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Synthesis of novel azo-resveratrol, azo-oxyresveratrol and their derivatives as potent tyrosinase inhibitors

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ABSTRACT

Ten azo compounds including azo-resveratrol (**5**) and azo-oxyresveratrol (**9**) were synthesized using a modified Curtius rearrangement and diazotization followed by coupling reactions with various phenolic analogs. All synthesized compounds were evaluated for their mushroom tyrosinase inhibitory activity. Compounds **4** and **5** exhibited high tyrosinase inhibitory activity (56.25% and 72.75% at 50 μM, respectively). The results of mushroom tyrosinase inhibition assays indicate that the 4-hydroxyphenyl moiety is essential for high inhibition and that 3,5-dihydroxyphenyl and 3,5-dimethoxyphenyl derivatives are better for tyrosinase inhibition than 2,5-dimethoxyphenyl derivatives. Particularly, introduction of hydroxyl or methoxy group into the 4-hydroxyphenyl moiety diminished or significantly reduced mushroom tryosinase inhibition. Among the synthesized azo compounds, azo-resveratrol (**5**) showed the most potent mushroom tyrosinase inhibition with an IC_{50} value of $IC_{50} = 36.28 \pm 0.72$ μM, comparable to that of resveratrol, a well-known tyrosinase inhibitor.

Resveratrol (Fig. 1) is a naturally occurring phytoalexin with a stilbene structure that is found in grapes, berries, peanuts, and red wine[1] and has attracted much attention for many years due to its diverse biological effects. There have been many reports proving its effects such as antioxidant, anti-aging, anti-inflammation, anticancer, cardio-protection and anti-tyrosinase activities.[2] Resveratrol is well-known as one of the most potent antioxidants against ROS and oxidative stress. Some of the biological activities of resveratrol, such as neuroprotective, cardioprotective, and anticancer effects, are attributed to its anti-oxidative property.[3–5] Its anti-oxidative action is reported to be exerted via hydrogen transfer from its hydroxyl group to reactive free radicals, converting it into a long-lived and less reactive ArO**.** radical.[6] Recently, it was shown that resveratrol can have anti-hypertensive properties through both endothelium-independent and endothelium-mediated vasorelaxing effects.[7–9] All these beneficial effects on human health have prompted the use of resveratrol as a therapeutic agent. Therefore, it is of interest to develop a series of resveratrol analogs that show resveratrol-like biological activities.

Generally, resveratrol and its analogs contain a double bond that may undergo *E*/*Z*-isomerization and two phenolic rings located on each end of the double bond (Fig. 1).[10,11] In previous studies, resveratrol analogs were mainly synthesized by modifying the substituents on both phenyl rings, not the double bond of the stilbene scaffold.[5,12] Benzene-fusioned (**I**) and heterocycle-fusioned (**II** and **III**) analogs of resveratrol were also recently synthesized and found to have antitumor, vasorelaxing or anti-tyrosinase activity (Fig. 1). The structural feature of these analogues is that their benzene, furan and thiazole rings serve as a double bond connecting two aromatic rings.[9] Oxyresveratrol, which is a resveratrol analog with an additional hydroxyl group, also showed comparable anti-tyrosinase activity to resveratrol, a well-known tyrosinase inhibitor. Especially, benzene-fusioned analogs, including compound **I** developed by our laboratory exhibited even more potent anti-tyrosinase activity than resveratrol.[13] Tyrosinase exists widely in bacteria, fungi, plants, insects, vertebrates and invertebrates[14] and is the rate limiting enzyme in the biosynthesis of melanin pigments responsible for coloration of hair, skin and eyes, the undesired enzymatic browning of fruits and vegetables and the developmental and defensive functions of insects.[15] Therefore, tyrosinase inhibitors have become increasingly important in the agricultural (for insecticides and browning inhibitors of vegetables and fruits), medicinal (for treatment of hyperpigmentation diseases) and cosmetic (for whitening agents) industries.

