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Synthesis and study of new paramagnetic resveratrol analogues

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Dedicated to Professor László Szekeres on the occasion of his 90th birthday

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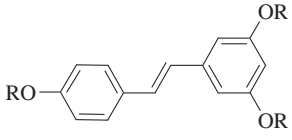
ABSTRACT

New resveratrol analogues containing five- and six-membered nitroxides and isoindoline nitroxides were synthesized. These new compounds were compared to resveratrol based on their ABTS radical scavenging ability as well on their capacity to suppress inflammatory process in macrophages induced by lipopolysaccharides. The ABTS and ROS scavenging activities of new molecules were the same or weaker than that of resveratrol, but some of paramagnetic resveratrol derivatives suppressed nitrite and TNFα production more efficiently than resveratrol. Based on these results the new nitroxide and phenol containing hybrid molecules can be considered as new antioxidant and anti-inflammatory agents.

1. Introduction

Resveratrol **1** (3,5,4′-trihydroxystilbene) (Fig. 1.) is a naturally occurring phytoalexin produced by wide range of plants in response to environmental stress or pathogenic attack and it was first isolated from the roots of white hellebore lily (Veratrum Grandiflorum O. Loes).[1] Resveratrol has been reported to exert a variety of biological activities including antioxidant, anti-inflammatory, anti-obesity, anti-viral, anti-microbial, cardioprotective, neuroprotective and cancer chemopreventive effects.[2] These effect are mediated through several biological receptors, including cyclooxygenase (COX), lipooxygenase (LOX), nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), quinone reductase 1 (QR1) quinine reductase 2 (QR2), ornithine decarboxylase (ODC) and aromatase.[3]

Due to its occurrence in red wines[4] the antioxidant activity of resveratrol was studied in details.[5,6] Several synthetic analogues of resveratrol were reported, in an attempt to determine the structural requirements for resveratrol-like biological activities also aiming extending the chemical diversity of polyphenols in view of possible improvements of their pharmacological properties.[7–11] Nitroxides are stable free radicals that rapidly cross cell-membranes, preempt free-radical formation by oxidising redox-active metal ions (Eq. 1, Fig. 2), and function both as intra- and extracellular SOD mimics (Eq. 2, 3).[12] The reduced form of the nitroxide, hydroxylamine, also has antioxidant activity (Eq. 4). Several studies indicated that modificating cardiopotective agents,[13] PARP-inhibitors[14] with nitroxides had a beneficial influence on their activity as supplemented by 'in status nascendi acting' antioxidants and radical scavengers. Chromones and flavones[15,16] modified by nitroxides were reported previously from our laboratory, although the biological activity of these 'hybrid' compounds was not outstanding. To access the influence of the nitroxide on antioxidant activity of resveratrol, paramagnetic analogues of resveratrol were synthesized. Our aim is to incorporate a nitroxide moiety into 'resveratrol-like' molecules according to one of the following strategies: (1) replacement of the phenol ring of resveratrol with a nitroxide ring, (2) insert the pyrroline ring between the phenol and resorcine ring, (3) replacement both phenol and resorcine rings with nitroxides. In these paper we report the synthesis of this new resveratrol analogues with a preliminary study of their antioxidant and antiinflammatory activities.



	R
1	H
2	CH_3

1, 2

Figure 1. Structure of resveratrol and 3,5,4′-trimethoxystilbene.

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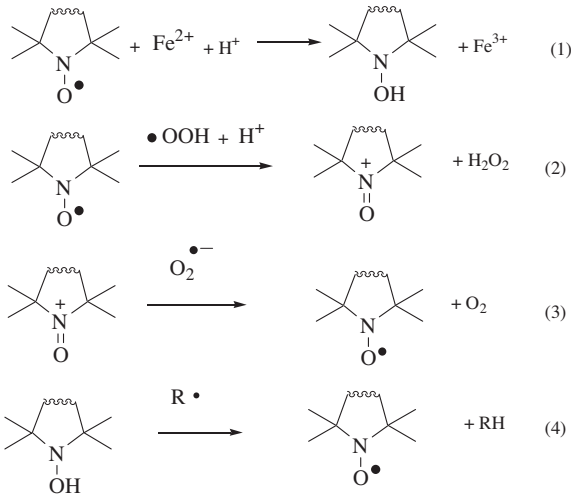


Figure 2. Antioxidant mechanisms of nitroxides and hydroxylamines.

2. Chemistry

For synthesis of new resveratrol analogues with E (trans) geometry we decided to use Heck coupling, as a well-established method furnishing E isomer stilbenes only.[17] Coupling of 3,5-dimethoxystyrene (**3**)[18] with **4** paramagnetic vinyliodide[19] in the presence of $Pd(OAc)_2$ as a catalyst and K_2CO_3, Bu_4NBr in DMF gave compound **5a**. An NMR study of the Heck coupled O-acetyl[20] derivative (**5b**) proved that only E isomer was formed. Reduction of **5a** with iron powder in glacial acetic acid gave compound **5c**, which was found to be also an E isomer exclusively after ^1H NMR study. (Scheme 1).

To prepare the corresponding 3,5-dihydroxy derivatives the Heck coupling were performed with paramagnetic vinyl compounds and 3,5-diacetoxy iodobenzene (**7**). This latter was prepared by acetylation of 3,5-dihydroxy-iodobenzene[21] **6** in CH_2Cl_2 with acetic anhydride in the presence of 4-dimethylaminopyridine.[7] The reaction of compound **7** with the corresponding paramagnetic vinyl compounds **8**[22] and **12** in DMF in the presence of $Pd(OAc)_2$, KOAc and Bu_4NBr furnished compound **9** and **13** after removing the protecting groups with NaOMe. The same procedure was used for the synthesis of **10** biradical. Compound **12** was synthesized by treatment of **11** aldehyde[23] with triphenylmethylphosphonium iodide in the presence of K_2CO_3, KOH and 18-crown-6 in refluxing dioxane (Scheme 2).

