

Evaluation of the radical scavenging activity of resorcinarenes by DPPH• free radical assay

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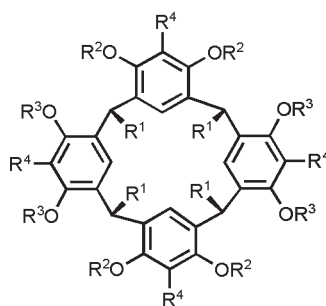
A series of methyl-pendant resorcinarene derivatives have been synthesised and evaluated for radical scavenging potential by using a colorimetric DPPH• assay to reveal their utility as potent radical scavengers. An evaluation of the efficiencies in relation to the number of DPPH• molecules reduced by one molecule of antioxidant establishes that resorcinarene can reduce approximately eight DPPH• molecules at a fairly lower stoichiometric concentration. This behaviour is compared to that of well known radical scavengers (α -tocopherol, BHT and resorcinol) frequently used as reference compounds in scientific evaluations.

Keywords: resorcinarenes, radical scavenging, reaction stoichiometry, antioxidant activity

Resorcinarenes are cavity-containing macrocyclic metacyclophanes with distinct hydrophobic and hydrophilic regions in their molecular architecture. They can be easily functionalised to provide a wide variety of molecular receptors for ionic and molecular recognition.¹ A large volume of research work has been published on their chemistry and biological applications.^{2,3} Calix[4]resorcinarene receptors have been utilised for the electrochemical detection of dopamine^{4, 5} and acetylcholine in micellar aqueous sodium dodecyl sulfate (SDS). Though different aspects of resorcinarenes have been examined in detail, surprisingly very little information is available on their antioxidant or radical scavenging potential and these are taken up in the present investigation. The information pooled from voluminous literature data available on the higher radical scavenging activities of phenolic and polyphenolic antioxidants^{6–12} prompted us to determine and corroborate the antioxidant activity of resorcinarenes. Salient features of our work on the subject are reported in this communication.

From various methods for estimation of antioxidant activity, we chose to use the widely documented 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical^{13–19} as it is a stable free radical and can accept an electron or a hydrogen radical to form a stable chemical species. It has a strong absorption band at 515 nm which disappears after reduction of the radical species to allow facile monitoring. The radical scavenging effect of the synthesised compounds was evaluated by the assay reported by Williams *et al.*¹⁴ wherein the reaction rates correlate directly with antioxidant activity such that the higher the rate, the more effective is the antioxidant.

Compound **1** was obtained by the acid-catalysed condensation of resorcinol and acetaldehyde.²⁰ It was then reacted to yield its aminomethylated,²¹ dansylated²² and ester²³ derivatives (see Scheme 1) whose chemical and physical characteristics tallied well with those reported in the cited literature. Compound **3** has been prepared for the first time as a yellow solid in 50% yield and has been characterized by recording its



Compound	R ¹	R ²	R ³	R ⁴
1	CH ₃	H	H	H
2	CH ₃	H	H	–H ₂ C–N ₂ –O
3	CH ₃		H	H
4	CH ₃	CH ₂ COOC ₂ H ₅	CH ₂ COOC ₂ H ₅	H

dl- α -Tocopherol	Resorcinol	BHT

Scheme 1 Structures of resorcinarene derivatives and the reference compounds investigated in the present study.

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NMR, IR and FAB mass spectra. Since compound **3** has four dansyl groups, apparently attached to two opposite phenyl rings of the resorcinarene, their position needs to be established with more rigour than is suggested here on the basis of NMR data and optical inactivity of the molecule which revealed it to be a symmetric molecule. In the present study, α -tocopherol, di-*tert*-butyl-4-methylphenol (BHT) and resorcinol were used as reference antioxidant compounds for ascertaining the antioxidant potential of the synthesised resorcinarenes.

It was determined that when the reaction of DPPH• with **1**, under pseudo-first order conditions²⁴ (by taking DPPH• in excess) was performed, it gave a typical set of absorption spectra (Fig. 1) with isobestic points at 341 and 428 nm. The course of the reactions between all the test and reference compounds with DPPH• was then followed quantitatively at 515 nm by adding 0.05 mL of different molar ratios of test compounds in methanol to 1.95 mL of freshly prepared methanolic solution of DPPH• (6.0×10^{-5} M) in quartz cuvettes and following the decrease in absorbance at 515nm every minute in triplicates till the reaction reached a plateau.¹⁴ A reference curve of absorbance against DPPH• concentration in methanol ([DPPH] M) was obtained to calculate the DPPH• concentration at various reaction times

$$\text{Abs}_{515\text{nm}} = 9500.869 \times (C_{\text{DPPH}\cdot}) - 3.73 \times 10^{-3} \quad (R^2=0.9999).$$

Thirteen different concentrations of **1** were prepared to study the antioxidant activity. For all molar ratios of **1**, the percentage of DPPH• remaining in the reaction mixture was plotted as a function of time (Fig. 2).