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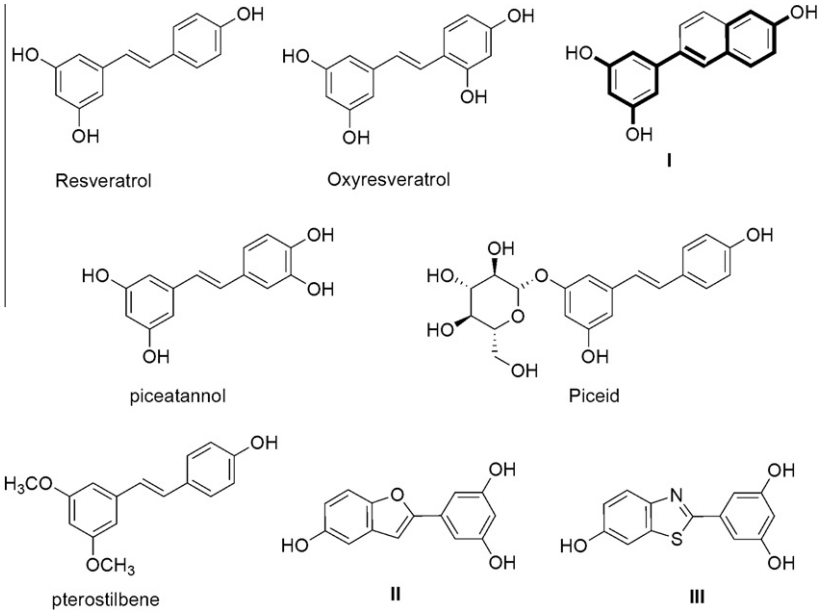


Figure 1. Chemical structures of resveratrol and its derivatives.

Aromatic azo compounds are widely used in various fields as organic dyes, radical initiators, therapeutics and drug delivery agents.[16–18] Moreover, azo compounds are also involved in a number of biological reactions such as inhibition of DNA, RNA and protein synthesis, carcinogenesis and nitrogen fixation.[19,20]

In this work, modification to convert an ethylene linker connecting two phenyl rings of resveratrol into an azo linker was attempted (Fig. 2). More varied and increased effects on anti-tyrosinase, as well as antioxidant and other activities, can be expected by azo-resveratrol or azo-oxyresveratrol due to the combination of the chemical properties of azo compounds and resveratrol or oxyresveratrol.

The double bond of resveratrol, oxyresveratrol or their derivatives was converted into an azo linkage but, in most compounds, the *para*-hydroxyl group on their phenyl ring was maintained because the *para*-hydroxyl group is more responsible for the radical-scavenging activity than the *meta*-hydroxyl group in the antioxidant reaction.[6,11,21]

In this work, ten non-symmetric azo compounds including azo-resveratrol and azo-oxyresveratrol were synthesized. Generally, symmetric aromatic azo compounds are prepared by the oxidation of aromatic amines using transition metals or by the reduction of nitro aromatics using metals.[22,23] In order to prepare non-symmetric aromatic azo compounds, a coupling reaction of aryl diazonium salts with electron-rich aromatics such as phenolic compounds was employed.[24] The synthesis of azo-resveratrol (**5**), azo-oxyresveratrol (**9**) and their *O*-methyl analogs is depicted in Scheme 1. For the preparation of azo-resveratrol (**5**) and its analogs, 3,5-dimethoxyaniline (**3**) was synthesized from methyl 3,5-dihydroxybenzoate. *O*-methylation of the phenolic hydroxyl groups of methyl 3,5-dihydroxybenzoate with dimethyl sulfate

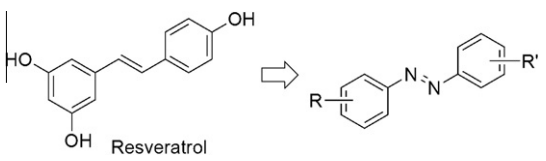
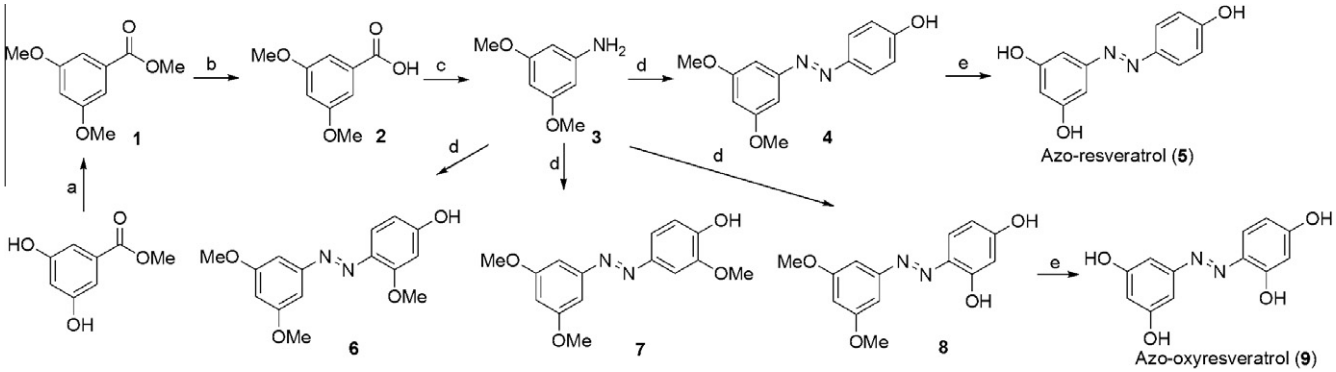