For the synthesis of *Z*-resveratrol derivative, compound **14**[24] was coupled with 1 equiv 3,5-dimethoxyphenylboronic acid[25] in aq. dioxane in the presence of excess Na_2CO_3 and 7% $Pd(PPh_3)_4$ catalyst[26] a to give mixture of compounds **15** (24%) and **16** (32%). Reduction of compound **16** with iron powder in acetic acid[27] furnished sterically hindered amine **17**. Suzuki-coupling of 4-hydroxyphenylboronic acid with compound **15** in aqueous dioxane, 5% $Pd(PPh_3)_4$ catalyst and Na_2CO_3 gave compound **18**. The hydroxylamine salt of **18** was demethylated in CH_2Cl_2 with large excess of BBr_3 to furnish the paramagnetic *Z*-resveratrol **19**, unfortunately in a very low (9%) yield This can be explained by partial irreversible destruction of nitroxide moiety. Unfortunately, regular procedures for reoxidation with activated MnO_2 or treatment with HNO_2[28] could not be applied in the presence of polyphenols (Scheme 3).

3. Biological evaluation

3.1. ABTS Radical scavenging assay

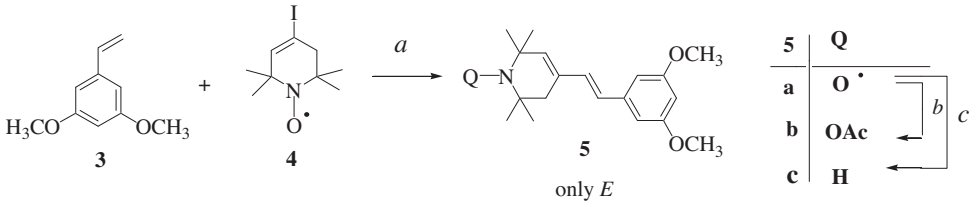
The antioxidant activity of new resveratrol derivatives was tested using an ABTS (2,2′azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay in ethanol or in 0.05 M PBS (pH 7.4) compared to Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as antioxidant standard.[29] The assays were run in ethanol, because nitroxides exhibited limited solubilities in PBS. For PBS measurements hydroxylamine salt of nitroxides were prepared. The antioxidant activity of resveratrol (**1**), 3,5,4′-trimethoxystilbene (**2**)[30] and compounds **5a**, **5c, 9**, **10**, **13, 19** is given therefore as Trolox Equivalent Antioxidant Capacity (TEAC) units in Table 1.

We can conclude that compounds with two phenolic hydroxyl groups in the presence of nitroxide can exhibit the same antioxidant activity as resveratrol itself in EtOH. Nitroxide in compound **5a** also exhibits some antioxidant activity (0.2 TEAC) compared to 3,5,4′-trimethoxy-stilbene **2** (no activity). Sterically hindered amine **5c**, has minimal antioxidant activity (0.3 TEAC), which can be contributed to their HCl salt proton donor activity in the absence of buffer, but no antioxidant capacity was observed in buffer. In PBS all the new compounds exhibited less ABTS radical scavenging activity than the resveratrol itself.

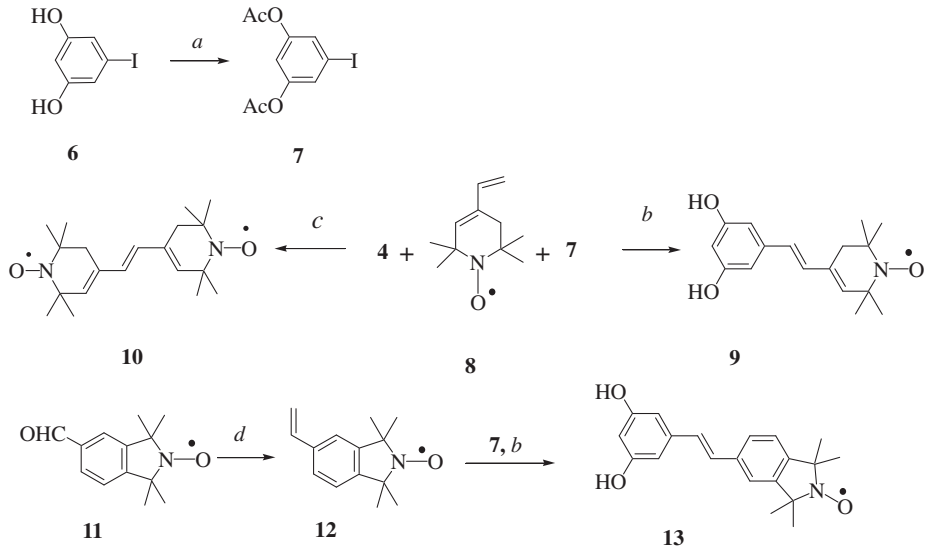
3.2. Antiinflammatory assays

Inflammatory processes are primarily mediated by rapidly activating cells of the innate immunity such as macrophages. After recognising the invaders, these cells produce a large number of reactive agents including RNS and ROS with many different inflammatory cytokines.[31] However, these molecules are able to fight against the pathogens or induce other immune cells, higher concentrations can cause systemic inflammation leading to tissue damage and to the symptoms of highly lethal septic shock.[32] Therefore, to investigate the anti-inflammatory effect of paramagnetic analogues compared with that of the parent molecule resveratrol, we applied RAW264.7 mouse macrophage cells and induced them with the bacterial endotoxin, lipopolysaccharide (LPS).