It was observed that in the case of **1**, 63.12% and 6.41% of DPPH• remained at steady state at molar ratios of 0.04 and 42.74 respectively. However, when **1** was replaced by **4** under

the same conditions, insignificant consumption of the DPPH• (4.0-5.0%) was observed even at the steady state (Fig. 3a). In the case of **2** which contained four morpholine substituents and the same number of -OH functions as **1**, 78.38% and 8.21% of DPPH• was left unconsumed at steady state at the same molar ratios of 0.042 and 42.74 respectively (Fig. 3b). Dansylated resorcinarene derivative **3** could inhibit much lesser amount of DPPH• and at a much slower rate such that 84.44% and 15.25% of DPPH• remained unconsumed at steady state at the same molar ratios of 0.042 and 42.73 respectively (Fig. 3c).

The other reference antioxidants were able to reduce DPPH• in a concentration dependent manner²⁵ when subjected to similar experiments (Fig. 4). While BHT showed higher radical scavenging potential than α -tocopherol up to a molar ratio of 0.26, α -tocopherol could exert a superior scavenging effect beyond this level. However, it was determined that resorcinol required a relatively higher molar ratio of 0.74 to neutralise 50% of the available DPPH•.

From the reaction curves of test and reference compounds, the percentage of DPPH• remaining at steady state versus the molar ratio of antioxidant to DPPH• was plotted (Fig. 5).

The numbers of DPPH• molecules reduced by one molecule of the antioxidant (σ) were calculated as the inverse of reaction stoichiometry obtained by doubling the IC_{50} of antioxidant¹⁴ (Table 1) for the test and reference compounds. The parent compound **1** exhibited excellent antioxidant activity by reducing approximately eight DPPH• molecules at a fairly lower stoichiometric value of 0.134. Among the derivatives of **1**, the one having morpholine moieties attached at four positions (**2**) could reduce approximately four DPPH• molecules at almost double the stoichiometric concentration of **1**. While in this case, the radical scavenging activity was superior to that of

