), 73.8 (CH, C-2″, C-2‴, and C-2⁗), 73.7 (CH, C-2′), 70.3 (CH, C-4⁗), 70.2 (CH, C-4′), 70 (CH, C-4″ and C-4‴), 69.4 (CH_2, C-6′, C-6″, and C-6‴), 61.4 (CH_2, C-6⁗), 56.8 (CH_3, OCH_3); HRFABMS m/z 789.2661 $[M + H]^+$ (calcd for $C_{31}H_{49}O_{23}$ 789.2665).

4-Hydroxy-3-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (4): white powder; $[\alpha]^{24}_D$ −47.5 (c 0.07, H_2O); UV (11% MeOH and 10 mM HOAc in H_2O) λ_{max} (log ϵ) 196 (4.58), 220 (3.76), 284 (3.44); ^{1}H NMR (D_2O, 600 MHz) δ 6.88 (1H, d, J = 8.9 Hz, H-5), 6.84 (1H, d, J = 2.6 Hz, H-2), 6.69 (1H, dd, J = 8.9, 2.6 Hz, H-6), 5.05 (1H, d, J = 7.7 Hz, H-1′), 4.46 (1H, d, J = 8.0 Hz, H-1″), 4.18 (1H, dd, J = 11.8, 1.6 Hz, H-6a′), 3.89 (1H, dd, J = 11.8, 6.0 Hz, H-6b′), 3.87 (1H, obsc., H-6a″), 3.87 (3H, s, OCH_3), 3.77 (1H, m, H-5′), 3.67 (1H, dd, J = 12.1, 5.5 Hz, H-6b″), 3.60 (1H, t, J = 8.9 Hz, H-3′), 3.54 (1H, t, J = 8.9 Hz, H-2′), 3.53 (1H, t, J = 8.9 Hz, H-4′), 3.41 (1H, t, J = 8.4 Hz, H-3″), 3.36 (1H, t, J = 9.3 Hz, H-4″), 3.32 (1H, m, H-5″), 3.27 (1H, t, J = 8.3 Hz, H-2″); ^{13}C NMR (D_2O, 100 MHz) δ 151.4 (C, C-1), 148.9 (C, C-3), 141.5 (C, C-4), 116.5 (CH, C-5), 109.7 (CH, C-6), 104.0 (CH, C-2), 103.3 (CH, C-1″), 101.9 (CH, C-1′), 76.7 (CH, C-5″), 76.5 (CH, C-3″), 76.3 (CH, C-3′), 76.2 (CH, C-5′), 74.0 (CH, C-2″), 73.8 (CH, C-2′), 70.5 (CH, C-4″), 70.2 (CH, C-4′), 69.0 (CH_2, C-6′), 61.5 (CH_2, C-6″), 56.9 (CH_3, OCH_3); HRFABMS m/z 465.1629 $[M + H]^+$ (calcd for $C_{19}H_{29}O_{13}$ 465.1608).