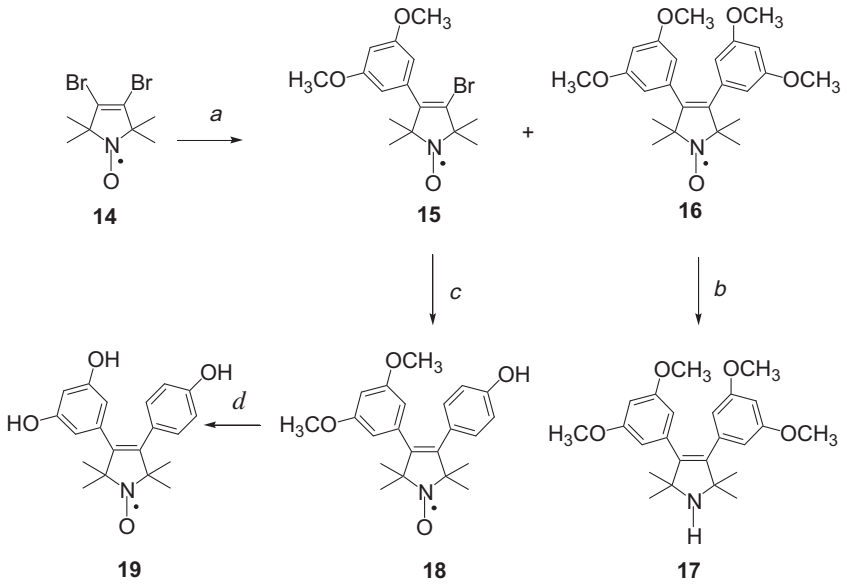
Via the activation of the inflammatory enzyme inducible NO-Synthase, the LPS-induced macrophages produce high concentrations of the RNS, nitric-oxide (NO). NO rapidly oxidises to its more stable form nitrite (NO_2^-), which accumulates in the media of treated cells.[33] All of the paramagnetic analogues were found to



Scheme 1. Reaction and conditions: (a) K_2CO_3 (3.0 equiv), $Pd(OAc)_2$ (0.05 equiv), Bu_4NBr (1.0 equiv), DMF, 80 °C, 5 h, 69%; (b) ascorbic acid (5 equiv), dioxane, water 40 °C, 20 min under N_2, then extraction with $CHCl_3$, Et_3N (1.1 equiv), AcCl (1.1 equiv) 0 °C–rt 1 h, 43–55%; (c) Fe (10 equiv), AcOH, 60 °C, then basify with K_2CO_3 extraction with $CHCl_3$, 45%.



Scheme 2. Reagents and conditions: (a) Ac_2O (3.0 equiv), DMAP (0.1 equiv), pyridine (3.0 equiv), CH_2Cl_2, rt 10 h, 74%; (b) KOAc (3.0 equiv), $Pd(OAc)_2$ (0.05 equiv), Bu_4NBr (1.0 equiv), DMF, 80 °C, 10 h, then NaOMe (0.1 equiv), MeOH, 1 h., rt, 55–78%; (c) K_2CO_3 (3.0 equiv), $Pd(OAc)_2$ (0.05 equiv), Bu_4NBr (1.0 equiv), DMF, 80 °C, 5 h, 57%; (d) PCH_3Ph_3I, K_2CO_3 (1.0 equiv), KOH (0.1 equiv), 18-crown-6 (0.05 equiv), dioxane, reflux, 48 h, 62%.



Scheme 3. Reagents and conditions: (a) $Pd(PPh_3)_4$ (0.07 equiv), under N_2, rt 15 min, dioxane, then 10% aq. Na_2CO_3 (excess), 3,5-dimethoxyhenylboronic acid (1 equiv.), reflux, 4 h, 24% for **15** and 32% for **16**; (b) Fe (10 equiv),AcOH, 60 °C, then basify with K_2CO_3 extraction with $CHCl_3$, 29%; (c) $Pd(PPh_3)_4$ (0.14 equiv), under N_2, rt 15 min, dioxane then 10% aq. Na_2CO_3 (excess), 4-hydroxyphenylboronic acid (1,5 equiv), reflux, 8 h, 59%; (d) EtOH/HCl, reflux 15 min, evaporation of ethanol then BBr_3 (5 equiv), CH_2Cl_2, −78 °C–rt, 24 h then BBr_3 (5 equiv), −78 °C–rt, 24 h, 9%.

Table 1
TEAC activity of resveratrol and its paramagnetic analogues

Compound	TEAC[a] in EtOH	TEAC[a] in PBS	Compound	TEAC[a] in EtOH	TEAC[a] in PBS
1	1.0 ± 0.05	1.3 ± 0.20	**9** (HO-4415)	0.92 ± 0.08	0.62 ± 0.10[b]
2	0.04 ± 0.02	ND	**10** (HO-4569)	0.22 ± 0.01	0.37 ± 0.08[b]
5a (HO-4408)	0.2 ± 0.07	0.28 ± 0.05[b]	**13** (HO-4450)	0.98 ± 0.02	0.67 ± 0.12[b]
5c (HO-4409)	0.32 ± 0.05	0.02 ± 0.01	**19** (HO-4486)	1.02 ± 0.03	ND

ND, not determined.
[a] $n = 3$.
[b] *N*-hydroxylamine HCl salt.

possess a concentration dependent (12.5–100 μM) inhibitory effect against LPS-induced NO_2^- production, as measured 24 h after treatment (Fig. 3). In the case of the compounds **9, 10, 13** but mostly in case of **5a, 5c** this inhibition was remarkably more powerful, than that of resveratrol, applied in the same concentrations and under the same experimental conditions. Among the

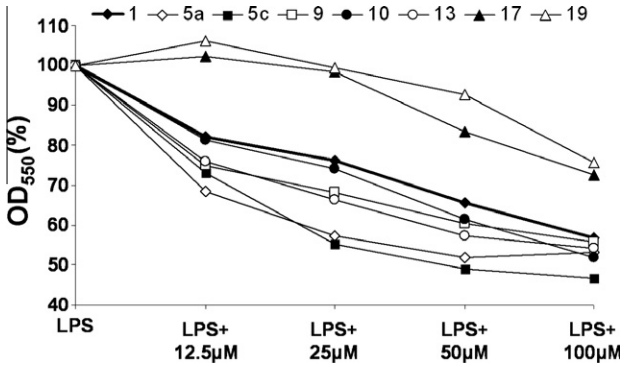


Figure 3. Effect of resveratrol and its analogues on nitrite production of LPS-induced macrophage cells: RAW264.7 cells were treated with LPS (100 ng/mL) alone or together with resveratrol or its analogues (12.5–100 μM). After 24 h incubation nitrite production was measured with Griess-reagent. Values are presented as means of OD_{550} ± SEM (n = 6) in percentage of the mean of LPS-treated cells. Maximal value of SEM was less than 2%. Experiments were repeated three times.

