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Antioxidant and antiradical activities of resorcinarene tetranitroxides

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ABSTRACT

Resorcinarene oxazines bearing four TEMPO fragments at the wide rim of the macrocycle were prepared through the aminomethylation of resorcinarene octols with 4-amino-TEMPO and formaldehyde. Tetra-TEMPO resorcinarenes are efficient scavengers of 1,1-diphenyl-2-picrylhydrazyl radicals. The model studies revealed that macrocyclic structure and intramolecular hydrogen bonding make considerable contribution to antiradical activity of these compounds. Tetra-TEMPO resorcinarenes show also superoxide dismutase-like activity and efficiently inhibit ABAP-induced peroxidation of linoleic acid.

Highly reactive oxygen species and lipid peroxyl radicals are produced in living cells in course of aerobic metabolism and are involved in a number of life sustaining biochemical processes.[1] The failures of the protective antioxidant systems of the cells lead to oxidative stress causing cardiovascular disease, cancer, chronic inflammatory and other pathologies.[2] Therefore, there is a demand for new antioxidants and antiradical agents, which may be used in vivo for scavenging free radicals. Remarkable antioxidant activity of stable cyclic nitroxides[3] was attributed to their ability to scavenge superoxide, peroxide and alkyl radicals. Some synthetic and natural phenolic compounds that can react with free radicals are highly efficient antioxidant agents.[4] Preorganization of several antioxidant or (and) antiradical groups on the macrocyclic platform such as calixarene[5] or resorcinarene[6] was shown to be an efficient strategy for rational design of bioactive compounds. For example, calixarenes bearing several fragments of hydroxycinnamic acid at the wide rim of the macrocycle had shown radical scavenging and antioxidant activity.[7] On the other hand, several calixarenes[8] and one resorcinarene[9] bearing nitroxyl radical groups at the wide or narrow rim of the macrocycle were synthesized and fully characterized, however, their antiradical and antioxidant properties were not studied.

In the crystalline state and in solutions readily available resorcinarene octols **1a**,**b** (Fig. 1) exist in a crown conformation stabilized by four intramolecular hydrogen bonds between the adjacent OH groups.[10] Similar hydrogen bonding stabilizes a crown conformation of C_4-symmetrical tetraoxazine derivatives **2** which can be readily obtained from compounds **1** through the fourfold Mannich-type cyclocondensation. It was anticipated that such a polyhydroxy compounds as octols **1** could have antiradical properties because of intramolecular hydrogen bonding which might promote the hydrogen atom abstraction by free radicals.[4d,11] On the other hand, the attachment of four free radical TEMPO fragments at the wide rim of **2a**,**b** was expected to result in an enhancement of antioxidant activity.

Known procedures were applied for the synthesis of compounds **1a**,**b**[12] and **2a–c**.[9,13] The reaction of resorcinarenes **1** with four equivalents of 4-amino-TEMPO and eight equivalents of formaldehyde resulted in the fourfold Mannich-type heterocyclizations[14] to give derivatives **2a** and **2b** (Fig. 1).

The EPR spectra of compounds **2a**,**b** measured in ethanol and 1:1 ethanol:water mixture contain a characteristic triplet ($g = 2.006$, hyperfine coupling constants of 15.9 G). The analysis of EPR spectra of tetraradicals **2a**,**b** and a spectrum of four equivalents of 4-amino-TEMPO revealed no significant effects caused by intramolecular exchange interactions between radical fragments attached to the resorcinarene platform. The ^{1}H NMR spectra of known diamagnetic tetraoxazine resorcinarenes[13] of type **2** contain one singlet for the CH and OH protons of the resorcinol rings and one set of signals for the methyl and methine protons of the bridges. The methylene protons of the oxazine rings emerge as *AB*-doublets owing to their diastereotopicity. This pattern was shown to correspond to chiral C_4-symmetrical orientation of the oxazine rings. Broadened ^{1}H NMR signals of paramagnetic com-