4-Hydroxy-3,5-dimethoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (5): white powder; UV (11% MeOH and 10 mM HOAc in H_2O) λ_{max} (log ϵ) 204 (4.65), 226 (sh) (3.80), 280 (3.30); ^{1}H NMR (D_2O, 600 MHz) δ 6.55 (2H, s, H-2 and H-6), 5.09 (1H, d, J = 8.0 Hz, H-1′), 4.45 (1H, d, J = 8.0 Hz, H-1″), 4.18 (1H, d, J = 11.8 Hz, H-6a′), 3.91 (1H, dd, J = 11.8, 6.0 Hz, H-6b′), 3.87 (1H, obsc., H-6a″), 3.86 (6H, s, OCH_3), 3.79 (1H, m, H-5′), 3.67 (1H, dd, J = 12.2, 5.9 Hz, H-6b″), 3.61 (1H, t, J = 9 Hz, H-3′), 3.54 (1H, t, J = 8.3 Hz, H-2′), 3.53 (1H, t, J = 9 Hz, H-4′), 3.39 (1H, t, J = 9.0 Hz, H-3″), 3.34 (1H, t, J = 9.0 Hz, H-4″), 3.30 (1H, m, H-5″), 3.25 (1H, t, J = 8.5 Hz, H-2″); ^{13}C NMR (D_2O, 150 MHz) δ 150.6 (C, C-1), 148.8 (C, C-3 and C-5), 130.5 (C, C-4), 96.4 (CH, C-2 and C-6), 103.0 (CH, C-1″), 101.6 (CH, C-1′), 76.5 (CH, C-5″), 76.3 (CH, C-3″), 76.1 (CH, C-3′), 76.0 (CH, C-5′), 73.7 (CH, C-2″), 73.5 (CH, C-2′), 70.1 (CH, C-4″), 69.9 (CH, C-4′), 68.8 (CH_2, C-6′), 61.3 (CH_2, C-6″), 57.0 (CH_3, OCH_3); HRFABMS m/z 495.1697 $[M + H]^+$ (calcd for $C_{20}H_{31}O_{14}$ 495.1714).

4-Hydroxy-3,5-dimethoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (6): white powder; $[\alpha]^{24}_D$ −41.7 (c 0.16, H_2O); UV (11% MeOH and 10 mM HOAc in H_2O) λ_{max} (log ϵ) 204 (4.64), 226 (sh) (3.80), 280 (3.28); ^{1}H NMR (D_2O, 600 MHz) δ 6.57 (2H, s, H-2 and H-6), 5.06 (1H, d, J = 7.8 Hz, H-1′), 4.46 (1H, d, J = 8.0 Hz, H-1″), 4.38 (1H, d, J = 7.8 Hz, H-1‴), 4.17 (1H, d, J = 12.0 Hz, H-6a′), 4.16 (1H, d, J = 11.5 Hz, H-6a″), 3.92 (1H, dd, J = 12.0, 6.6 Hz, H-6b′), 3.87 (6H, s, OCH_3), 3.85 (1H, obsc., H-6a‴), 3.84 (1H, obsc., H-5′), 3.76 (1H, dd, J = 11.5, 4.8 Hz, H-6b″), 3.62 (1H, dd, J = 12.0, 6.0 Hz, H-6b‴), 3.60 (1H, t, J = 9 Hz, H-3′), 3.54 (1H, dd, J = 8.1, 7.7 Hz, H-2′), 3.49 (1H, t, J = 9.3 Hz, H-4′), 3.46 (1H, m, H-5″), 3.43 (1H, t, J = 8.9 Hz, H-4″), 3.42 (2H, t, J = 9 Hz, H-3″ and H-3‴), 3.34 (1H, m, H-5‴), 3.28 (1H, t, J = 9 Hz, H-4‴), 3.25 (1H, t, J = 8 Hz, H-2‴), 3.24 (1H, t, J = 8.1 Hz, H-2″); ^{13}C NMR (D_2O, 100 MHz) δ 151.2 (C, C-1), 149.0 (C, C-3 and C-5), 130.8 (C, C-4), 96.9 (CH, C-2 and C-6), 103.8 (CH, C-1″), 103.5 (CH, C-1‴), 102.1 (CH, C-1′), 76.7 (CH, C-5‴), 76.5 (CH, C-3″ and C-3‴), 76.4 (CH, C-3′), 76.1 (CH, C-5′), 75.7 (CH, C-5″), 74.0 (CH, C-2″ or C-2‴), 73.9 (CH, C-2″ or C-2‴), 73.8 (CH, C-2′), 70.5 (CH, C-4′ or C-4‴), 70.4 (CH, C-4′ or C-4‴), 70.1 (CH, C-4″), 70.0 (CH_2, C-6′), 69.2 (CH_2, C-6″), 61.6 (CH_2, C-6‴), 57.3 (CH_3, OCH_3); HRFABMS m/z 657.2198 $[M + H]^+$ (calcd for $C_{26}H_{41}O_{19}$ 657.2242).