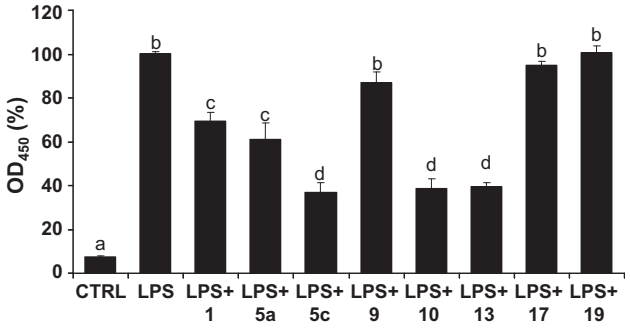


Figure 5. Effect of resveratrol and its analogues on TNFα production of LPS-induced macrophage cells: RAW264.7 cells were treated with LPS (100 ng/mL) alone or together with resveratrol or its analogues (50 μM). After 1.5 h incubation TNFα production was measured. Values are presented as means of OD_{450} ± SEM (n = 3) in percentage of the mean of LPS-treated cells. Experiments were repeated three times. Means without a common letter differ, p<0.05.

analogues, compound **17, 19** showed the weakest inhibiting potency, since they were not able to approach the effect of the parent molecule (Fig. 3). This observation can be explained that six-membered nitroxides **5a, 9, 10** and isoindoline nitroxide **13** with lower redox potential is more potent RNS scavenger than the five-membered nitroxide **19**. It is known for instance that NO_2 forms the respective oxoammonium cation with nitroxide and it more readily happens with nitroxides possessing lower redox potential.[34]

The other group of highly reactive agents, which are capable of damaging lipids, proteins and the nucleic acids of the cells, are the ROS. They may have originated from many different sources, like mitochondrial respiration or the enzymes NADPH-oxidase and cyclooxigenase.[35] In our experimental model, we detected high levels of ROS in LPS-induced macrophages 24 h after the treatment, which were decreased by the paramagnetic analogues in a concentration dependent manner (125–100 μM) (Fig. 4). Despite of the high efficacy of our compounds in inhibiting NO_2^- production, we found only a moderate effect in decreasing ROS levels compared with resveratrol, applied under the same experimental conditions. Similarly to the nitrite measurement, compound **17, 19** were found to have the weakest antioxidant potential and only compound **5a, 5c, 9** and **13** were capable of presenting a comparable antioxidant effect (Fig. 4). This observation correlates well with ABTS radical scavenging activity in PBS. In this case the six-membered nitroxides and isoindoline nitroxide are more efficient again

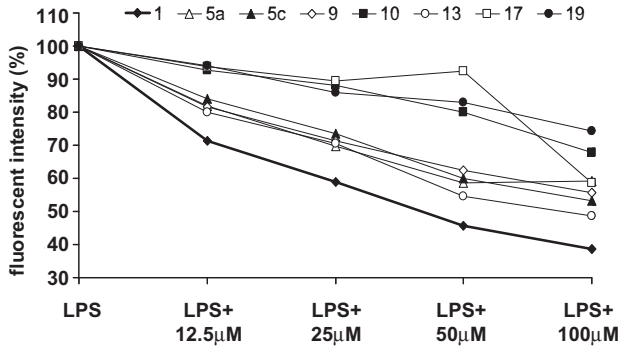


Figure 4. Effect of resveratrol and its analogues on ROS production of LPS-induced macrophage cells: RAW264.7 cells were treated with LPS (100 ng/mL) alone or together with resveratrol or its analogues (12.5–100 μM). After 24 h incubation ROS production was measured with C400. Values are presented as means of the fluorescent intensity ± SEM (n = 6) in percentage of the mean of LPS-treated cells. Maximal value of SEM was less than 3%. Experiments were repeated three times.

in ROS scavenging than pyrroline nitroxide **19**, but ROS scavenging activity seemingly requires the presence of aromatic rings. Biradical **10** was not found to be an efficient ROS scavenger.

Not only directly damaging, but immunomodulating agents—cytokines, such as interleukins and interferons—are synthesized by activated macrophages.[36] One of the most important cytokines is TNFα, which is basically responsible for the induction of inflammatory processes in the human organism.[37] 1.5 h after LPS challenge we measured a 10–12-fold increase in TNFα concentration in the media of the treated cells (Fig. 5). All of the used paramagnetic analogues (50 μM) and resveratrol (50 μM) decreased the amount of TNFα and compound **5c, 10, 13** exhibited the strongest effect. Resveratrol analogues **9, 17** decreased the amount of TNFα, but this reduction was proved to be statistically not significant and compound **19** had no effect on TNFα production at all (Fig. 5).