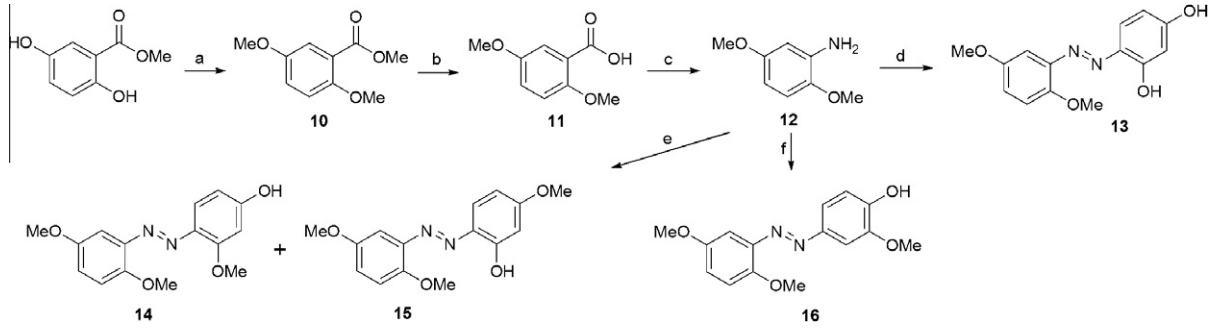
Figure 2. Chemical structure of stilbene with an azo linker designed from resveratrol.

followed by hydrolysis with 1 N-NaOH afforded 3,5-dihydroxybenzoic acid (**2**). A modified Curtius rearrangement of the resulting acid **2** with diphenylphosphoryl azide (DPPA)[25] in refluxing 1,2-dichloroethane gave a rather stable isocyanate intermediate that required further hydrolysis with sodium hydroxide to generate the corresponding amine **3**, a key material for preparation of the diazonium salt. Treatment of **3** with sodium nitrite in an aqueous HCl solution generated arenediazonium salt,[26] which was reacted with various electron-rich phenols such as phenol, 3-methoxyphenol, 2-methoxyphenol, and resorcinol (1,3-dihydroxybenzene) to give non-symmetric azo aromatic derivatives **4**–**8**, respectively. Coupling between aryl diazonium salt and phenols takes place almost exclusively at the *para* position if it is open. *Para*-products were exclusively generated in all coupling reactions. *O*-demethylation of **4** was performed using boron tribromide at room temperature to afford azo-resveratrol (**5**) contaminated with borate salts. Crude azo-resveratrol was acetylated with acetic anhydride to remove the borate salts, and finally the corresponding acetylated azo-resveratrol was deacetylated using sodium methoxide to generate pure azo-resveratrol (**5**). Azo-oxyresveratrol (**9**) was also prepared from *O*-demethylation of **8** using BBr_3 followed by the same purification processes used in the purification of azo-oxyresveratrol (**5**).