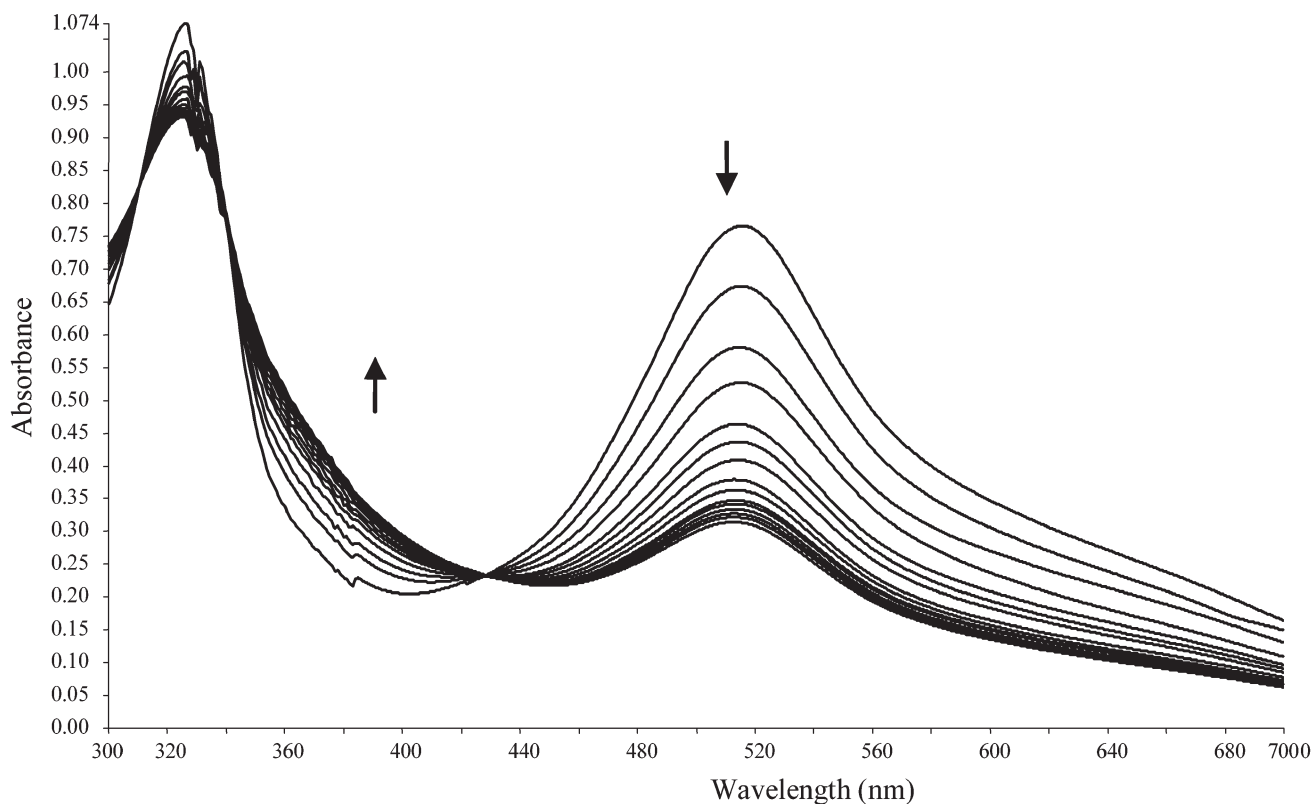


Fig. 1 Time dependence of DPPH• absorption spectra upon reaction with **1** in methanol; DPPH•: 7.77×10^{-5} M (excess), **1**: 6.25×10^{-6} M. Times after mixing: 0,2,4,6,8,10,12,14,16,18,20,22 and 24 minutes. The arrows indicate direction of absorbance changes.