4. Conclusions

In conclusion, new paramagnetic resveratrol analogues were synthesized by Heck reaction and Suzuki-coupling. Compounds with two phenolic hydroxyl groups and a nitroxide moiety exhibited less TEAC (in buffer) and ROS scavenging activity than resveratrol (**1**). However, LPS induced inflammation processes compounds **5a, 5c, 9** and **13** suppressed nitrite formation and compounds **5c, 10, 13** reduced the amount of TNFα The 3,4-disubstituted pyrroline nitroxide **19** and **17** amine did not exhibited remarkable biological activity. The structure–activity relationships studies suggest that in a resveratrol-like compound substitution of an aromatic ring with a tetrahydropyridine or an isoindoline nitroxide is more advantageous than incorporating a pyrroline nitroxide ring. Based on previous results,[38] we believe that isoindoline and tetrahydropyridine nitroxides attached to an aromatic ring (**5a, 10, 13**) or pre-nitroxide **5c** have an advantage over polyphenols because of their ability to alter the cellular redox state by scavenging free radicals and this ability in vivo is recycled. A further study of the most active antioxidants is in progress.

5. Experimental

5.1. Synthesis

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on Fisons EA 1110 CHNS elemental analyser. The IR (Specord 75) spectra were in each case consistent with

the assigned structure. Mass spectra were recorded on a Thermoquest Automass Multi instrument in the EI mode. ^{1}H NMR spectra were recorded with Varian UNITYINOVA 400 WB spectrometer. Chemical shifts are referenced to Me_4Si. Measurements were run at 298 K probe temperature in $CDCl_3$ solution. ^{1}H NMR spectra of all diamagnetic new compounds can be found in the supplementary material. ESR spectra were taken on Miniscope MS 200 in 10^{-4} M $CHCl_3$ solution and all monoradicals gave triplet line a_N = 14.4–15.1 G, **10** biradical gave quintet line a_{N1} = 7.7 G, a_{N2} = 15.5 G. Flash column chromatography was performed on Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially available plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel GF254. Compounds **2**,[30] **3**,[19] **4**,[20] **6**,[21] **8**,[22] **11**,[23] **14**,[24] and 3,5-dimethoxyphenylboronic acid[25] were prepared according to published procedures and **1**, 3,5-dimethoxybenzaldehyde, Trolox, ABTS and other reagents were purchased from Aldrich.

5.1.1. (*E*)-4-(3,5-Dimethoxystiryl)-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-1-yloxyl radical (5a) and (*E*)-4,4′-(Ethene-1,2-diyl)-bis(2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-1-yloxyl) Biradical (10)

To a solution of 3,5-dimethoxystyrene **3** (820 mg, 5.0 mmol) or compound **8** (900 mg, 5.0 mmol) and **4** vinyl iodide (1.40 g, 5.0 mmol), $Pd(OAc)_2$ (112 mg, 0.5 mmol), Bu_4NBr (1.61 g 5.0 mmol in DMF (20 mL) K_2CO_3 (2,07 g, 15.0 mmol) was added and the mixture was stirred at 80 °C for 5 h under N_2. The solvent was evaporated off; the residue was partitioned between EtOAc (20 mL) and water (10 mL). The aqueous phase was washed with EtOAc (10 mL), the combined organic phase was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography with hexane/Et_2O 2:1 to elute the remaining of starting materials, then with hexane/EtOAc, 2:1 to get the title compounds.

Compound **5a**: 1.09 g (69%) orange crystals, mp 63–65 °C, R_f 0.57 (hexane/EtOAc, 2:1). IR (nujol): 1590 (C=C) cm^{-1}. MS (EI) *m*/*z* (%): 316 (M^+, 46), 286 (10), 271 (55), 243 (62), 151 (53), 77 (100). Anal Calcd for $C_{19}H_{26}NO_3$: C 72.12; H 8.28; N 4.43; found: C 72.10; H 8.15; N 4.39.

Compound **10**: 946 mg (57%) brownish crystals, mp 208–210 °C, R_f 0.63 (hexane/EtOAc, 2:1). IR (nujol): 1640 (C=C) cm^{-1}. MS (EI) *m*/*z* (%): 332 (M^+, 18), 303 (16), 287 (41), 272 (45), 149 (100). Anal Calcd for $C_{20}H_{32}N_2O_2$: C 72.25; H 9.70; N 8.43; found: C 72.12; H 9.65; N 8.31.

5.1.2. (*E*)-1-Acetoxy-4-(3,5-dimethoxystyryl)-2,2,6,6-tetrameth yl-1,2,3,6-tetrahydropyridine (5b)

To a stirred solution of compound **5a** (948 mg, 3.0 mmol, obtained by Heck reaction) in dioxane (20 mL) ascorbic acid (2.64 g, 15.0 mmol) in water (10 mL) was added and the mixture was stirred under N_2 for 15 min at 40 °C. The pale yellow solution was extracted with $CHCl_3$ (2 × 20 mL) and dried under N_2 on $MgSO_4$. Acetyl chloride (260 mg, 3.3 mmol) was added, followed by the addition of Et_3N (333 mg, 3.3 mmol) at 0 °C. Stirring was continued for 1 h at rt then the mixture was filtered and the filtrate was evaporated in vacuo to dryness. The residue was partitioned between brine (10 mL) and EtOAc (15 mL) and the aqueous phase was washed with EtOAc (2 × 10 mL). The combined organic phase was dried ($MgSO_4$), filtered and evaporated and after flash chromatography (hexane/EtOAc, 2:1) the title compound was obtained as a white crystalline solid 592 mg (55%), mp 87–89 °C, R_f 0.52 (hexane/EtOAc, 2:1), IR (nujol) 1755 (C=O), 1580 (C=C). MS (EI) *m*/*z* (%): 359 (M^+, 1), 344 (16), 302 (100), 43 (39). ^{1}H NMR (CD_3OD) 6.78 (d, 1H, *J* = 16 Hz), 6.59 (s, 2H), 6.42 (d, 1 H, *J* = 16 Hz), 6.34 (s, 1H), 5.65 (s, 1H), 3.76 (s, 6H), 2.50–2.28 (m, 2H), 2.12 (s, 3H) 1.32 (s, 3H), 1.19 (s, 6H), 1.17 (s, 3H). Anal Calcd for $C_{21}H_{29}NO_4$: C 70.17; H 8.13; N 3.90; found: C 70.25; H 8.09; N 3.79.