As shown in Scheme 2 and 2,5-dimethoxyphenyl azo compounds **13**–**16** were also synthesized from methyl 2,5-dihydroxybenzoate using the same modified Curtius rearrangement and diazotization followed by a coupling reaction with phenols. It is interesting to note that the coupling reaction of 2,5-dimethoxyphenyl diazonium ion with 3-methoxyphenol did not give the corresponding *para*-product exclusively. Instead, the *para*- and *ortho*-products were obtained as an inseparable mixture. According to the ^{1}H NMR experiments, the ratio of the *para*- and *ortho*-products was 2:1. Acetylation of the mixture with acetic anhydride in the absence of tertiary amine produced a less polar spot as a sole product on TLC along with the unreacted starting material. The less polar spot turned out to be an acetylated mixture of **14** and **15** by NMR analysis; notably, the unreacted starting material was compound **15**. This result suggests that the phenolic hydroxyl group of **14** is more favorable to acetylation than that of **15**, probably due to an intramolecular hydrogen bond between the hydroxyl group of **15** and the neighboring

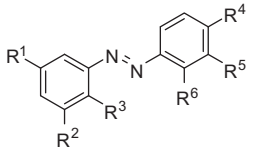


Scheme 1. Synthesis of azo-resveratrol, azo-oxyresveratrol and their analogs. Reagents and conditions: (a) $(CH_3)_2SO_4$, K_2CO_3, CH_3CN, rt, 88%; (b) 1 N-NaOH, 1,4-dioxane, rt, 98%; (c) (i) DPPA, Et_3N, 1,2-dichloroethane, reflux, (ii) 2 N-NaOH, THF, rt, 87%; (d) (i) $NaNO_2$, 1 N-HCl, THF, 0–5 °C, (ii) phenols, 1 N-NaOH, 0–5 °C, 80% for **4**, 72% for **6**, 73% for **7**, 66% for **8**; (e) (i) BBr_3, CH_2Cl_2, 0 °C to rt, (ii) Ac_2O, pyridine, rt, (iii) 1 N-NaOMe, MeOH, rt, 54% for **5**, 61% for **9**.



Scheme 2. Synthesis of 2,5-dimethoxyphenyl azo compounds. Reagents and conditions: (a) $(CH_3)_2SO_4$, K_2CO_3, CH_3CN, rt, 81%; (b) 1 N-NaOH, 1,4-dioxane, rt, 92%; (c) (i) DPPA, Et_3N, 1,2-dichloroethane, reflux, (ii) 2 N-NaOH, THF, rt, 76%; (d) (i) $NaNO_2$, 1 N-HCl, THF, 0–5 °C, (ii) resorcinol, 1 N-NaOH, 0–5 °C, (iii) Ac_2O, pyridine, rt, (iv) 1 N-NaOMe, MeOH, rt, 49%; (e) (i) $NaNO_2$, 1 N-HCl, THF, 0–5 °C, (ii) 3-methoxyphenol, 1 N-NaOH, 0–5 °C, (iii) Ac_2O, CH_2Cl_2, rt, 11% for **15**; (f) (i) $NaNO_2$, 1 N-HCl, THF, 0–5 °C, (ii) guiacol, 1 N-NaOH, 0–5 °C, 18%.

Table 1
Tyrosinase inhibition and substitution pattern of the substituted azo analogs **4**–**9**, **13**, **15** and **16**



Compound	R^1	R^2	R^3	R^4	R^5	R^6	Tyrosinase inhibition[a] (%)
4	OMe	OMe	H	OH	H	H	56.25 ± 0.45
5	OH	OH	H	OH	H	H	72.75 ± 1.60
6	OMe	OMe	H	OH	H	OMe	8.90 ± 1.87
7	OMe	OMe	H	OH	OMe	H	1.03 ± 9.08
8	OMe	OMe	H	OH	H	OH	48.50 ± 3.47
9	OH	OH	H	OH	H	OH	41.46 ± 2.17
13	OMe	H	OMe	OH	H	OH	27.80 ± 1.14
15	OMe	H	OMe	OMe	H	OH	6.38 ± 2.79
16	OMe	H	OMe	OH	OMe	H	0.44 ± 1.65

Values represent means ± S.E. of three experiments.
[a] Tyrosinase inhibition was measured using L-tyrosinase as the substrate at 50 μM.

nitrogen, as well as steric hindrance. In the coupling reaction of diazonium salt with resorcinol and 2-methoxyphenol, the *para*-products **13** and **16** were formed exclusively. All synthesized compounds were characterized by melting point, ^1H and ^{13}C NMR and low/high resolution mass experiments.

