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Selective Synthesis and Biological Evaluation of Sulfate-Conjugated Resveratrol Metabolites

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Abstract

Five resveratrol sulfate metabolites were synthesized and assessed for activities known to be mediated by resveratrol: inhibition of tumor necrosis factor (TNF)-α-induced NFκB activity, cylcooxygenases (COX-1 and COX-2), aromatase, nitric oxide production in endotoxin-stimulated macrophages, and proliferation of KB or MCF7 cells, induction of quinone reductase 1 (QR1), accumulation in the sub-G_1 phase of the cell cycle, and quenching of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Two metabolites showed activity in these assays; the 3-sulfate exhibited QR1 induction, DPPH free radical scavenging, and COX-1 and COX-2 inhibitory activities, and the 4′-sulfate inhibited NFκB induction, as well as COX-1 and COX-2 activities. Resveratrol, as well as its 3′-sulfate and 4-sulfate, inhibit NO production by NO scavenging and down-regulation of iNOS expression in RAW 264.7 cells. Resveratrol sulfates displayed low antiproliferative activity and negligible uptake in MCF7 cells.

Introduction

Resveratrol (**1**, 3,5,4′-trihydroxystilbene) is a naturally occurring phytoalexin produced by various plants in response to environmental stress or pathogenic attack. It is present in peanuts, mulberries, blueberries, and grapes[1–3] and possesses numerous biological activities that result in antioxidant,[4] anti-inflammatory,[5–7] anti-ischemic,[8–10] neuroprotective,[11,12] anti-aging,[13–15] anti-obesity,[16] antiviral,[17] cardioprotective,[18–20] anticancer,[21] and cancer chemopreventive effects.[1,22–24] As a cancer chemopreventive agent, resveratrol has been shown to interfere with or inhibit all three stages of carcinogenesis: initiation, promotion, and progression.[1] Interestingly, it is apparent that resveratrol can elicit these effects even though serum concentrations are low.[25] Although resveratrol is efficiently absorbed on oral administration, rapid metabolism leads to the production of sulfates and glucuronides.[25–34] These facts cast doubt on the physiological relevance of the high resveratrol concentrations typically used for in vitro studies, and suggest at least some, if not most, of the biological effects elicited by resveratrol may be attributed to resveratrol metabolites.

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Several resveratrol absorption and metabolism studies have been performed in rodent models. Initially, an isolated rat small intestine perfusion model was used.[29] Kuhnle and coworkers reported that orally administered resveratrol is mainly converted to glucuronide conjugates.[29] The metabolism of resveratrol was also investigated by Yu et al., who carried out oral and intraperitoneal injections with rats and mice.[31] Using synthetic standards, they identified *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-sulfate in mouse serum.[31] Similar to previous findings, the glucuronide and sulfate conjugates of resveratrol were detected in rat urine and mouse serum, with minimal amounts of unchanged resveratrol.[31] Wenzel et al. established that all five possible resveratrol sulfate metabolites, as well as the 3-*O*-β-D-glucuronide, are produced in rats.[25]

Resveratrol metabolism studies with human beings have produced similar results to those obtained with rodent models. De Santi et al. reported the sulfation and glucuronidation of resveratrol in human liver samples,[26,27] and also observed sulfation in a human duodenum preparation.[28] Goldberg and collaborators demonstrated that after oral or iv injection, the majority of resveratrol detected in serum and urine was glucuronide and sulfate conjugates.[35] Following that study, comparable data were reported by Meng et al., who found that no more than 2.3% of the administered resveratrol is unchanged.[33] Walle et al. investigated the absorption, bioavailability, and metabolism of resveratrol by administering ^{14}C-resveratrol to human subjects, confirming the findings that resveratrol is metabolized quickly and extensively.[34] All of these in vivo studies support the idea of resveratrol being predominantly converted to its glucuronic acid and sulfate conjugates after oral, ip, or iv administration.[25,29,32–36]

Based on resveratrol metabolism studies, it is reasonable to suggest the in vitro data obtained using high concentrations of resveratrol lack direct in vivo relevance. Although administration of resveratrol has led to responses such as anticancer[21] and cancer chemopreventive[37–39] activities in animal models, it remains a fact that rapid and extensive metabolism leads to glucuronides and sulfates. Accordingly, response data could be explained by 1) local chemopreventive effects in the GI tract before metabolism occurs;[37,39] 2) the conversion of resveratrol sulfates and glucuronides back to resveratrol in target organs such as the liver;[25,32] 3) enterohepatic recirculation involving biliary secretion of resveratrol metabolites followed by deconjugation by gut microflora and then reabsorption;[36] and 4) the possible biological activities of the resveratrol metabolites themselves. The latter has been suggested for other compounds, such as quercetin, (−)-epicatechin, and (+)-catechin.[40–42]

Selective chemical syntheses of glucuronide conjugates have been reported,[43,44] and syntheses of sulfated resveratrol are known as well. However, these are non-regioselective syntheses that requires HPLC separation of mono-, di-, and tri-sulfated conjugates.[25,45] As such, these procedures impede the preparation of sufficient quantities of sulfates required for a systematic investigation of their biological activities. In order to address this limitation, we have synthesized the previously identified resveratrol sulfate metabolites. All five metabolites have been prepared and isolated as their salts, and the biological effects of each metabolite have been investigated in a set of assays that are associated with cancer chemopreventive activity.

Results and Discussion

Chemistry

The synthesis of the five resveratrol sulfate metabolites is complicated by the fact that there are two different mono- and two different disulfated resveratrol compounds. This means that, regardless of the type of synthetic scheme that is utilized, there needs to be a way to selectively protect the three hydroxyl groups present in the resveratrol structure. Thus, the

4′-sulfate **6** was chosen as the first target due to the ease of selective protection of the 3,5-hydroxyl groups.

Compounds **10** and **11** were selected as the two reactants to undergo the Heck coupling. TBDMS and acetate groups were chosen in order to increase the yield[46] and enable selective cleavage under different conditions (Scheme 1). As reported previously,[46] acetyl migrations have been observed during the Heck reaction; nevertheless, successful formation of compound **12** was confirmed. Following this, a catalytic amount of NaOMe was used to cleave the acetate group to generate **13**. The sulfation of **13** posed several problems. Since intermediate **14** is a sulfate sodium salt, it was not practical to perform organic extraction to remove inorganic impurities. It was therefore purified by applying the concentrated reaction mixture to a silica gel column, using EtOAc and MeOH as the solvent system. Deprotection of **14** with TBAF provided the tetra-*n*-butylammonium salt **6**.

The synthesis of **6** made it apparent that it is not easy to prepare and purify sulfated resveratrol metabolites. The published literature correctly states that the multiple sulfation reaction is "a synthetic nightmare."[47] For that reason the synthesis of trisulfated resveratrol **2** was attempted next in order to optimize the sulfation reaction, sulfate salt formation, and sulfate salt purification steps using commercially available resveratrol as the starting material (Scheme 2).

The first difficulty encountered in the process of synthesizing **2** was the method used to monitor the progress of the sulfation reaction. Switching the sulfation reagent from SO_3·pyridine complex to SO_3·NMe_3 facilitated the TLC monitoring of the reaction and made the work-up more convenient. Since trisulfated resveratrol is a very polar compound, the types of TLC plates used were changed from normal-phase to reversed-phase.

After driving the sulfation reaction to completion, the next challenge in the synthesis of tri-sulfated resveratrol metabolite **2** was forming the sulfate salts. Initial attempts using excess Na_2CO_3 made it clear that removal of inorganic salt from the product was very difficult because of the highly polar nature of the desired product. Use of a Dowex 50X8-200 column that had been converted to the K^+ form enabled formation of the tripotassium salt while limiting the introduction of excess K^+ into the reaction mixture. The final traces of inorganic impurities were removed by size exclusion chromatography.