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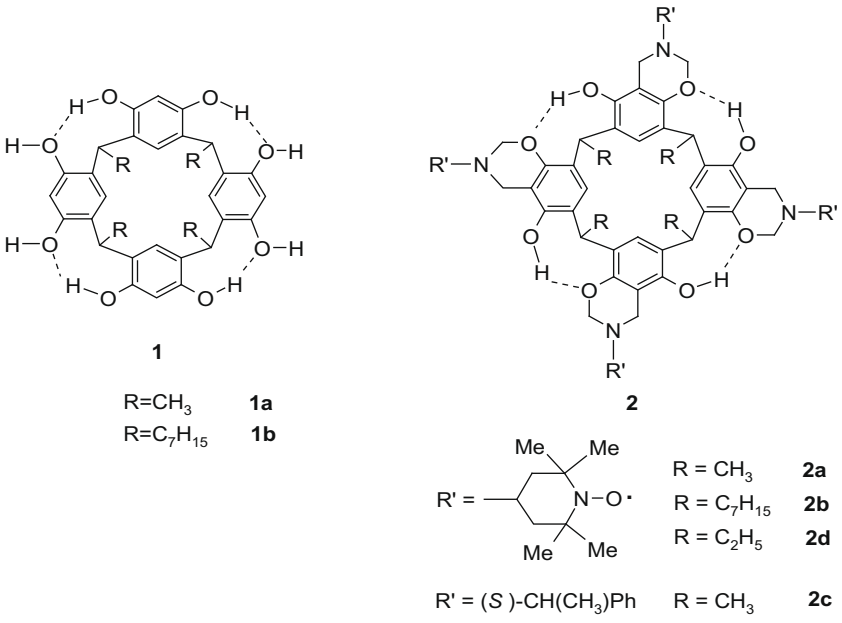

Figure 1. Chemical structures of spin labeled resorcinarenes and model compounds.

pounds **2a**,**b** are similar to those of **2c**[13c] and known tetraradical **2d** whose structure was unambiguously determined by single-crystal X-ray analysis.[9]

Antiradical properties of **1a** and **2a–c** were monitored by the decrease in absorbance of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH).[15,16] The free radical scavenging activity of **2a** (Fig. 2) is comparable to that of **2b**,**c** and **1a**. These compounds were found to be about 100 times more efficient radical scavengers than resorcinol or 4-hydroxy-TEMPO (Table 1). Thus it seems likely that the

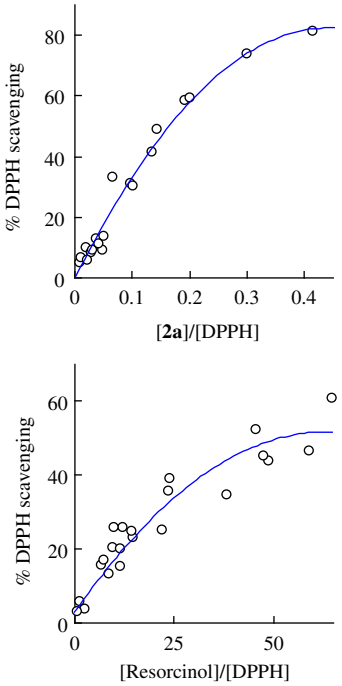

Figure 2. Antiradical activities of resorcinarene tetranitroxide **2a** (top) and resorcinol (bottom), measured as percent scavenging of DPPH after 5 min reaction time and plotted versus the ratio [Scavenger]/[DPPH]. The reaction was monitored through the decrease in DPPH absorbance at 517 nm.

Table 1
Apparent second-order rate constants of the reactions of resorcinarene tetranitroxides and model compounds with superoxide (k_2), ECR_{50} values in the DPPH-test, relative antioxidant efficiency (RAE) values and stoichiometric factors (n) in ABAP-induced linoleic acid peroxidation test[a]

Compound	ECR_{50}[b]	$k_2 \times 10^{-6}$ (M^{-1} c^{-1})	RAE[c]	n
2a	0.19 ± 0.04	2.2 ± 0.5	17 ± 4	30 ± 9
2b	0.21 ± 0.03	0.10 ± 0.07	16 ± 5	31 ± 5
2c	0.18 ± 0.01	n.d.	n.d.	n.d.
1a	0.29 ± 0.04	n.d.	1.7 ± 0.8	4.9 ± 0.8
4-Hydroxy-TEMPO	57 ± 8	0.49 ± 0.19[d]	1.4 ± 0.4	2.8 ± 0.6
Resorcinol	64 ± 17	n.d.[e]	0.4 ± 0.04	2.4 ± 0.2

[a] Data are means ± SD.
[b] ECR_{50} = [Scavenger]/[DPPH] producing a 50% scavenging of DPPH after 5 min reaction time.[16]
[c] RAE – the ratio of the inhibition ability of the compound tested to that of Trolox C which used as a reference inhibitor.
[d] Measurements in Ref. 18 gave $k_2 = 0.65 \times 10^6$ M^{-1} c^{-1} (50 mM phosphate buffer, pH 7, 23 °C).
[e] n.d. – not determined.

abstraction of hydrogen atom from resorcinarene **1a** results in radical stabilized by intramolecular OH···O^- hydrogen bond whereas the positive charge and the spin are delocalized over the resorcinol ring. The reactivities of **1a** and **2a–c** are in keeping with the known fact that H-atom abstraction is feasible from intramolecularly H-bonded molecules rather than intermolecularly H-bonded or non-bonded ones.[4d,11] Due to geometrical reasons the OH groups of resorcinol can form only linear intermolecular hydrogen bonds.