5.1.3. General procedure for reduction of nitroxides to sterically hindered amines (5c, 17)

To a solution of nitroxide **5a** or **16** (2.0 mmol) in AcOH (10 mL) Fe powder (1.12 g, 20.0 mmol) was added and the mixture was warmed to 60 °C until the reaction started. The mixture was stirred at room temperature for 1 h, diluted with water (30 mL), decanted, and the decanted aq. solution made alkaline with solid K_2CO_3 (intense foaming!). The mixture was extracted with $CHCl_3$/MeOH (9:1) (3 × 15 mL), dried ($MgSO_4$), filtered, evaporated and chromatographic purification ($CHCl_3$/MeOH) gave compound **8** or **19** (29–45%).

5.1.3.1. (*E*)-4-(3,5-Dimethoxystyryl)-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridine(5c). 270 mg (45%), white solid, mp: 201–203 °C, R_f 0.59 ($CHCl_3$/MeOH, 2:1), Ms (EI) *m*/*z* (%): 301 (M^+, 23), 286 (100), 136 (26). IR (nujol): 3220 (NH), 1630, 1570 (C=C) cm^{-1}. ^{1}H NMR (CD_3OD) 6.91 (d, 1H, *J* = 16 Hz), 6.65 (s, 2H), 6.43 (d, 1 H, *J* = 16 Hz), 6.35 (s, 1H), 5.83 (s, 1 H), 3.75 (s, 6H), 2.45 (s, 2H), 2.03 (s, 6H), 1.15 (s, 6H). Anal. Calcd for $C_{19}H_{27}NO_2$: C 75.71; H 9.03; N 4.65; found: C 75.55; H 9.00; N 4.50.

5.1.3.2. 3,4-Bis(3,5-dimethoxyphenyl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole (17). 230 mg (29%), mp 252–254 °C (HCl salt), R_f 0.40 ($CHCl_3$/MeOH, 9:1). Ms (EI) *m*/*z* (%): 397 (M^+, 1), 382 (100), 367 (7), 184 (27). IR (nujol): 3100 (NH), 1570 (C=C) cm^{-1}. ^{1}H NMR (CD_3OD) 6,38 (s, 2H,), 6.30 (s, 4H), 3.68 (s, 12 H), 1.65 (s, 12 H). Anal. Calcd for $C_{34}H_{31}NO_4$ C 72.52; H 7.86; N 3.52; found: C 72.36; H 7.77; N 3.38.

5.1.4. 5-iodo-1,3-phenylene diacetate (7)

Acetic anhydride (3.06 g, 30.0 mmol) was added dropwise to a solution of compound **6** (2.36 g, 10.0 mmol), pyridine (2.37 g, 30.0 mmol) and DMAP (122 mg, 1.0 mmol) in CH_2Cl_2 (20 mL) at 0 °C. The reaction mixture was then stirred overnight at rt and the solution was concentrated in a vacuum, then the residue was purified by flash column chromatography (hexane/Et_2O, 2:1) to provide the title compound as white crystalline solid 2.36 g (74%), mp 46–48 °C, R_f 0.36 (hexane/Et_2O, 2:1). Ms (EI) *m*/*z* (%): 320 (M^+, 5), 278 (21), 236 (52), 43 (100). IR (nujol): 1760 (C=O), 1575 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) 7.35 (s, 2H), 6.91 (s, 1H), 2.72 (s, 6H). Anal. Calcd for $C_8H_9IO_2$ C 36.39; H 3.44 found C 36.25, H 3.29.

5.1.5. 1,1,3,3-Tetramethyl-5-vinylisoindolin-2-yloxyl Radical (12)

A solution of aldehyde **11** (1.09 g, 5.0 mmol), K_2CO_3 (690 mg, 5.0 mmol), triphenylmethylphosphonium iodide (2.02 g, 5.0 mmol), KOH (28 mg, 0.5 mmol) and 18-crown-6 (66 mg, 0.25 mmol) in dioxane (25 mL) was stirred and refluxed for 48 h. After cooling, the inorganic salt was filtered off, the dioxane was evaporated at reduced pressure and the residue was partitioned between water (10 mL) and Et_2O (30 mL). the organic phase was separated, dried ($MgSO_4$), filtered and evaporated and the residue was purified by flash column chromatography (hexane:Et_2O, 2:1) to give the title compound as a yellow solid 669 mg (62%), mp 74–76 °C, R_f 0.49 (hexane/Et_2O). Ms (EI) *m*/*z* (%): 216 (M^+, 81), 186 (88), 171 (100), 110 (93). 91 (50). IR (nujol): 1580 (C=C) cm^{-1}. Anal. Calcd for $C_{14}H_{18}NO$: C 77.74; H 8.39; N 6.48; found: C 77.74; H 8.22; N 6.39.

5.1.6. General procedure for Heck coupling with 5-iodo-1,3-phenylene diacetate (9, 13)

To a stirred solution of compound **8** (360 mg, 2.0 mmol) or compound **12** (432 mg, 2.0 mmol), tetrabutylammonium bromide (2.0 mmol 644 mg), $Pd(OAc)_2$ (22 mg, 0.1 mmol) and potassium acetate (588 mg, 6.20 mmol) in DMF (20 mL) **7** iodo compound

(640 mg, 2.0 mmol) was added at rt under N_2. The reaction mixture was warmed to 80 °C for 3 h and then cooled to room temperature. The solvent was evaporated, the residue was partitioned between water (10 mL) and $CHCl_3$ (25 mL). The organic phase was separated, dried ($MgSO_4$), filtered and evaporated and the residue was dissolved in MeOH (20 mL) and NaOMe solution (freshly made from 5 mg sodium and 2 mL MeOH) was added and the mixture was stirred for 1 h at room temperature. Then the solvent was evaporated, the residue was purified by flash column chromatography ($CHCl_3/Et_2O$, 2:1) to give the title compounds in 55–78% yield. These derivatives must be kept in deep freezer (−18 °C) otherwise decompose within several days.