The synthesis of mono- and di-sulfated resveratrol metabolites requires the preparation of four different precursors (Scheme 3). These precursors require selective protection of three hydroxyl groups that are present on the stilbene framework. The protected intermediates necessary to synthesize mono- and di-sulfated resveratrol were prepared and separated (Scheme 3; compounds **13**, **15**-**17**) following the procedure established by Zhang et al.[43] Using the previously determined reaction conditions, each of the four TBDMS-protected resveratrol compounds was sulfated. At this point, these sulfated intermediates were not converted to potassium sulfates due to observation that not forming potassium salts before the deprotection step enhanced the solubilities of the intermediate with both non-polar TBDMS groups and polar sulfate groups. Although the difference appears minimal, not forming potassium salts greatly improved the solubilities of the intermediates, and made it possible to effectively remove organic impurities from the reaction mixture before the TBDMS deprotection reaction. The TBDMS deprotection reactions were carried out with KF instead of TBAF in order to avoid formation of the tetra-*n*-butylammonium salts. By combining these optimization efforts, four different potassium salts **3**–**5** and **7** of sulfated resveratrol metabolites were successfully formed as shown in Scheme 3.

The NMR peak assignments for all possible sulfated resveratrol metabolites are compared in Table 1. As expected, the signals move downfield as sulfates are added. The ^{1}H NMR

chemical shifts and coupling constants, along with the mass spectrometry data, allowed the unambiguous assignments of the structures of all five metabolites. In particular, the equivalence or non-equivalence of the protons attached to C-2 and C-6 was diagnostic.

Biological Results

One of the most extensively studied biological activities of resveratrol investigated during the past few years has been its cancer-chemopreventive potential.[48] This stilbene has been demonstrated to block the multistep process of carcinogenesis at the various stages of initiation, promotion, and progression. Some possible mechanisms involve down regulation of the inflammatory response through inhibition of synthesis and release of pro-inflammatory mediators, modification of eicosanoid synthesis, inhibition of activated immune cells, or inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) via modulation of NFκB. To explore the activities of the synthetic resveratrol sulfate derivatives, they were tested in a set of assays indicative of chemoprevention, including inhibition of TNF-α-induced NFκB activity, COX-1 and COX-2 inhibition, inhibition of nitric oxide production by iNOS in LPS-induced macrophage cells, aromatase inhibition, QR1 induction, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical quenching, and cytotoxicity in KB and MCF7 cells. The results are summarized in Table 2.

The role of NFκB in many cellular processes is well studied. Deregulated activity of the NFκB pathway has been observed and linked to the progression of cancer and several human ailments. Our test system assesses inhibition of NFκB induction by TNF-α in a stably transfected 293/NFκB-Luc human embryonic kidney cell line.[49] Presumably, the sulfate metabolites could be transported intact into the kidney cells by organic anion transporters.[50–53] All of the metabolites retained some level of activity in this assay, but potency was reduced, relative to resveratrol. The most active metabolite was the 4′-sulfate **7**, and the two least active were **2** and **4**. In general, the ability of these stilbenes to inhibit the induction of NFκB is surprisingly insensitive to the substituents present, or to their arrangement.

The involvement of prostaglandins (PG) and other eicosanoids in the development of human cancer is well known.[54] Importantly, an increase in PG synthesis may influence tumor growth in human beings and experimental animals. Cleaved from membrane phospholipids by phospholipases, arachidonic acid (AA) is converted through the cyclooxygenase (COX) pathway to produce PGs.[55] Therefore, inhibition of COX and the subsequent reduction of PG synthesis provides a viable strategy for the prevention of cancer.[56–60] Accordingly, resveratrol and resveratrol sulfates were tested for inhibition of both COX-1 and COX-2. Resveratrol inhibited COX-1 and -2 with IC_{50} values of 6.65 and 0.75 μM, respectively. The 3-sulfate **5** and the 4′-sulfate **7** inhibited COX-1 with IC_{50} values comparable to resveratrol (3.60 and 5.55 μM, respectively). In COX-2 inhibition assays, the 3-sulfate **5** demonstrated an IC_{50} of 7.53 μM and the 4′-sulfate **7** had an IC_{50} of 8.95 μM.

Nitric oxide (NO), a product of nitric oxide synthase (NOS), mediates diverse physiological processes (e.g., vasodilation, immune response) as a signaling molecule. However, continuous and excessive production of NO by inducible nitric oxide synthase (iNOS) causes pathophysiological problems such as chronic inflammatory diseases and cancer development.[61] In addition, up-regulation of nitric oxide (NO) synthesis may contribute to tumor growth by facilitating angiogenesis.[62] Inhibitors of inducible nitric oxide synthase (iNOS) may have chemopreventive activity due to antiproliferative effects,[56] and resveratrol has been reported to function in this capacity.[63] Therefore, the abilities of resveratrol and resveratrol sulfates to inhibit the production of NO by iNOS in macrophage cells were determined. With an IC_{50} value of 15 μM, resveratrol was the most potent inhibitor of nitric oxide synthase. Modest activity was observed with 4′-sulfate **7**, followed by the 3,4′-

disulfate **3** and the 3-sulfate **5**, which were equipotent. The least potent nitric oxide synthase inhibitor was the 3,5-disulfate **4**, which was actually less potent than the trisulfate **2**.

To determine whether resveratrol sulfates induce nitric oxide production by themselves, these compounds were tested under LPS-free circumstances. None of the resveratrol sulfates showed significant enhancement of NO production when tested at a concentration of 34 μM (data not shown).

Several publications have indicated that polyphenols like resveratrol[64] and epigallocatechin gallate (EGCG)[65] inhibit NO and peroxynitrite formation due to antioxidant activity.[66] Therefore, NO scavenging activity of each resveratrol sulfate was measured using the NO generating reagent sodium nitroprusside (SNP). At a concentration of 60 μM, compounds **1**, **5** and **7** showed slight NO scavenging activity, with 17.4 ± 4.3, 24.7 ± 1.2, 10.3 ± 4.3% inhibition, while compounds **2**, **3**, and **4** were not active, demonstrating 1.6 ± 3.8, 0.0 ± 3.3, and 1.2 ± 4.9% inhibition, respectively

Several polyphenols, including 6-gingerol, epigallocatechin gallate (EGCG), indole-3-carbinol, and oroxylin A, as well as resveratrol itself, have been reported to inhibit iNOS expression in LPS-treated RAW 264.7 cell lines.[67] We performed Western blot analyses to determine if compounds **1**, **5**, **7** inhibit nitrite production via down-regulation of iNOS expression. As shown in Figure 1, LPS increased the protein expression of iNOS in comparison with untreated RAW 264.7 cells. Under the same conditions, resveratrol sulfates **1**, **5**, **7** moderately suppressed the expression of iNOS compared to LPS-treated control. In sum, compounds **1**, **5**, **7** showed moderate inhibition in NO production by NO scavenging activity and down-regulation of iNOS protein expression. Although these responses are not strong, since up-regulation of iNOS is correlated with activation of upstream NFκB pathways,[61] and some inhibitors of NO production function through NFκB regulation,[68] a compound such as **7** could possibly mediate a stronger response in a broader biological milieu.

Using the same experimental approach simultaneously we investigated protein expression of COX-2 (Figure 1). None of tested compounds, at the concentration 34 μM, inhibited COX-2 expression, which is consistent with a previous report.[69]

Resveratrol is known to induce programmed cell death (apoptosis) in a variety of cell lines, including lung, colon, prostate, and breast.[70–73] Since a $subG_1$ cell population is an indicator of cell death by apoptosis or necrosis, we explored the potential of resveratrol and resveratrol sulfates to induce accumulation of HL-60 human acute leukemia cells in the $subG_1$ compartment. Consistent with previous results,[74] resveratrol was active in this process, but the sulfate metabolites were inactive.

The lack of cytotoxicity of the sulfates **2**–**4** and **7** in MCF7 and KB cells (Table 2), as well as low activity in other cell-based assays, suggests a lack of uptake as well as a lack of hydrolysis. To test for cellular uptake, the 3,5-disulfate **4** and resveratrol were incubated separately with MCF7 cells, and LC-MS-MS was used to measure intracellular levels of resveratrol and its various sulfate conjugates. After incubation with resveratrol as a control, MCF7 cells were found to contain resveratrol and the disulfate **4**, but not the 4′-sulfate **7**. This indicated that resveratrol entered MCF7 cells and was metabolized to form **7**. When MCF7 cells were incubated with the 4′-sulfate **7**, no absorption of **7** was detected, and neither resveratrol **1** nor the disulfate **4** were detected intracellularly. These results indicate lack of hydrolysis and uptake of the 4′-sulfate 7 by MCF7 cells. Based on the cytotoxicity results (Table 2), the other sulfates are probably also not hydrolyzed or absorbed to an appreciable extent by MCF7 cells.