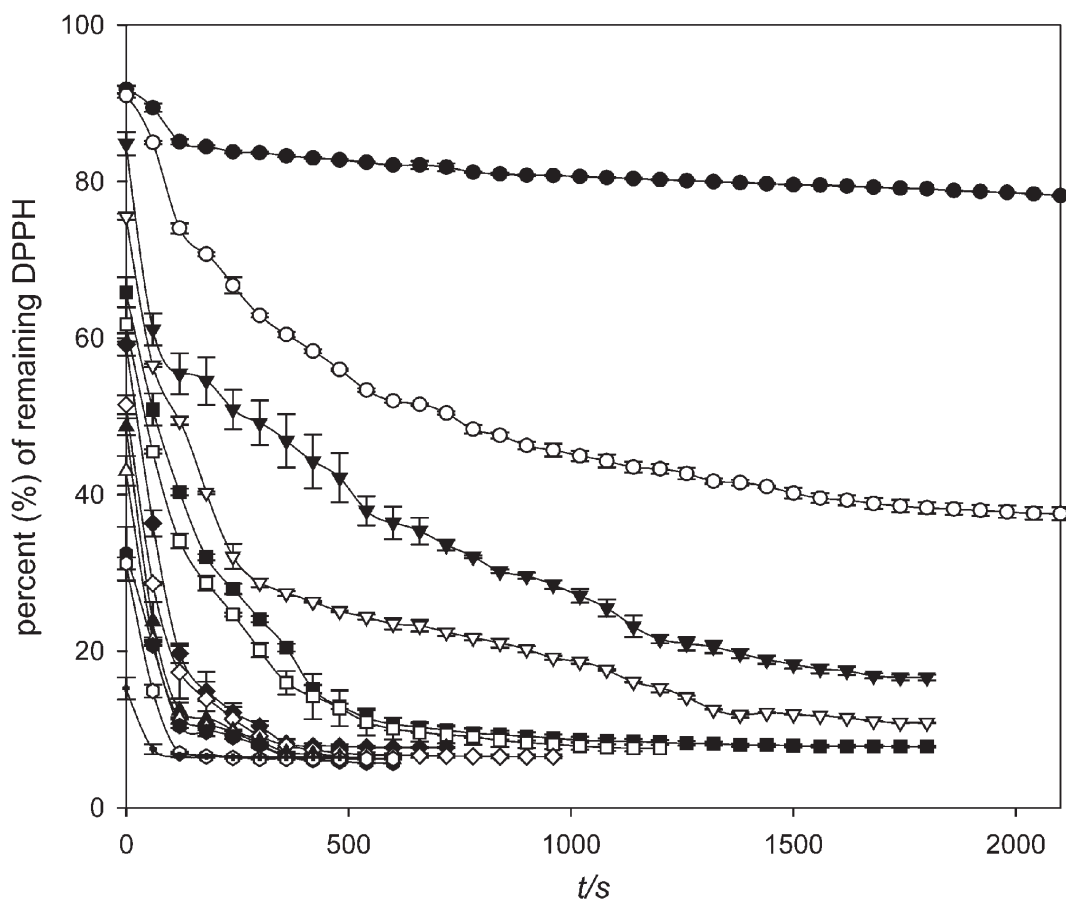


Fig. 2 Reaction curves between 6×10^{-5} M DPPH[•] solution and different concentrations of **1** at molar ratios (●) 0.042, (○) 0.106, (▼) 0.213, (▽) 0.320, (■) 0.427, (□) 1.068, (◆) 2.136, (◇) 3.205, (▲) 4.273, (△) 10.683, (●) 21.37, (○) 32.05, (◊) 42.74.

α -tocopherol and BHT, it was lower by 50% than that of **1**. The results obtained can be ascribed to the presence of the $-\text{CH}_2$ group attached to the morpholyl unit that makes the hydroxyl proton less labile.²⁶ Indeed, compound **3** which bears four $-\text{OH}$ groups could reduce merely two DPPH[•] molecules at almost four times the stoichiometric concentration of **1**. While in this case, the radical scavenging activity was superior to resorcinol, it was lower again by 50% than that of **2**. When all the eight $-\text{OH}$ groups were blocked by esterification (as in **4**), no free radical scavenging activity was observed even at molar ratios as high as 10.68. These results were found to be in agreement with earlier literature reports on resorcinol.¹¹

Thus the radical scavenging effect of resorcinarene derivatives and reference compounds followed the descending order, **1** > **2** > BHT > dl- α -tocopherol > **3** > resorcinol > **4** and the resorcinarene ester analog **4** was found to exhibit negligible radical scavenging activity. We conclude that compound **1** with a measured stoichiometry of about eight can be used as an excellent radical scavenger analogous to BHT, tocopherol and resorcinol. Further work to understand the kinetics and mechanism of reaction is in progress.

Experimental

All the reagents used in the study were purchased from Sigma-Aldrich or Merck and were chemically pure. The solvents used were distilled. Column chromatography was performed on silica gel (60–120 mesh) obtained from Merck. ¹H NMR ¹³C NMR spectra were recorded on a 300 MHz Bruker DPX 300 instrument at room temperature using tetramethylsilane (TMS) as an internal standard. IR spectra were recorded on a Nicolet Protégé 460 spectrometer in KBr disks while

the FAB mass spectra were recorded on a JEOL SX 102/DA-6000 Mass spectrometer/Data System using Argon/Xenon (6 kV, 10mA) as the FAB gas. Melting points were determined on an electrothermal melting point apparatus obtained from M/S Toshniwal and were uncorrected. Elemental analysis was carried out on a Perkin Elmer's 240C-CHN analyser.

Preparation of **3**

To a solution of resorcinarene (1 mmol) in acetonitrile (20 mL), triethylamine (4 mmol) was added in one portion with vigorous stirring. A yellow precipitate formed and the reaction mixture was stirred for 15 min. A solution of dansyl chloride (4 mmol for tetra dansylated derivatives) in acetonitrile (25 mL) was added to the suspension in one portion and the reaction mixture was rigorously stirred to facilitate dissolution of the precipitate. The reaction mixture was stirred at room temperature for 72 h. The precipitate formed was filtered off, washed with acetonitrile (30 mL) and water (50 mL). The crude product was purified by column chromatography on silica using chloroform as eluent to give dansylated resorcinarene. M.p. 210, IR (ν_{max} , KBr): 3481, 2918, 1397, 1146, 1034–689 cm^{-1} . ¹H NMR spectrum (300 MHz, CDCl_3 , δ in ppm) 11.6 (4H, s, OH), 8.61 (4H, d, Dns-H), 8.37 (4H, d, Dns-H), 8.087 (4H, d, Dns-H), 7.61 (4H, t, Dns-H), 7.51 (4H, t, Dns-H), 7.19 (4H, d, Dns-H), 6.48 (2H, s, ArH), 6.43 (2H, s, ArH), 6.15 (2H, s, ArH), 6.08 (2H, s, ArH), 4.23 (4H, q, ArCHAr), 2.82 (24H, s, $\text{N}(\text{CH}_3)_2$), 1.62 (12H, d, CH_3). ¹³C NMR spectrum (75 MHz, CDCl_3) δ (ppm): 153.2, 151.9, 145.6, 138.8, 132.5, 131.1, 129.7, 128.9, 126.5, 125.5, 123.35, 119.2, 119.0, 115.3, 102.2, 45.2, 33.1, 29.6, 20.0. Anal. Calcd for $\text{C}_{80}\text{H}_{76}\text{N}_4\text{O}_{16}\text{S}_4$: C, 65.02; H, 5.18; N, 5.18. Found: C, 65.35; H, 5.06; N, 3.54; FAB-MS [(M+1)⁺] peak at 1477 (also recrystallised from chloroform. Anal. Calcd for $\text{C}_{80}\text{H}_{76}\text{N}_4\text{O}_{16}\text{S}_4\text{CHCl}_3$: C, 60.92; H, 4.86; N, 3.51. Found: C, 61.21; H, 4.96; N, 3.56).