4-Hydroxy-2-methoxyphenyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (7): white powder; UV (11% MeOH and 10 mM HOAc in H_2O) λ_{max} (log ϵ) 196 (4.57), 220 (3.83), 284 (3.40); ^{1}H NMR (D_2O, 600 MHz) δ 7.13 (1H, d, J = 8.7 Hz, H-6), 6.64 (1H, d, J = 2.7 Hz, H-3), 6.48 (1H, dd, J = 8.7, 2.7 Hz, H-5), 5.01 (1H, d, J = 7.4 Hz, H-1′), 4.46 (1H, d, J = 7.9 Hz, H-1″), 4.44 (1H, d, J = 7.9 Hz, H-1‴), 4.18 (1H, d, J = 11.6 Hz, H-6a″), 4.17 (1H, dd, J = 12.0 Hz, H-6a′), 3.90 (1H, obsc., H-6b′), 3.89 (1H, obsc., H-6a‴), 3.86 (3H, s, OCH3), 3.81 (1H, dd, J = 11.6, 5.2 Hz, H-6b″), 3.74 (1H, m, H-5′), 3.69 (1H, dd, J = 12.3, 6.0 Hz, H-6b‴), 3.58 (2H, obsc., H-2′ and H-3′), 3.51 (1H, t, J = 9 Hz, H-4′), δ 3.48 (1H, obsc., H-5″), 3.45 (2H, obsc., H-4″ and H-3‴), 3.41 (1H, obsc., H-3″), 3.39 (1H, m, H-5‴), 3.35 (1H, t, J = 9.4 Hz, H-4‴), 3.28 (1H, dd, J = 9.4, 7.9 Hz, H-2″), 3.27 (1H, dd, J = 9.0, 8.0 Hz, H-2‴); ^{13}C NMR (D_2O, 150 MHz) δ 152.7 (C, C-4), 150.9 (C, C-2), 139.7 (C, C-1), 119.4 (CH, C-6), 107.8 (CH, C-5), 103.6 (CH, C-1″ and C-1‴), 102.2 (CH, C-1′), 102.1 (CH, C-3), 76.6 (CH, C-5‴), 76.5 (CH, C-3″ and C-3‴), 76.4 (CH, C-3′), 76.3 (CH, C-5′), δ 75.7 (CH, C-5″), 74.0 (CH, C-2″ and C-2‴), 73.9 (CH, C-2′), 70.5 (CH, C-4‴), 70.4 (CH, C-4′), 70.2 (CH, C-4″), 69.4 (CH_2, C-6″), 69.3 (CH_2, C-6′), 61.6 (CH_2, C-6‴), 56.8 (CH_3, OCH_3); HRFABMS m/z 627.2199 $[M + H]^+$ (calcd for $C_{25}H_{39}O_{18}$ 627.2136).

Induction and Analysis of DNA Single-Strand Breaks. Plasmid pBR322 (100 ng) (Invitrogen AB, Lidingö, Sweden) was incubated for 1 h at 37 °C in 100 mM sodium phosphate buffer (pH 7.4) in the presence of 0.3% H_2O_2 and 30, 100, 300 ng or 1 μg of compound **1** (total volume of each reaction mixture was 12 μL). After the reaction, 2.4 μL of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol) was added to each reaction tube, 10 μL aliquots were loaded onto a 0.7% agarose gel, and electrophoresis was run for 1.5 h at 80 V. Gels were stained by 0.5 μg/mL ethidium bromide solution for 15 min and washed with water, and the photographs of gels were obtained under UV light. The degree of DNA damage, as indicated by the plasmid DNA molecules with single-strand breaks (SSBs), was estimated by the naked eye.

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