To test the stability of resveratrol and its 4′-sulfate **7**, each compound was incubated separately for 24 h at 37 °C in the cell culture medium used for MCF7 cellular uptake studies. Based on LC-MS-MS analyses, the 4′-sulfate **7** was stable under these incubation conditions. On the other hand, resveratrol **1** degraded approximately 20% during this time period. Therefore, metabolic sulfation of resveratrol forms stable derivatives that can be excreted in bile or urine. Since enterohepatic recirculation of resveratrol occurs,[36] resveratrol sulfates will be deconjugated by gut microflora, and then resveratrol will be reabsorbed to prolong its anticancer effects.

CYP19 (aromatase) converts C19 androgens to aromatic C18 estrogens through three consecutive hydroxylation reaction steps.[75] Aromatase transcription is mediated by IκB kinaseβ (IKKβ), a kinase previously known for cancer-promoting activity.[76] Under some situations (e.g., post menopause), aromatase is a key player in estrogen production, and inhibitors have been shown to function as chemopreventive agents. Based on an in vitro test system, resveratrol and its sulfates were found to be relatively weak inhibitors. The most active of the metabolites was the 4′-sulfate **7**, which produced 30% inhibition at a concentration of 34 μM.

Induction of NAD(P)H:quinone reductase 1 (QR1) is a well established mechanism for cancer chemoprevention.[77–80] Induction of QR1 commonly coincides with the induction of other phase II detoxifying enzymes.[81] Therefore, a rapid and sensitive QR1 cellular assay[82] was used to evaluate resveratrol and resveratrol metabolites. The results summarized in Table 2 include the induction ratio (IR), which is the ratio of the observed QR1 activity resulting from treatment with 34 μM of the test compound vs. DMSO control. In addition, the concentrations to double the activity of QR1 (CD) are listed. The 3-sulfate **5** was more potent than resveratrol in this assay, while the other sulfate metabolites were all less potent. However, all of the sulfates retained some degree of activity.

The cancer chemopreventive effects of resveratrol and related phenolic natural products may be due, in part, to quenching unstable free radicals and reducing damage to DNA by reactive oxygen species (ROS).[83–85] The free-radical scavenging activities of the test compounds were examined by measuring ability to quench the DPPH radical. The activity of the 3-sulfate **5** was comparable to that of the parent compound **1**, while the activity of the 4′-sulfate **7** was somewhat lower. The remaining disulfates **3** and **4** were much less active as free radical scavengers and, as expected from the absence of any phenolic hydroxyl groups, the trisulfate **2** was inactive.

Conclusion

Resveratrol exerts chemopreventive activity and a host of targets have been established.[86] We selected a variety of in vitro and cell-based targets (Table 2) to determine the activity displayed by resveratrol relative to sulfate metabolites. Overall, the sulfate metabolites are less active than resveratrol, with some exceptions, such as resveratrol 3-sulfate (**5**), which mediates comparable or even greater QR1 induction, DPPH radical scavenging, and COX-1 inhibition. Not surprisingly, in general, the activities of the sulfate metabolites decrease as the degree of sulfation increases, although there are exceptions (e.g., the activities of **2** vs. **4** on inhibition of nitric oxide synthase). Since serum concentrations of sulfated metabolites are higher than the serum concentrations of resveratrol, the ability of the metabolites to typically retain some degree of activity may be of relevance.

Experimental Section

Melting points were determined using capillary tubes with a Mel-Temp apparatus and are uncorrected. The proton nuclear magnetic resonance spectra were recorded using an

ARX300 300 MHz Bruker NMR spectrometer. IR spectra were recorded using a Perkin-Elmer 1600 series FTIR spectrometer. Flash and gravity chromatographic purification were performed using 230–400 mesh silica gel unless otherwise noted. Chemicals and solvents were reagent grade and obtained from commercial sources without further purification. Synthetic compounds were analyzed at the Purdue University Campus-Wide Mass Spectrometry Center using a Finnigan MAT LCQ Classic mass spectrometer system equipped with electrospray. Combustion microanalyses were performed at the Purdue University Microanalysis Laboratory using a Perkin-Elmer Series II CHNS/O model 2400 analyzer and all reported values are within 0.4% of calculated values. These elemental analyses confirmed ≥ 95% purity.

Resveratrol Tripotassium 3,5,4′-Sulfate (2)

A mixture of $SO_3{\cdot}NMe_3$ (1.097 g, 7.886 mmol) in Et_3N (2.50 mL, 13.14 mmol) was added to a well-stirred mixture of **1** (0.100 g, 0.438 mmol) in anhydrous MeCN (5.0 mL) at room temperature under argon. The resulting reaction mixture was heated at reflux under argon for 120 h. The reaction mixture was cooled to room temperature, decanted and concentrated under reduced pressure. Water (5.0 mL) was added to the reaction mixture and the resulting mixture was stirred for 30 min at room temperature. The water layer was concentrated to approximately 2.0 mL and applied to a column of cation-exchange resin (Dowex 50WX8-200, H^+ form, 9 g, 2 × 19 cm) prepared by eluting solvents in the following order: water (300 mL), saturated K_2CO_3 solution (400 mL), and water (300 mL). The crude product was eluted with water and fractions containing the desired intermediate were combined and concentrated and applied to a column of reversed-phase C-18 silica gel (eluent: 0-20% MeOH-H_2O, reversed-phase C-18 silica gel, 6 g, 2 × 11 cm). Fractions containing the desired compound were combined and concentrated. The crude product was applied to a size-exclusion chromatography column (eluent: H_2O, Sephadex® G-10, 14 g, 2 × 20 cm) to afford **2** as a white solid (0.054 g, 21%: mp >350 °C. ^{1}H NMR (300 MHz, D_2O) δ 7.49 (d, *J* = 8.7 Hz, 2 H), 7.26 (d, *J* = 2.1 Hz, 2 H), 7.15 (d, *J* = 8.7 Hz, 2 H), 7.14 (d, *J* = 16.5 Hz, 1 H), 7.03 (d, *J* = 16.5 Hz, 1 H), 6.99 (t, *J* = 2.1 Hz, 1 H); positive ESIMS *m/z* (rel intensity) 621 (MK^+, 100). Anal. Calcd for $C_{14}H_9K_3O_{12}S_3{\cdot}1.25H_2O$: C, 27.78; H, 1.92; S, 15.89. Found: C 27.54; H, 1.77; S, 15.62.

Resveratrol Dipotassium 3,4′-Sulfate (3)

$SO_3{\cdot}NMe_3$ (0.487 g, 3.504 mmol) and Et_3N (0.81 mL, 5.83 mmol) were added to a well-stirred mixture of **16** (0.100 g, 0.292 mmol) in anhydrous MeCN (5.0 mL) at room temperature under argon. The resulting reaction mixture was heated to reflux under argon for 48 h. The reaction mixture was cooled to room temperature, decanted and concentrated under reduced pressure and applied to a column of reversed-phase C-18 silica gel (eluent: 10–25 % CH_3CN-H_2O, 5 g, 2 × 10 cm). Fractions containing the desired compound and some impurities were combined, concentrated and dissolved in 30% aqueous MeOH (10 mL). KF (0.051 g, 0.875 mmol) was added to the solution, which was stirred vigorously at room temperature under argon for 12 h and concentrated under reduced pressure. The crude product was applied to a column of cation-exchange resin (Dowex 50WX8-200, H^+ form, 9 g, 2 × 19 cm) prepared by eluting solvents in the following order: water (300 mL), saturated K_2CO_3 solution (400 mL), and water (300 mL). The crude product was eluted with water and fractions containing the desired intermediate were combined, concentrated, and applied to a column of reversed-phase C-18 silica gel (eluent: 0–20% MeOH-H_2O, 6 g, 2 × 11 cm). Fractions containing the desired compound and some impurities were combined, concentrated, and applied to a size-exclusion chromatography column (eluent: H_2O, Sephadex® G-10, 14 g, 2 × 20 cm) to afford **3** as a white solid (0.015 g, 26%): mp >300 °C. ^{1}H NMR (300 MHz, D_2O) δ 7.46 (d, *J* = 8.4 Hz, 2 H), 7.13 (d, *J* = 8.1 Hz, 2 H), 7.05 (d, *J* = 16.5 Hz, 1 H), 6.94 (d, *J* = 16.5 Hz, 1 H), 6.71 (s, 1 H), 6.65 (s, 1 H), 6.38 (t, *J* = 2.1 Hz,

1 H); positive ESIMS *m/z* (rel intensity) 487 (MNa^+, 100). Anal. Calcd for $C_{14}H_{10}K_2O_9S_2 \cdot 2H_2O$: C, 33.59; H, 2.82; S, 12.81. Found: C, 33.42; H, 2.38; S, 12.83.