Tyrosinase inhibition by the synthesized azo-phenols **4**–**9**, **13**, **15** and **16** was examined with an expectation that the compounds would show good anti-tyrosinase activity. The inhibitory activities were examined using mushroom tyrosinase as described previously with minor modification.[27] The inhibition of mushroom tyrosinase using the synthesized azo-phenol derivatives is summarized in Table 1.[28] Whereas compounds **6**, **7**, **15** and **16** exhibited weak or no inhibition, compounds **4**, **8** and **13** showed moderate inhibitory activity against mushroom tyrosinase, with 56.25%, 48.50% and 27.80% inhibition at 50 μM, respectively. Azo-oxyresveratrol (**9**) also showed moderate inhibitory potency with

Table 2
Inhibitory effects of compound **4**, azo-resveratrol (**5**) and resveratrol on mushroom tyrosinase activity

Compound	Conc. [a] (μM)	Tyrosinase inhibition (%)	IC_{50} [b] (μM)
4	5.0	8.40 ± 0.70	50.2 ± 1.01
	20.0	46.04 ± 0.82	
	50.0	56.31 ± 0.51	
	80.0	61.41 ± 0.72	
Azo-resveratrol (**5**)	15.0	7.60 ± 1.09	36.28 ± 0.72
	30.0	44.29 ± 1.13	
	50.0	72.75± 1.60	
Resveratrol	0.5	13.67 ± 4.24	26.63 ± 0.55
	1.0	16.26 ± 0.75	
	5.0	27.09 ± 7.79	
	10.0	38.05 ± 1.84	
	20.0	47.29 ± 1.07	
	30.0	47.78 ± 1.30	

[a] Values represent means ± S.E. of three experiments.
[b] 50% inhibitory concentration.

41.46% inhibition at 50 μM. Azo-resveratrol (**5**) exerted the most potent inhibitory activity against mushroom tyrosinase, with 72.75% inhibition at the same concentration. The inhibitory activities decreased in the order of **5** > **4** > **8** > **9** > **13** > **6**, **15** > **7**, **16**. These results suggest that 3,5-dimethoxyphenyl and 3,5-dihydroxyphenyl derivatives are superior to 2,5-dimethoxy derivatives in the inhibition of tyrosinase (**4**, **5**, **8** and **9** vs **13**, **15** and **16**) and that 4-hydroxyphenyl is an essential group for potent inhibitory activity (**4** and **5**). It is worthy to note that introduction of the second substituent into the 4-hydroxyphenyl moiety decreases mushroom tyrosinase inhibition, depending on the substituent and position. Insertion of a methoxy group into the 3-position dramatically diminished the tyrosinase inhibitory effect (**7** and **16**). Introduction of a methoxy group into the 2-position (**6**) lagged behind introduction into the 3-position in decreasing order of inhibitory potency. It is also notable that 4-hydroxyphenyl azo compounds exhibited more significant inhibitory activity against mushroom tyrosinase than 2,4-dihydroxyphenyl azo compounds (**5** vs **9** and **4** vs **8**). Interestingly, this result is contrary to that observed in phenyl-benzo[*d*]thiazole, phenyl-thiazolidine-4-carboxylic acid and phenyl-pyrrolidine-2,5-dione analogs, in which 2,4-dihydroxyphenyl derivatives exhibited higher mushroom tyrosinase inhibition than 4-hydroxyphenyl derivatives.[29] The bioactivities of compounds **4** and **5** were investigated in greater detail. As depicted in Table 2, resveratrol, a positive control, inhibited mushroom tyrosinase activity in a concentration-dependent manner. The data showed that compound **4** (IC_{50} = 50.20 ± 1.00 μM) is 2-fold less potent than resveratrol (IC_{50} = 26.63 ± 0.55 μM) as an inhibitors of mushroom tyrosinase. However, azo-resveratrol (**5**, IC_{50} = 36.28 ± 0.72 μM) exhibited almost equipotent tyrosinase inhibitory activity to resveratrol. Compounds **4** and **5** displayed dose-dependent inhibition of mushroom tyrosinase. Using the Chemdraw Ultra 10.0 program, the Log*P* values for resveratrol and azo-resveratrol are 3.03 and 3.30, respectively, suggesting that azo-resveratrol (**5**)[30] may be easily absorbed into skin than resveratrol if the compounds are used as an external application for treatment of various diseases associated with hyperpigmentation. Research on the antioxidant, anti-aging, anti-inflammation and anticancer activities of azo-resveratrol, azo-oxyresveratrol[30] and their analogs is currently underway and the results will be presented in due course.