5.1.7. (*E*)-5-(2-(1-Oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)vinyl)benzene-1,3-diol Radical (9)

317 mg (55%) beige solid, mp 66–68 °C, R_f 0.36 ($CHCl_3/Et_2O$, 2:1), Ms (EI) *m*/*z* (%): 288 (M^+, 3), 274 (6), 243 (3), 138 (100) 109 (91). IR (nujol): 3200 (OH), 1590 (C=C) cm^{-1}. Anal. Calcd for $C_{17}H_{22}NO_3$: C 70.81; H 7.69; N 4.86; found: C 70.66; H 7.60; N 4.79

5.1.8. (*E*)-5-[2-(2-Oxyl-1,1,3,3-tetramethylisoindolin-5-yl)vinyl]benzene-1,3-diol Radical (13)

Yellow solid, 505 mg (78%), mp 215–217 °C. R_f 0.32 ($CHCl_3/Et_2O$, 2:1). Ms (EI) *m*/*z* (%): 324 (M^+, 62), 294 (100), 277 (62), 138 (93). IR (nujol): 3240 (OH), 1580 (C=C) cm^{-1}. Anal. Calcd for $C_{20}H_{22}NO_3$: C 73.82; H 7.12; N 4.30; found: C 73.75; H 7.05; N 7.09.

5.1.9. 3-Bromo-4-(3,5-dimethoxyphenyl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl Radical (15) and 3,4-Bis-(3,5-dimethoxyphenyl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl Radical (16)

To a deoxygenated (N_2 was bubbled for 10 min) solution of compound **14** (1.49 g, 5.0 mmol) in dioxane (25 mL) $Pd(PPh_3)_4$ (444 mg, 0.35 mmol) was added and the solution was stirred for 15 min at rt, 3,5-dimethoxyphenylboronic acid (910 mg, 5.0 mmol) and 10% aq. Na_2CO_3 solution (10 mL) were added, the mixture was stirred and heated at reflux temperature under N_2 for 4 h. The mixture was allowed to cool and the solvents were evaporated. The residue was suspended in water (10 mL) and pH 3 was adjusted with 5% H_2SO_4 (intense foaming!). The aqueous solution was extracted with $CHCl_3$ (2 × 20 mL). The combined phases were dried ($MgSO_4$), filtrated and evaporated. The residue was purified by flash column chromatography (hexane/EtOAc 2:1). The first band was the unreacted compound **14**, the second band was 3,3′,5,5′-tetramethoxy-1,1′-biphenyl, and the third was compound **15**: yellow brown crystals 426 mg (24%); mp: 90–93 °C, R_f 0.53 (hexane/EtOAc, 2:1). Ms (EI) *m*/*z* (%): 356/354 (M+, 6:6), 274 (100), 245 (81), 230 (25), 107 (52). IR (nujol): 1590 cm^{-1}. Anal. Calcd for $C_{16}H_{21}BrNO_3$: C 54.10; H 5.96; N 3.94; found: C 53.92; H 6.18; N 3.86. The fourth band was compound **16** as yellow crystals 659 mg (32%), R_f 0.35 (hexane/EtOAc, 2:1), mp: 175–177 °C. Ms (EI) *m*/*z* (%): 412 (M^+, 55), 397 (17), 382 (10), 274 (100). Anal. Calcd for $C_{24}H_{30}NO_5$: C 69.88; H 7.33; N 3.40; found: C 69.79; H 7.22; N 3.31.

5.1.10. 3-(3,5-dimetoxyphenyl)-4-(4-hydroxyphenyl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl Radical (18)

To a deoxygenated (N_2 was bubbled through for 10 min) solution of compound **15** (710 mg 2.0 mmol) in dioxane (25 mL) $Pd(PPh_3)_4$ (58 mg, 0.05 mmol) was added. The solution was stirred for 15 min at rt then 4-hydroxyphenylboronic acid (413 mg 3.0 mmol) and 10% aq. Na_2CO_3 solution (20 mL) were added. The mixture was stirred and heated at reflux temperature under N_2 for 4 h. The mixture was allowed to cool and the solvents were evaporated. To residue was suspended in water (10 mL) and 5% aq. H_2SO_4 was added to adjust the pH to 3 (intense foaming!) and the aqueous solution was extracted with $CHCl_3$ (2 × 20 mL). The combined phases were dried ($MgSO_4$), filtrated and evaporated. The residue was purified by flash column chromatography (hexane/EtOAc 2:1) to yield compound **18** as a yellow crystalline solid 217 mg (59%), mp: 228–230 °C, R_f 0.26 (hexane/EtOAc, 2:1). Ms (EI) *m*/*z* (%): 368 (M^+, 82), 353 (41), 338 (27), 323 (37), 167 (100). Anal. Calcd for $C_{22}H_{26}NO_4$: C 71.72; H 7.11; N 3.80; found: C 71.58; H 7.03; N 3.62.