Resveratrol Tripotassium 3,5-Sulfate (4)

$SO_3 \cdot NMe_3$ (0.487 g, 3.504 mmol) and Et_3N (0.81 mL, 5.83 mmol) were added to a well-stirred mixture of **17** (0.100 g, 0.292 mmol) in nhydrous MeCN (5.0 mL) at room temperature under argon. The resulting reaction mixture was heated to reflux under argon for 48 h. The reaction mixture was cooled to room temperature, decanted and concentrated under reduced pressure and applied to a column of reversed-phase C-18 silica gel (eluent: 10–25% CH_3CN-H_2O, 5 g, 2 × 10 cm). Fractions containing the desired compound and some impurities were combined and concentrated and dissolved in 30% aqueous MeOH (10 mL). KF (0.051 g, 0.875 mmol) was added to the solution, which was stirred vigorously at room temperature under argon for 12 h and concentrated under reduced pressure. The crude product was applied to a to a column of cation-exchange resin (Dowex 50WX8-200, H^+ form, 9 g, 2 × 19 cm) prepared by eluting solvents in the following order: water (300 mL), saturated K_2CO_3 solution (400 mL), and water (300 mL). The crude product was eluted with water and fractions containing the desired intermediate and some impurities were combined and concentrated and applied to a column of reversed-phase C-18 silica gel (eluent: 0–20% MeOH-H_2O, 6 g, 2 × 11 cm). Fractions containing the desired compound and some impurities were combined and concentrated and applied to a size-exclusion chromatography column (eluent: H_2O, Sephadex® G-10, 14 g, 2 × 20 cm) to afford **4** as a white solid (0.015 g, 20%): mp >300 °C. ^{1}H NMR (300 MHz, D_2O) δ 7.31 (d, *J* = 8.7 Hz, 2 H), 7.18 (d, *J* = 2.1 Hz, 2 H), 7.01 (d, *J* = 16.2 Hz, 1 H), 6.94 (t, *J* = 2.1 Hz, 1 H), 6.83 (d, *J* = 16.5 Hz, 1 H), 6.70 (d, *J* = 8.7 Hz, 2 H); positive ESIMS *m/z* (rel intensity) 487 (MNa^+, 100). Anal. Calcd for $C_{14}H_9K_3O_9S_2 \cdot \mathbf{2H_2O}$: C, 31.22; H, 2.43; S, 11.91. Found: C, 30.94; H, 2.26; S, 11.73.

Resveratrol Potassium 3-Sulfate (5)

$SO_3 \cdot NMe_3$ (0.548 g, 3.948 mmol) and Et_3N (0.92 mL, 6.56 mmol) were added to a well-stirred mixture of **15** (0.300 g, 0.656 mmol) in anhydrous MeCN (5.0 mL) at room temperature under argon. The resulting reaction mixture was heated to reflux under argon for 48 h. The reaction mixture was cooled to room temperature, decanted and concentrated under reduced pressure and applied to a column of reversed-phase C-18 silica gel (eluent: 10–30% CH_3CN-H_2O, 5 g, 2 × 10 cm). Fractions containing the desired compound and some impurities were combined and concentrated and dissolved in anhydrous MeOH (10 mL). KF (0.051 g, 0.875 mmol) was added to the solution, which was stirred vigorously under argon at room temperature for 12 h and concentrated under reduced pressure. The resulting crude reaction mixture was applied to a column of cation-exchange resin (Dowex 50WX8-200, H^+ form, 8 g, 2 × 18 cm) prepared by eluting solvents in the following order: water (300 mL), saturated K_2CO_3 solution (400 mL), and water (300 mL). The crude product was eluted with water and fractions containing the desired intermediate and some impurities were combined and concentrated and applied to a column of reversed-phase C-18 silica gel (eluent: 0–30% MeOH-H_2O, 6 g, 2 × 11 cm). Fractions containing the desired compound and some impurities were combined and concentrated and applied to a size-exclusion chromatography column (eluent: H_2O, Sephadex® G-10, 14 g, 2 × 20 cm) to give **5** as a white solid (0.010 g, 15%): mp >300 °C. ^{1}H NMR (300 MHz, D_2O) δ7.34 (d, *J* = 8.7 Hz, 2 H), 7.02 (d, *J* = 16.5 Hz, 1 H), 6.88 (s, 1 H), 6.84 (d, *J* = 16.5 Hz, 1 H), 6.77 (s, 1 H), 6.74 (d, *J* = 8.4 Hz, 2 H), 6.54 (t, *J* = 2.1 Hz, 1 H); positive ESIMS *m/z* (rel intensity) 386 (MK^+, 100). Anal. Calcd for $C_{14}H_{11}KO_6S$: C, 48.54; H, 3.20; S, 9.26. Found: C, 48.24; H, 3.12; S, 8.90.

Resveratrol Tetrabutylammonium 4′-Sulfate (6)

TBAF (0.53 mL, 2.189 mmol) was added to a well-stirred mixture of **14** (0.100 g, 0.178 mmol) in MeOH (5.0 mL) at room temperature under argon. The resulting reaction mixture was stirred at room temperature under argon for 12 h. The reaction mixture was concentrated under reduced pressure and the crude product was purified by recrystallization from methanol to afford the product **6** as a white solid (0.015 g, 15%): mp 182–184 °C. ^{1}H NMR (300 MHz, MeOH-d_4) δ 7.38 (d, J = 8.7 Hz, 2 H), 7.17 (d, J = 8.4 Hz, 2 H), 6.92 (d, J = 16.5 Hz, 1 H), 6.81 (d, J = 16.2 Hz, 1 H), 6.37 (d, J = 2.1 Hz, 2 H), 6.07 (t, J = 2.1 Hz, 1 H), 3.11 (t, J = 8.4 Hz, 8 H), 1.59–1.49 (m, 8 H), 1.36–1.23 (m, 8 H), 0.90 (t, J = 7.3 Hz, 12 H); positive ESIMS m/z (rel intensity) 353 (MNa^+, 100). Anal. Calcd for $C_{30}H_{47}NO_6S$: C, 65.54; H, 8.62; N, 2.55; S, 5.83. Found: C, 65.47; H, 8.63; N, 2.44; S, 5.74.

Resveratrol Potassium 4′-Sulfate (7)

SO_3·NMe_3 (0.533 g, 3.829 mmol) and Et_3N (0.73 mL, 5.22 mmol) were added to a well-stirred mixture of **13** (0.330 g, 0.722 mmol) in anhydrous MeCN (8.0 mL) at room temperature under argon. The resulting reaction mixture was heated to reflux under argon for 48 h. The reaction mixture was cooled to room temperature, decanted and concentrated under reduced pressure and applied to a column of reversed-phase C-18 silica gel (eluent: 10–30% CH_3CN-H_2O, 5 g, 2 × 10 cm). Fractions containing the desired compound and some impurities were combined and concentrated and dissolved in anhydrous MeOH (10 mL). KF (0.054 g, 0.935 mmol) was added to the solution, which was stirred vigorously under argon at room temperature for 12 h and concentrated under reduced pressure. The resulting crude reaction mixture was applied to a column of cation-exchange resin (Dowex 50WX8-200, H^+ form, 8 g, 2 × 18 cm) prepared by eluting solvents in the following order: water (300 mL), saturated K_2CO_3 solution (400 mL), and water (300 mL). The crude product was eluted with water and fractions containing the desired intermediate and some impurities were combined, concentrated and applied to a column of reversed-phase C-18 silica gel (eluent: 0–30% MeOH-H_2O, 6 g, 2 × 11 cm). Fractions containing the desired compound and some impurities were combined and concentrated and applied to a size-exclusion chromatography column (eluent: H_2O, Sephadex® G-10, 14 g, 2 × 20 cm) to give **7** as a light-brown solid (0.025 g, 10%): mp >300 °C. ^{1}H NMR (300 MHz, D_2O) δ 7.40 (d, J = 8.4 Hz, 2 H), 7.11 (d, J = 8.4 Hz, 1 H), 6.97 (d, J = 16.5 Hz, 1 H), 6.84 (d, J = 16.5 Hz, 2 H), 6.47 (d, J = 2.1 Hz, 2 H), 6.13 (t, J = 2.1 Hz, 1 H); negative ESIMS m/z (rel intensity) 307 (100). Anal. Calcd for $C_{14}H_{11}KO_6S$: C, 48.54; H, 3.20; S, 9.26. Found: C, 48.19; H, 3.05; S, 8.95.