In summary, on the basis of structures of resveratrol and oxyresveratrol that show a variety of biological activities, azo-resveratrol, azo-oxyresveratrol and their analogs were first synthesized using a modified Curtius rearrangement and diazotization followed by a coupling reaction with various phenolic compounds. Tyrosinase inhibition by the synthesized compounds was examined. 4-Hydroxyphenyl derivatives suppressed mushroom tyrosinase activity more effectively than the other derivatives including 2,4-dihydroxyphenyl, 2-methoxy-4-hydroxyphenyl and 2-hydroxy-4-methoxyphenyl. 4-Hydroxy-3-methoxyphenyl analogs significantly reduced the antityrosinase effect. Generally, 3,5-dihydroxyphenyl and 3,5-dimethoxyphenyl analogs were more potent tyrosinase inhibitors than 2,5-dimethoxyphenyl analogs. Azo-resveratrol (**5**) exhibited the most potent mushroom tyrosinase inhibition. The IC_{50} value of azo-resveratrol was comparable to that of resveratrol. Higher Log*P* value of azo-resveratrol compared with resveratrol might provide merit superior to resveratrol as a lead compound for the development of whitening agents and pharmaceutical drugs to treat hyperpigmentation. Synthetic methodology and structure-activity relationships of azo-resveratrol (**5**), azo-oxyresveratrol (**9**) and their analogs will be extensively utilized in designing and synthesizing potent tyrosinase inhibitors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.050.

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28. *Assay to measure inhibitory effects on mushroom tyrosinase*: Mushroom tyrosinase was used to measure tyrosinase activity for the entire study. Tyrosinase activity was determined as described previously with minor modification.[27] Briefly, 20 μL of aqueous mushroom tyrosinase solution (1000 units) was added to a 96-well microplate (Nunc, Denmark) in a 200 μL assay mixture containing 1 mM l-tyrosine solution and 50 mM phosphate buffer (pH 6.5). The assay mixture was incubated at 25 °C for 30 min. Following incubation, the amount of dopachrome produced was determined spectrophotometrically at 492 nm (OD_{492}) using a microplate reader (Hewlett Packard, Palo Alto, CA, USA). IC_{50}, or inhibitory concentration 50, is the concentration of a compound that inhibits the maximal enzyme velocity by half. IC_{50} is derived from the *X*-axis on an inhibitor concentration versus product formation curve and is determined from the alignment of a dose-response curve on the dependent *Y*-axis. In the present study, dose-dependent inhibition experiments were performed in triplicate to determine the IC_{50} of compounds. According to the inhibition percentage of three doses in each experiment, the log-linear curves and their equations were determined. Individual IC_{50} values were then calculated as the concentration at which the *Y*-axis equaled 50% inhibition. The results from three experiments are shown.
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30. *(E)-5-((4-Hydroxyphenyl)diazenyl)benzene-1,3-diol (azo-resveratrol,* ***5****)*: Yellow solid; melting point, 179–181 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 7.77 (d, 2H, *J* = 9.0 Hz), 6.90 (d, 2H, *J* = 9.0 Hz), 6.80 (d, 2H, *J* = 2.0 Hz), 6.37 (t, 1H, *J* = 2.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 160.8, 158.8, 155.0, 146.2, 124.6, 115.5, 104.3, 101.0; HRMS (ESI) *m*/*z* $C_{12}H_9N_2O_3$ $(M-H)^-$ Calcd 229.0613, obsd 229.0620; *(E)-4-((3,5-Dihydroxyphenyl)diazenyl)benzene-1,3-diol (azo-oxyresveratrol,* ***9****)*: Orange-colored solid; melting point, 197–199 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 7.63 (d, 1H, *J* = 8.5 Hz), 6.73 (d, 2H, *J* = 2.0 Hz), 6.50 (dd, 1H, *J* = 2.0, 8.5 Hz), 6.34 (t, 1H, *J* = 2.0 Hz), 6.30 (d, 1H, *J* = 2.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 163.0, 159.2, 157.1, 152.3, 134.4, 132.4, 109.0, 104.1, 102.9, 99.8; LRMS (ESI) *m*/*z* $(M-H)^-$ 245; HRMS (ESI) *m*/*z* $C_{12}H_9N_2O_4$ $(M-H)^-$ Calcd 245.0562, obsd 245.0578.