The superoxide scavenging activity of macrocycles **2a**,**b** was studied by spectrophotometric monitoring the competition between antioxidant and nitroblue tetrazolium for superoxide radical generated enzymatically in a xanthine–xanthine oxidase system.[17,18] Preliminary studies revealed that compounds **2a**,**b** do not affect the rate of the enzymatic oxidation of xanthine into uric acid. The initial rates of superoxide induced reduction of nitroblue tetrazolium in the absence (V) and in the presence (v) of **2a,b** and 4-hydroxy-TEMPO was monitored by measuring the absorbance at 560 nm.[19] The data show that V/v depend linearly on [nitroxide]/[NBT] (Fig. 3) according to equation: $V/v = 1 + k_2$[nitroxide]/k_2'[NBT]. Second-order rate constants k_2 were evaluated from this

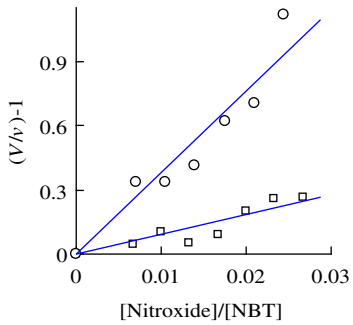

Figure 3. Evaluation of the apparent second order rate constants (k_2) for superoxide dismutation by resorcinarene tetranitroxide **2a**. The initial rates of reactions of superoxide with nitroblue tetrazolium were measured in the absence (V) and in the presence (v) of **2a** (○) or 4-hydroxy-TEMPO (□), and values of {(V/v) − 1} were plotted versus the ratio [nitroxide]/[NBT].

dependence using the known value of k_2' for the reaction of nitroblue tetrazolium with superoxide.[17b] The obtained k_2 values for the reaction of compound **2a** is about 4 times higher than for the reaction with 4-hydroxy-TEMPO (Table 1) whereas model compound **2c** has no effect on the reduction rate of nitroblue tetrazolium at the concentrations studied.

The rate limiting step of the $O2^{-\cdot}$ dismutation may involve the pH-dependent oxidation of the TEMPO fragments of **2a**,**b** to the corresponding nitronium cations, which may be rapidly reduced to the nitroxides by superoxide.[18] It should be noted that nitronium cation can also react with the two-electron reducing agents such as NADH and NADPH.[18,20] The second-order rate constants for the reaction of the nitronium cation with NADH and superoxide were estimated to be $>1 \times 10^7$ and $(3.4 \pm 0.2) \times 10^9\ M^{-1}\ c^{-1}$, respectively.[21] Since the formation of nitronium cation may limit the overall reaction rate in the presence of NADH,[20] the kinetics of decay of **2a** and 4-hydroxy-TEMPO were studied by EPR spectrometry in the presence of NADH.[22] Statistically corrected initial decay rates of tetraradical **2a** and 4-hydroxy-TEMPO are nearly the same, suggesting that the TEMPO fragments of **2a** react with superoxide independently from each other. The activity of lipophilic resorcinarene **2b** bearing four *n*-heptyl residues at the narrow rim of the macrocycle is considerably lower than that of the less lipophilic compound **2a** (Table 1). It seems likely that the hydrophobic association of molecules **2b** in polar reaction medium may hamper the reactions of the nitroxide residues with superoxide.

The free radical resorcinarenes were also expected to inhibit a model lipid peroxidation reaction.[23] Compounds **2a**,**b** were used as inhibitors of the formation of conjugated dienes from linoleic acid in micelles in the presence of 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) as an initiator. Relative antioxidant efficiency (RAE) values[24] were obtained from the plots of the rate of the conjugated diene formation versus 1/[Inhibitor] for compound tested and Trolox C (Fig. 4).[25] As shown in Table 1 resorcinarene nitroxide tetraradicals **2a**,**b** suppress linoleic acid peroxidation about 10 times more efficiently than octol **1a** and 4-hydroxy-TEMPO. The stoichiometric factors of compounds **2a** and **2b** characterize their high radical trapping efficiency. In contrast, resorcinol was found to be relatively inefficient inhibitor of linoleic acid peroxidation. The aptitude of nitroxides to protect oxidative damage in the model system can be explained by scavenging both the carbon-centered and peroxyl radicals.[3c,26] The reaction between nitroxide and peroxyl radicals may proceed through the formation of trioxide intermediate.[26a,b] The trioxide fragments attached to the resorcinarene platform may undergo several transformations to give alkoxyl radicals, dioxygen and regenerate TEMPO fragments. This