3,5-bis(*tert*-Butyldimethylsilyloxy)benzaldehyde (9)

TBDMSCl (2.73 g, 14.5 mmol) was added to **8** (1.00 g, 7.24 mmol) in DMF (10 mL) at 0 °C under argon. The reaction mixture was stirred for 16 h at room temperature. Water (50 mL) was added to the reaction mixture and the mixture was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over sodium sulfate and concentrated. The resulting crude product was purified by column chromatography (eluent: hexanes-EtOAc 9:1, silica gel) to afford the product **9** as an orange oil (2.12 g, 80%). IR (film) 3072, 2956, 2931, 2886, 1704, 1384, 1259, 1031 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.86 (s, 1 H), 6.95 (d, J = 2.1 Hz, 1 H), 6.58 (t, J = 2.1 Hz, 1 H), 0.98 (s, 18 H), 0.21 (s, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.6, 157.6, 138.8, 118.6, 114.6, 25.9, 18.5, −4.1; EIMS m/z (rel intensity) 366 (M^+, 100), 309 (96), 267 (58), 239 (52), 84 (78), 73 (70). Anal. Calcd for $C_{19}H_{34}O_3Si_2$: C, 62.24; H, 9.41. Found: C, 61.90; H, 9.41.

(5-Vinyl-1,3-phenylene)bis(oxy)bis(*tert*-butyldimethylsilane) (10)

A reaction mixture containing $MePPh_3Br$ (2.93 g, 8.20 mmol), $NaNH_2$ (0.319 g, 8.20 mmol), and dry ether (12.0 mL) was stirred under argon at room temperature for 20 h. The reaction mixture was cannulated into a well-stirred mixture of **9** (0.300 g, 0.820 mmol) in dry ether (1.0 mL) at −10 °C under argon. After 10 min, the ice bath was removed and the reaction mixture was stirred at room temperature for 5 h. The crude product was concentrated and purified by column chromatography (eluent: hexanes, silica gel) to provide the product **10** as a clear oil (0.21 g, 72%). IR (film) 3056, 2930, 1584, 1471, 1255, 1061 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (dd, *J* = 17.8, 10.8 Hz, 1 H), 6.55 (d, *J* = 2.1 Hz, 2 H), 6.28 (t, *J* = 2.4 Hz, 1 H), 5.68 (dd, *J* = 17.8, 0.9 Hz, 1 H), 5.22 (dd, *J* = 10.8, 0.9 Hz, 1 H), 1.00 (s, 18 H), 0.24 (s, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.0, 139.8, 137.2, 114.3, 112.1, 111.9, 26.1, 18.7, −3.9; positive ESIMS *m/z* (rel intensity) 365 (MH^+, 100). Anal. Calcd for $C_{20}H_{36}O_2Si_2$: C, 65.87; H, 9.95; Si, 15.40. Found: C, 65.48; H, 9.65, Si, 15.53.

4-Iodophenyl Acetate (11)

Ac_2O (3.44 mL, 36.36 mmol) was added to a well-stirred mixture of 4-iodophenol (4.00 g, 18.18 mmol) in dry pyridine (15 mL) at room temperature under argon. The resulting reaction mixture was stirred at room temperature under argon for 12 h. H_2O (40 mL) was added and the mixture was extracted with $CHCl_3$ (3 × 40 mL). The combined organic layers were washed with citric acid (10% w/v) to remove extra pyridine. The resulting crude product was purified by column chromatography (eluent: $CHCl_3$, silica gel) to provide the product as a clear oil (0.823 g, 53%). ^1H NMR (300 MHz, $CDCl_3$) δ 7.66 (d, *J* = 8.4 Hz, 2 H), 6.84 (d, *J* = 8.7 Hz, 2 H), 2.26 (s, 3 H); EIMS *m/z* (rel intensity) 262 (M^+, 23), 220 (100).

(*E*)-4-(3,5-bis(*tert*-Butyldimethylsilyloxy)styryl)phenyl Acetate (12).[46]

Et_3N (1.25 mL), $Pd(OAc)_2$ (0.008 g, 0.038 mmol), and PPh_3 (0.006 g, 0.025 mmol) were added to a well-stirred mixture of **10** (1.865 g, 5.114 mmol), **11** (1.00 g, 3.816 mmol), and CH_3CN (10 mL) at room temperature under argon. The reaction mixture was heated to reflux under argon for 35 h. The resulting suspension was extracted with Et_2O (3 × 30 mL). The combined organic layers were washed with water (1 × 20 mL) and brine (1 × 20 mL), dried over sodium sulfate, and concentrated to provide the product **12** as a clear oil (0.760 g, 40%). ^1H NMR (300 MHz, $CDCl_3$) δ 7.32 (d, *J* = 8.7 Hz, 2 H), 6.90 (d, *J* = 8.4 Hz, 2 H), 6.82 (d, *J* = 16.5 Hz, 1 H), 6.74 (d, *J* = 16.5 Hz, 1 H), 6.45 (d, *J* = 2.1 Hz, 2 H), 6.10 (t, *J* = 2.1 Hz, 1 H), 2.11 (s, 3 H), 0.81 (s, 18 H), 0.06 (s, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.9, 157.2, 150.5, 139.5, 135.5, 129.3, 127.9, 122.2, 112.1, 32.1, 26.2, 18.7, −3.9; positive ESIMS *m/z* (rel intensity) 521 (MNa^+, 100), 499 (MH^+, 82).

(*E*)-4-[3,5-bis(*tert*-Butyldimethylsilyloxy)styryl]phenol (13).[43]

NaOMe (0.002 g, 0.038 mmol) was added to a well-stirred mixture of **12** (0.760 g, 1.52 mmol) in dry MeOH (2 mL) at room temperature. The resulting reaction mixture was stirred at room temperature for 2 h and concentrated under reduced pressure. The crude product was purified by column chromatography (eluent: ether-hexanes 2:1, silica gel) to afford the product **13** as a clear oil (0.629 g, 92%). ^1H NMR (300 MHz, $CDCl_3$) δ 7.39 (d, *J* = 8.7 Hz, 2 H), 6.96 (d, *J* = 16.2 Hz, 1 H), 6.83 (d, *J* = 16.2 Hz, 1 H), 6.81 (d, *J* = 8.7 Hz, 2 H), 6.63 (d, *J* = 2.1 Hz, 2 H), 6.27 (t, *J* = 2.1 Hz, 1 H), 5.92 (s, 1 H), 1.00 (s, 18 H), 0.24 (s, 12 H); negative ESIMS *m/z* (rel intensity) 455 (100).

TBDMS-protected Resveratrol Sodium 4′-Sulfate (14)

SO_3·pyridine (0.349 g, 2.189 mmol) was added to a well-stirred mixture of **13** (0.500 g, 1.095 mmol) in dry pyridine (2.0 mL) at room temperature under argon. The reaction

mixture was heated to reflux under argon for 15 h. A solution of Na_2CO_3 (0.232 g, 2.189 mmol) in H_2O (2.0 mL) was added to the reaction mixture, which was stirred at 60 °C under argon for 1 h. The reaction mixture was cooled to room temperature and concentrated. The crude product was used directly for the next step, but the structure was confirmed by NMR. ^{1}H NMR (300 MHz, MeOH-d_4) δ 7.43 (d, J = 8.7 Hz, 2 H), 7.20 (d, J = 8.7 Hz, 1 H), 6.95 (d, J = 16.2 Hz, 1 H), 6.88 (d, J = 16.5 Hz, 1 H), 6.56 (d, J = 2.1 Hz, 2 H), 6.14 (t, J = 2.1 Hz, 1 H), 0.91 (s, 18 H), 0.13 (s, 12 H).