5.1.11. 3-(3,5-Dihydroxyphenyl)-4-(4-hydroxyphenyl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl Radical (19)

A solution of compound **18** (368 mg, 1.0 mmol) in EtOH (10 mL), saturated previously with HCl gas, was refluxed for 15 min. After cooling, the solvent was evaporated and the residue was suspended in CH_2Cl_2 (20 mL) and BBr_3 (2.50 g, 10.0 mmol) dissolved in CH_2Cl_2 (5 mL) was added at -78 °C and the mixture was allowed to warm to rt. and stirred for 24 h. Then the solvent was cooled to −78 °C and BBr_3 (2.50 g, 10.0 mmol) dissolved in CH_2Cl_2 (5 mL) was added again and the stirring was continued for further 24 h at rt. The mixture was poured onto mixture of crushed ice (100 g) and $NaHCO_3$ (5.04 g 60.0 mmol) with stirring (caution: intense foaming!), $CHCl_3$ (40 mL) was added and the mixture was stirred for 5 min. After separation of the organic phase, the aqueous phase was separated, saturated with NaCl and extracted again with $CHCl_3$/MeOH 9:1 mixture (20 mL). The combined organic phase was dried ($MgSO_4$) filtered, evaporated and the residue was purified by flash column chromatography ($CHCl_3$/MeOH, 9:1) to give the title compound as a beige solid 30 mg (9%) mp: 198–200 °C, R_f 0.39 ($CHCl_3/Et_2O$, 2:1). Ms (EI) *m*/*z* (%): 340 (M^+, 1), 326 (23), 186 (100), 77 (53). IR (nujol): 1580 (C=C) cm^{-1}. Anal. Calcd for $C_{20}H_{22}NO_4$ C 70.57; H 6.51; N 4.11; found: C 70.45; H 6.32; N 4.02. This derivative must be kept in deep freezer (−18 °C) otherwise it decomposes within a few days.

5.1.12. General procedure for hydroxylamine formation (5a/OH, 9/OH, 10/OH, 13/OH)

A solution of compound **5a** or **9** or **10** or **13** (0.5 mmol) in EtOH (10 mL) saturated previously with HCl gas was refluxed for 15 min. After cooling the solvent was evaporated, the residue was crystallized from acetone/Et_2O to give the hydroxylamine HCl salt for assays.

5.1.12.1. (*E*)1-Hydroxy-4-(3,5-Dimethoxystiryl)-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridine HCl Salt (5a/OH). White solid, 91 mg (52%), mp 70–72 °C.

5.1.12.2. (*E*)-5-(2-(1-hydroxy-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)vinyl)benzene-1,3-diol (9/OH). Beige solid, 32 mg (22%), mp 280 °C (dec.).

5.1.12.3. (*E*)-4,4′-(Ethene-1,2-diyl)bis(1-hydroxy-2,2,6,6-tetramethyl-1,2,3,6-tetrahydro) HCl salt (10/OH). White solid, 109 mg (65%), mp 170 °C (dec.).

5.1.12.4. (*E*)-5-(2-(2-hydroxy-1,1,3,3-tetramethylisoindolin-5-yl)vinyl)benzene-1,3-diol HCl salt (13 /OH). White solid, 77 mg (43%), mp 183–185 °C.

5.2. Biological assays

5.2.1. ABTS scavenging assay

ABTS was dissolved in water (7 mM) and ABTS radical cation ($ABTS^+$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^+$ solution was diluted with ethanol or

0.05 M PBS to an absorbance of 0.70 (±0.02) at 734 nm. The solution of antioxidant was added to $ABTS^+$ solution in 12.5, 10, 7.5, 2.5 μM concentration in ethanol or PBS. Trolox was also prepared in ethanol or PBS as a standard. Absorbance reading was taken at 25 °C after 6 min incubation with antioxidant. Experiments were performed on a Specord 40 spectrophotometer. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and of Trolox. To calculate the TEAC, the gradient of the plot of the percentage inhibition of absorbance versus concentration plot for antioxidant in question is divided by the gradient of the plot for Trolox. The TEAC values and standard deviations are the results of three independent experiments.

5.2.2. Determination of ROS production

RAW264.7 macrophage cells were harvested and seeded in $5*10^4$ cell/well density in 96-well plates and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich) containing 10% heat-inactivated faetal calf serum (FCS, Sigma–Aldrich) and L-Glutamin (Sigma–Aldrich) at 37 °C with 5% CO_2 overnight. Then culturing medium was replaced with a fresh one and macrophages were incubated with LPS (100 ng/mL) from (*Escherichia coli* 0127:B8, Sigma–Aldrich) alone or together with resveratrol or its analogues (12.5–100 μM) for 24 h. For the determination of ROS, 2,4-dichlorodihydrofluorescein-diacetat (C400, Sigma–Aldrich) (2 μg/mL) was added for an additional 2 h. Fluorescence was measured at 485 nm excitation and 555 nm emission wavelengths by using a Fluostar Optima (BMG Labtechnologies) fluorescent microplate reader. All experiments were performed in 6 parallels ($n = 6$) and repeated three times, which were found basically identical.

5.2.3. Determination of nitrite production

RAW264.7 macrophages were seeded, incubated and treated as described at the determination of ROS production. For the measurement of nitrite production, Griess-reagent (1% sulphanilamide, 0.1% naphthylethylenediamide in 5% phosphoric acid) was added to equal amount of culturing medium and light absorption was detected at 550 nm using an Anthos 2010 (Rosys) microplate reader. All experiments were performed in 6 parallels ($n = 6$) and repeated three times, which were found basically identical.

5.2.4. TNFα measurement

RAW264.7 macrophage cells were harvested and seeded in $2*10^5$ cell/well density in 24-well plates and incubated as described at the determination of ROS production. Then culturing medium was replaced with a fresh one and macrophages were incubated with LPS (100 ng/mL) alone or together with resveratrol or its analogues (50 μM) for 1.5 h. For the measurement of TNFα production, Mouse TNFα ELISA Ready-SET-Go kit (eBioscience) was used in accordance with the protocol of the manufacturer. After the whole procedure, light absorption was detected at 450 nm using the Anthos 2010 microplate reader. All experiments were performed in 3 parallels ($n = 3$) and repeated 3 times, which were found basically identical.

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Supplementary data

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