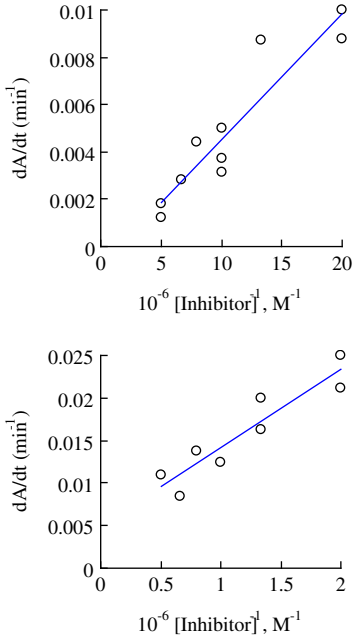

Figure 4. Relative antioxidant efficiency of resorcinarene tetranitroxide **2a**. The rate of conjugated diene formation in a model system consisting of linoleic acid and ABAP was determined by measuring the absorbance A as function of time at 234 nm. The plot of (dA/dt) versus $[\text{Inhibitor}]^{-1}$ yields a line with the slope S. The ratio of the slope S for Trolox C (bottom) to that for **2a** (top) gives the RAE value[24] for the inhibitor **2a**.

explains high values of the stoichiometric factors for compounds **2a** and **2b** (Table 1).

In conclusion, resorcin[4]arenes are promising scaffolds for rational design of multifunctional antiradical and antioxidant agents. Macrocyclic structure and intramolecular hydrogen bonding in resorcinarenes make a major contribution to their antiradical activity. Attaching four TEMPO fragments at the wide rim of resorcinarene results in efficient scavengers of superoxide and peroxyl radicals. Apparently, structural variations at the wide and narrow rim of hydrogen bonded resorcinarenes may result in new highly active compounds that may be applicable as protectors of cells against free radical damage.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.070.

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15. The reaction of **1a**, **2a–c** and model compounds with DPPH was studied at 25 °C in solution containing of 1.5 mL EtOH and 0.5 mL 0.2 M acetate buffer (pH 5.5). Various initial concentration of compounds **1a**, **2a–c** ranging from 0.5 to 25 μM, 4-hydroxy-TEMPO (0.01–7.5 mM), resorcinol (0.05–5 mM) and DPPH (50–100 μM) were used. The ECR_{50} were calculate from the plots of the percentage DPPH transformation after 5 min of reaction against the ratio [Scavenger]/[DPPH]. Values obtained are the average of 3–5 experiments.
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19. The reaction mixture (50 mM phosphate buffer, pH 7.4) contained 50 μM xanthine, 75 μM nitroblue tetrazolium, EDTA (0.1 mM) and 0.5–2 μM resorcinarenes **2a**,**b** or 4-hydroxy-TEMPO. Stock solution of compounds **2a**,**b** were prepared in methanol. The volume concentration of the organic solvent in reaction mixture (2 mL) was 2%. The reaction was started by adding 0.008 U of xanthine oxidase to the mixture incubated at 25 °C.
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22. The samples contained 0.1–0.2 mM NADH, 2.5 mM hypoxantine, 0.05 mM EDTA, 250 U catalase and 0.01 mM paramagnetic resorcinarenes **2a**,**b** or 0.04 mM 4-hydroxy-TEMPO in 50 mM phosphate buffer (pH 7.4). The reactions were initiated by adding of xanthine oxidase (0.04 U/mL). EPR signal intensity of nitroxide was compared to signal intensity measured after the same time in control sample without NADH. The EPR spectra were registered at 293 K using 2.5 mW microwave power, 100 kHz field modulation of 2.0 G and Mn^{2+}/MgO as an internal standard. Samples were accommodated in 0.8 mm diameter glass capillaries which were placed in a standard EPR tube.
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25. Kinetic measurements were performed as reported in Ref. 24. In our experiments, reaction mixture contained 0.05 M phosphate buffer (pH 7.4), 0.02% Lubrol, 0.63 mM linoleic acid and 1 mM ABAP. The sample was incubated under air atmosphere at 37 °C and the change in absorbance was monitored over time at 234 nm. Inhibition was started by adding of 5 μL of inhibitor solution to the reaction mixture with the constant rate of autooxidation. The results are expressed in terms of relative antioxidant efficiency defined as the ratio of the antioxidant efficiency of the inhibitor to that of Trolox C. The stoichiometric factors *n* were determined from the ratio of the lag times for the various concentrations of test antioxidant and Trolox C, the stoichiometric factor of which was taken to be 2. The values of RAE represent mean of three separate determinations and stoichiometric factors *n* are the average of 3–7 experiments.
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