(*E*)-3-(*tert*-Butyldimethylsilyloxy)-5-[4-(*tert*-butyldimethylsilyloxy)styryl] Phenol (15).[43]

TBDMSCl (0.347 g, 2.300 mmol) was added to a well-stirred mixture of resveratrol (**1**, 0.500 g, 2.190 mmol) and imidazole (0.186 g, 2.738 mmol) in anhydrous DMF (2.0 mL) at −10 °C under argon. The reaction mixture was warmed to ambient temperature. After 12 h, additional imidazole (0.186 g, 2.738 mmol) and TBDMSCl (0.347 g, 2.300 mmol) were added to the reaction mixture and stirring was continued for 12 h. The resulting reaction mixture was diluted with EtOAc (80 mL) and washed with H_2O (3 × 30 mL). The combined organic layers were dried with sodium sulfate and concentrated. The resulting crude product was purified by flash column chromatography (eluent: 10–25% hexanes-EtOAc, silica gel) to afford the product **15** as an orange oil (0.296 g, 33%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35 (d, J = 8.4 Hz, 2 H), 6.95 (d, J = 16.2 Hz, 1 H), 6.83–6.78 (m, 3 H), 6.57 (t, J = 1.5 Hz, 1 H), 6.53 (t, J = 1.8 Hz, 1 H), 6.24 (t, J = 2.1 Hz, 1 H), 5.24 (s, 1 H), 0.98 (s, 18 H), 0.20 (s, 12 H); negative ESIMS *m/z* (rel intensity) 455 (100).

(*E*)-3-(*tert*-Butyldimethylsilyloxy)-5-(4-hydroxystyryl)phenol (16).[43]

Continuation of the column chromatography of **15** afforded **16** as an orange oil (0.113 g, 15%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 (d, J = 8.4 Hz, 2 H), 6.95 (d, J = 16.2 Hz, 1 H), 6.83–6.78 (m, 3 H), 6.56 (t, J = 1.5 Hz, 1 H), 6.53 (t, J = 1.8 Hz, 1 H), 6.23 (t, J = 2.1 Hz, 1 H), 4.83 (s, 1 H), 4.71 (s, 1 H), 0.98 (s, 9 H), 0.20 (s, 6 H).

(*E*)-5-[4-(*tert*-Butyldimethylsilyloxy)styryl]benzene-1,3-diol (17).[43]

Continuation of the column chromatography of **16** afforded **17** as a clear oil (0.090 g, 12%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 (d, J = 8.7 Hz, 2 H), 6.94 (d, J = 16.2 Hz, 1 H), 6.81–6.76 (m, 3 H), 6.52 (d, J = 2.1 Hz, 2 H), 6.26 (t, J = 2.1 Hz, 1 H), 6.02 (s, 2 H), 0.98 (s, 9 H), 0.20 (s, 6 H); negative ESIMS *m/z* (rel intensity) 341 (100).

NFκB Luciferase Assay

Human embryonic kidney cells 293 were used to monitor any changes occurring along the NFκB pathway. This cell line contains chromosomal integration of a luciferase reporter construct regulated by the NFκB response element. Transcription factors can bind to the response element when stimulated by certain agents, allowing transcription of the luciferase gene. Following an incubation period of 48 h with TNFα and test compounds, cells were analyzed for luciferase activity using the Luc assay system from Promega.[49] Results were expressed as a percentage, relative to control (TNFα-treated) samples, and dose–response curves were constructed for the determination of IC_{50} values, which were generated from the results of five serial dilutions of test compounds and were the mean of two different experiments.

COX-1 and -2 Assays

COX-1 from sheep seminal vesicles and recombinant human COX-2 was purchased from Cayman Chemical, Ann Arbor, Michigan. The effect of test compounds on COX-1 and COX-2 was determined by measuring PGE_2 production produced in the COX reaction via an enzyme immunoassay. The reaction was initiated by adding arachidonic acid (AA), the

mixture incubated for 10 min at room temperature, and terminated with 27.8 μM indomethecin. PGE_2 was quantitated by an ELISA method. Diluted samples of the reaction mixture were transferred to a 96-well plate (Nunc-Immuno Plate Maxisorp, Fisher Scientific, Pittsburgh, PA) coated with goat anti-mouse IgG (Jackson Immuno Research Laboratories, West Grove, PA). The tracer (PGE_2-acetylcholinesterase, Cayman Chemical, Ann Arbor, MI) and primary antibody (mouse anti-PGE_2, Monsanto, St. Louis, MO) were added. PGE_2 was determined by the spectrophotometric method at 412 nm using Elman's reagent. A standard curve with PGE_2 (Cayman Chemical, Ann Arbor, MI) was generated on the same plate, which was used to quantify the PGE_2 levels produced in the sample-treated wells. Results were expressed as a percentage, relative to control (solvent-treated) samples, and dose–response curves were generated for the determination of IC_{50} values.[87]

Measurement of Nitric Oxide (NO) Production in LPS-stimulated Macrophages

This assay was performed as previously described.[88] Briefly, RAW 264.7 cells (1×10^5 cells/well) were incubated in 96-well culture plates for 24 h. The cells were treated with serially diluted compounds dissolved in phenol red-free DMEM for 30 min, followed by treatment with or without LPS (1 μg/mL) for an additional 20 h. NO is an unstable molecule and subsequently oxidized to a stable end product nitrite, therefore the amount of NO was estimated by the measurement of nitrite. After 20 h, nitrite released in the media was reacted with Griess reagent, and absorbance was measured at 540 nm. A standard curve was created by using known concentrations of sodium nitrite.

NO-Scavenging Activity

Compounds were diluted ten times with PBS and 20 μL of the diluted solution of each compound was incubated with 100 μL of 6 mM SNP in PBS for 3 h at room temperature. The final concentration of compounds and SNP were 60 μM and 5 mM, respectively. The Griess reaction was performed to estimate the amount of nitrite. Briefly, 180 μL of Griess reagent was added in each well and the absorbance was measured at 540 nm.[89] The results are expressed as average of % inhibition of triplicate determinations ± standard deviation.

Western Blot Analysis

RAW 264.7 cells were pretreated with samples for 15 min before 1 μg/mL LPS for 18 h to examine the expression of iNOS protein. Cells were lysed with lysis buffer. Total protein (30 μg) in each cell lysate was resolved using 8% SDS-PAGE, and electrotransferred to PVDF membranes. The membranes were incubated with 5% skimmed milk in 0.1% Tween 20 containing TBS (TBST) for 1 h at room temperature. Then, membranes were incubated with corresponding primary antibodies in 3% skimmed milk in TBS for 1 h at 37 °C. After washing with TBST for 5 min, three times, membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies for 1 h at 37 °C. Chemiluminescence (ECL) detection kit from Amersham Bioscience (Piscataway, NJ) was employed for the visualization according to the manufacturer's instructions.

Cell Cycle Analysis

HL-60 cells (2×10^5 cells/well) were treated with samples for 24 h. The media was discarded and nuclear isolation medium 4′,6-diamidino-2-phenylindole (NIM-DAPI; Beckman Coulter) solution was added just before the measurement using Cell Lab Quanta™ SC (Beckman Coulter) flow cytometer. NIM-DAPI-stained cells were analyzed after excitation with UV light source. The distribution of cells in each phase of cell cycle was exhibited in a DNA histogram and percentage in $subG_1$ was analyzed.

Cytotoxicity with Cultured Cells

The effect of compounds on cancer cell proliferation was evaluated using the sulforhodamine B (SRB) method.[90] Briefly, KB or MCF7 cells were plated in 96-well plates containing samples and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 72 h of incubation, cells were fixed with 10% trichloroacetic acid solution for 1 h and stained with 0.4% SRB in 1% acetic acid solution. Stained cells were suspended in 10 mM Tris buffer. The effect of compounds on cell viability was quantified by measuring absorbance at 515 nm.

Tandem Mass Spectrometry of Resveratrol 4′-Sulfate

Negative ion electrospray mass spectrometry was used for the analysis of resveratrol 4′-sulfate (**7**) using a high resolution Waters Synapt QqTOF mass spectrometer. The deprotonated molecules of resveratrol and resveratrol 4′-sulfate (**7**) were abundant at *m/z* 227 and *m/z* 307, respectively, and were used as precursor ions for product ion tandem mass spectrometry. Resveratrol 4′-sulfate anion eliminated SO_3 to form a base peak of *m/z* 227 corresponding to resveratrol anion. Other abundant ions in the tandem mass spectra of resveratrol 4′-sulfate (**7**) and resveratrol were observed at *m/z* 185 and *m/z* 143. The transition of *m/z* 307 to *m/z* 227 was used during LC-MS-MS (using a Thermo Finnigan Quantum triple quadrupole mass spectrometer) with selected reaction monitoring (SRM) for the quantitative analysis of resveratrol 4′-sulfate (**7**). The quantitative analysis of resveratrol in these studies was carried using SRM of the transition *m/z* 227 to *m/z* 185 as described previously.[31] Naringenin was used as an internal standard and measured by monitoring the SRM transition *m/z* 271 to *m/z* 151.

Stabilities of Resveratrol and Resveratrol 4′-Sulfate

The stabilities of resveratrol and resveratrol 4′-sulfate (**7**) were investigated for 24 h at 37 °C in RPMI 1640 (Invitrogen, Carlsbad, CA) cell culture medium which was used for the MCF-7 cellular uptake studies. These experiments were carried out three times. Resveratrol-4′-sulfate (**7**) was stable for 24 h (0 h, 100 ± 2.2%; 12 h, 95.1 ± 3.0%; 24 h, 98.8 ± 3.1%). However, resveratrol degraded ~50% during 24 h under these conditions (0 h, 100 ± 0.3%); 12 h, 71.4 ± 7.2%; 24 h, 46.8 ± 8.7%).

Cellular Uptake Studies of Resveratrol and Resveratrol 4′-Sulfate

MCF7 human breast cancer cells (4.5×10^5 cells/well in a 96-well plate) were incubated with 50 μM of resveratrol-4′-sulfate (**7**) at 37 °C for 24 h. The RPMI 1640 cell culture medium was removed, and the cells were rinsed three times with equal volumes of PBS. The cells in each well were harvested, treated with 120 μL lysis buffer for 30 s with mixing, and then sonicated for 5 s. Acetonitrile (370 μL) and 10 μL of naringenin (internal standard; 20 μM) were added, and the cell lysate was vortex mixed for 30 s. After centrifugation at 10,000 *g* at 4 °C for 15 min, the supernatant from each sample was removed, evaporated to dryness under a stream of nitrogen, and reconstituted in 100 μL methanol/water (1:4) for analysis using LC-MS-MS. MCF-7 cells treated with resveratrol 4′-sulfate contained no resveratrol 4′-sulfate, resveratrol 3,5-disulfate, or resveratrol after 24 h. A negative control experiment in which the cells were treated with only buffer also showed no resveratrol or resveratrol sulfates in the cells. MCF-7 cells treated with resveratrol (positive control) were found to contain resveratrol (54.7 ± 19.0 pmol/million cells) and resveratrol 3,5-disulfate (1028.0 ± 166.6 pmol/million cells) but no resveratrol 4′-sulfate.

Inhibition of Aromatase

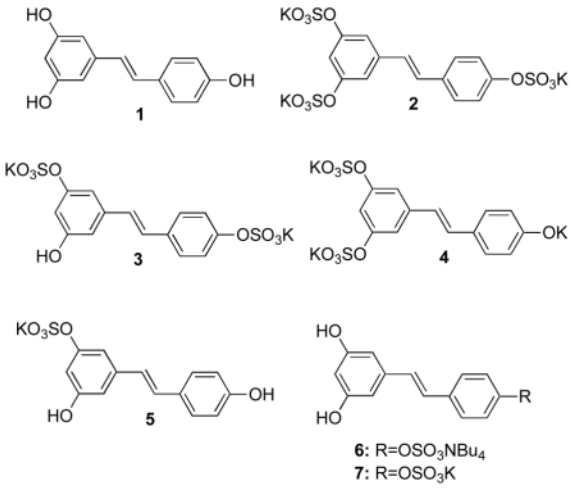
A high-throughput enzyme assay was used to screen samples for aromatase inhibition.[91] This assay employs dibenzylfuorescein as a substrate, and the level fluorescence due to the resultant fluorescein indicates the level of enzyme activity.

Determination of QR Activity in Cell Culture

Quinone reductase was assessed using Hepa 1c1c7 murine hepatoma cells as previously reported.[81] Quinone reductase activity was measured as a function of the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimetylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan. Protein content was determined via crystal violet staining of identical plates. Specific activity is defined as nmol of formazan formed per mg protein per min. The induction ratio (IR) of QR activity represents the specific enzyme activity of agent-treated cells compared with a DMSO-treated control. The concentration to double activity (CD) was determined through a dose-response assay for active substances (IR >2).

Evaluation of Antioxidant Capacity

To evaluate antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging was performed according to the method of Lee et al.[92] Briefly, 95 μL of DPPH radical solution (316 μM) was added in a 96-well plate containing 5 μL of each compound dissolved in 100% DMSO, and incubated for 30 min at 37 °C. The absorbance of each well was measured at 515 nm using a microplate reader. The DPPH radical scavenging activity of each sample was evaluated by calculating % of inhibition as follows: % inhibition = $(1-A_{sample}/A_{control}) \times 100$.



Acknowledgments

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Abbreviations

COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
DIPEA	diisopropylethylamine

DMSO	dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
FTIR	Fourier transform infrared spectroscopy
LPS	lipopolysaccharide
QR1	quinone reductase-1
SNP	sodium nitroprusside
TBAF	tetra-*n*-butylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
TBDMSCl	*tert*-butyldimethylsilyl chloride
TNF-α	tumor necrosis factor alpha

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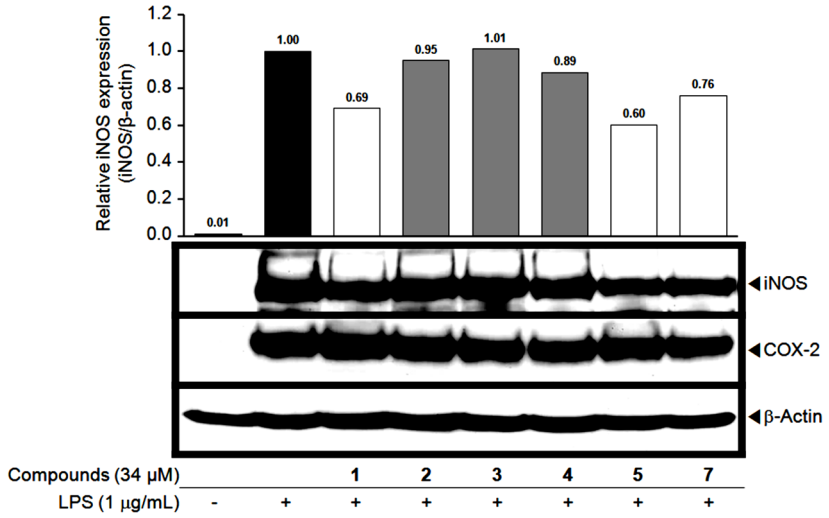
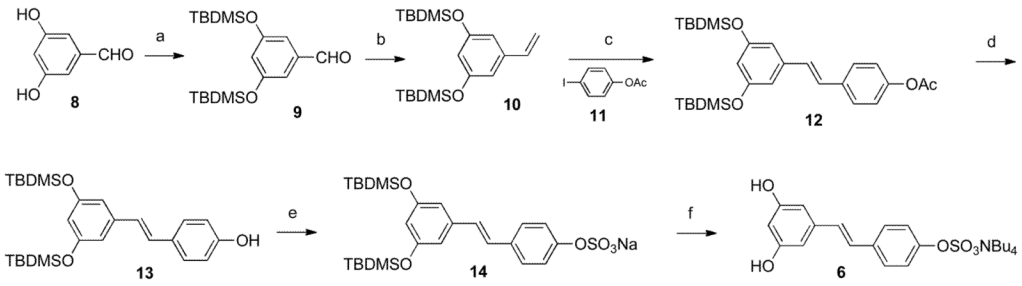


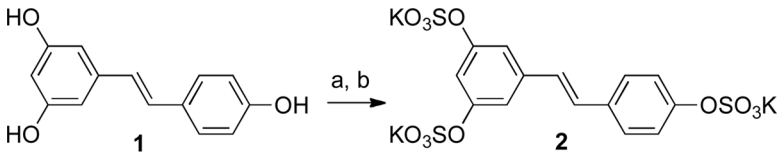
Figure 1.

Effects of resveratrol and its sulfate derivatives on iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with compounds 15 min prior to LPS (1 μg/mL) stimulation and further incubated for 18 h. Cells were lysed, and protein (30 μg) was subjected to 8% SDS-PAGE. The level of iNOS and COX-2 protein expression was examined by immunoblot analysis. β-Actin was used as an internal control. The experiment was performed in duplicate. The density of each band was measured by using an image analyzer system. Average values of relative iNOS protein expression are shown in comparison with the LPS-treated control (black bar). Compounds **1**, **5** and **7** (open bars) showed suppressive effects on the expression of iNOS protein with relative values of 0.62, 0.54 and 0.67, respectively. Compounds **2**, **3**, and **4** were not active, and COX-2 expression was not altered.



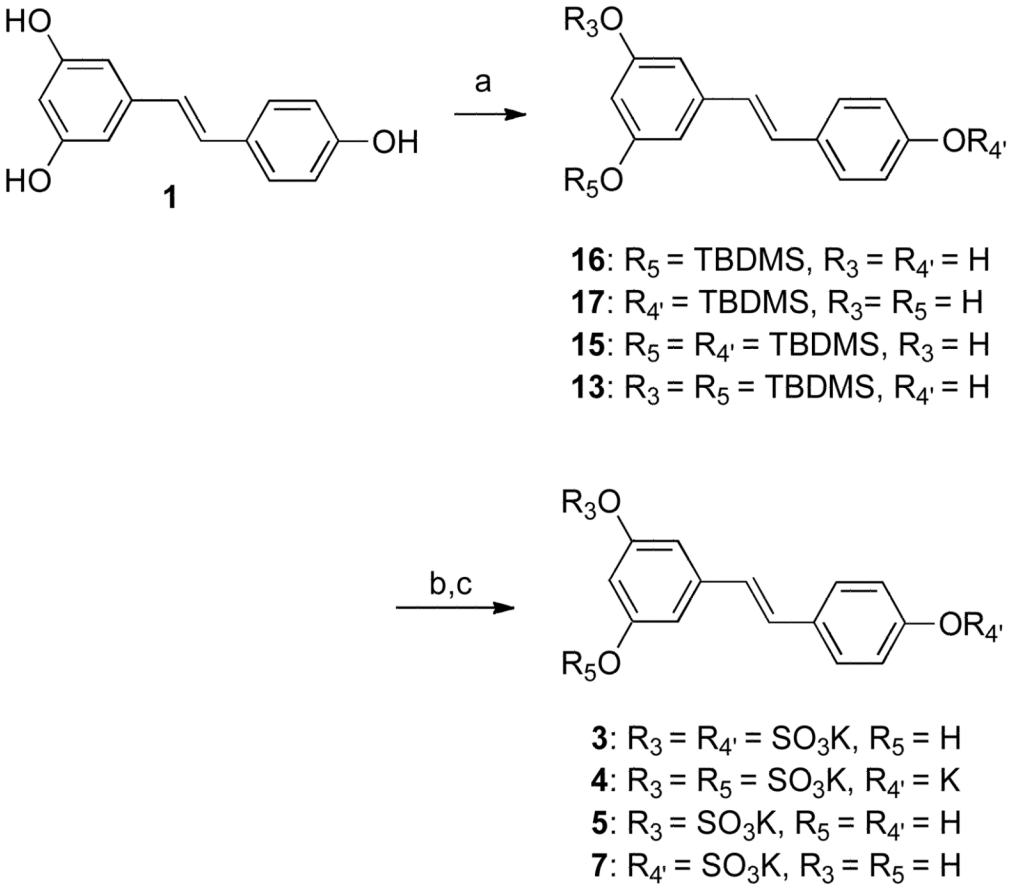
Scheme 1.

[a]Reagents and conditions: (a) TBDMSCl, DMF, 80%; (b) $NaNH_2$, $MePPh_3Br$, Et_2O, 72%; (c) $Pd(OAc)_2$, Et_3N, PPh_3, MeCN, 40%; (d) NaOMe, MeOH, 92%; (e) SO_3-pyridine, pyridine; (f) TBAF, MeOH, 15% (for e and f).



Scheme 2.

[a]Reagents and conditions: (a) SO_3-NMe_3, MeCN, Et_3N, reflux, 21%; (b) Dowex 50WX8-200 ion exchange column, K^+ form.

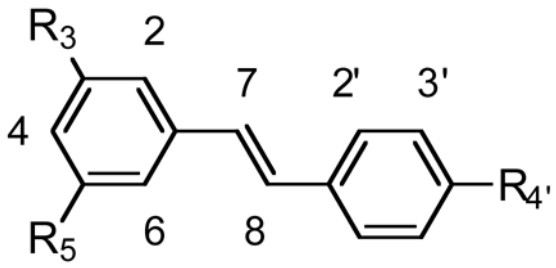


Scheme 3.

[a]Reagents and conditions: (a) TBDMSCl, imidazole, DMF, 12–33%; (b) SO_3-NMe_3, MeCN, Et_3N; (c) KF, MeOH/H_2O, 15–26% (for b and c).

Table 1

^{1}H NMR (in D_2O) Shifts (ppm) for Sulfated Resveratrols



	proton assignment						
compound	**4**	**2**	**6**	**7**	**8**	**2′**	**3′**
Resveratrol	5.41	5.68	5.68	6.60	6.33	6.94	6.20
2	7.03	7.26	7.26	7.14	7.03	7.49	7.15
3	6.38	6.65	6.71	7.05	6.94	7.46	7.14
4	6.94	7.18	7.18	7.01	6.83	7.31	6.70
5	6.54	6.77	6.88	7.02	6.84	7.34	6.74
6	Not soluble in D_2O.						
7	6.13	6.47	6.47	6.97	6.84	7.40	7.11

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Table 2

Biological Evaluation of Resveratrol and Resveratrol Sulfates in Bioassays Indicative of Cancer Chemoprevention

	compound tested					
assay	**Resveratrol**	**2**	**3**	**4**	**5**	**7**
NFκB						
% inhibition[a]	75.7 ± 2.12	36.7 ± 9.4	53.4 ± 2.9	42.5 ± 5.23	33.0 ± 4.81	64.0 ± 2.26
IC_{50} (μM)	0.173±0.05	–	–	–	–	18.2 ± 0.99
COX-1						
% inhibition[a]	75.2 ± 4.53	37.8 ± 0.71	23.3 ± 0.98	30.9 ± 2.69	74.3 ± 0.99	63.2 ± 3.39
IC_{50} (μM)	6.65 ± 2.5	–	–	–	3.60 ± 0.8	5.55 ± 1.73
COX-2						
% inhibition[a]	72.2±4.67	2.4 ± 2.0	16.5 ± 2.69	25.5 ± 5.52	62.0 ± 1.7	65.8 ± 7.64
Nitric Oxide						
% inhibition[a]	71.8 ± 3.5	24.8 ± 3.1	41.7 ± 5.5	4.8 ± 4.5	41.0 ± 0.7	56.8 ± 5.9
IC_{50} (μM)	15.0 ± 2.6	–	–	–	–	–
% $SubG_1$[a,b]	24.9 ± 0.149	–	–	–	–	2.22 ± 0.012
Cytotoxicity[c]						
KB % survival	47.8 ± 5.2	78.1 ± 7.8	99.3 ± 15.0	84.2 ± 13.1	70.3 ± 8.8	108.5 ± 10.3
MCF7 % survival	38.6 ± 3.5	106.7 ± 9.6	100.0 ± 11.3	97.2 ± 5.2	51.7 ± 0.2	103.4 ± 8.8
Aromatase						
% inhibition[a]	34.8 ± 1.21	28.5 ± 0.2	22.5 ± 0.64	20.5 ± 0.43	28.2 ± 1.12	30.4 ± 0.56
QR1						
CD (μM)[d]	21 ± 0.46	> 11.7	> 10.1	> 10.1	2.6 ± 0.38	> 6.9
DPPH						
% inhibition[e]	65.2 ± 2.0	0.4 ± 1.1	6.8 ± 1.0	14.5 ± 4.2	68.0 ± 1.9	42.8 ± 2.5
IC_{50} (μM)	178.5 ± 9.3	–	–	–	219.2 ± 3.1	–

[a] Determined at a compound concentration of 34 μM. The compounds were not cytotoxic at this concentration in HEK-293 cells.

[b] The percentage of $subG_1$ HL-60 cells, characteristic of apoptosis. The distribution in subG1 of vehicle-treated cells was 2.2 ± 0.0%.

[c] Determined at a concentration of 20 μg/mL.

[d] Concentration to double the expression of QR1.

[e] Determined at a compound concentration of 340 μM.