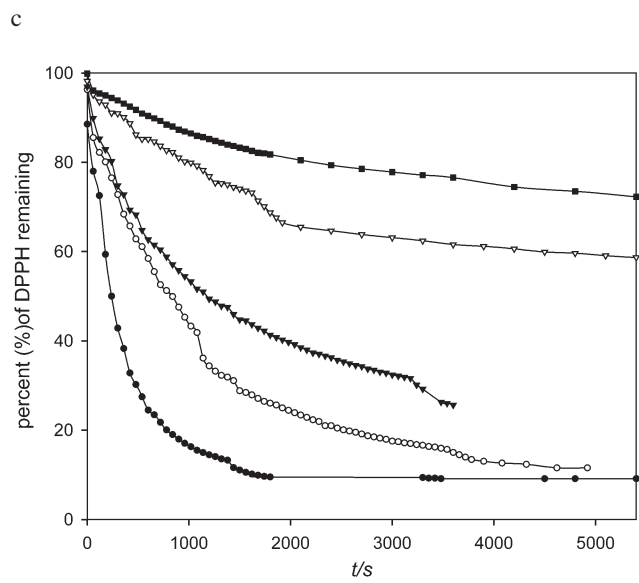
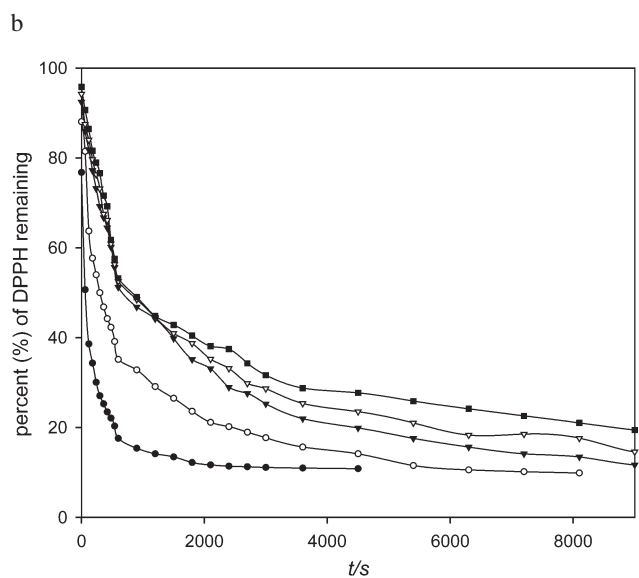
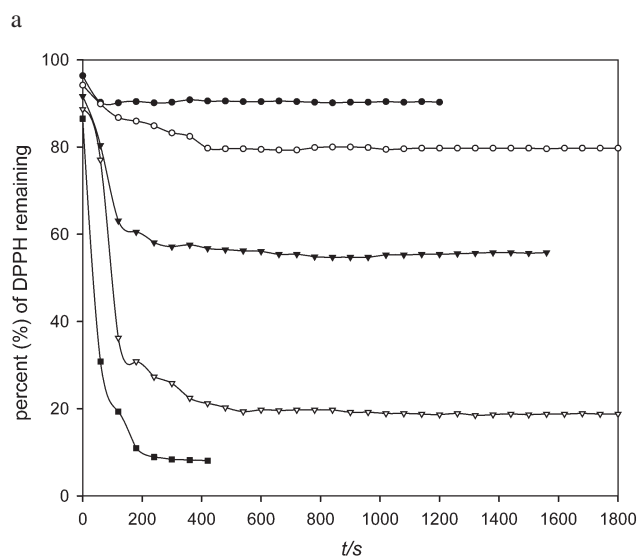
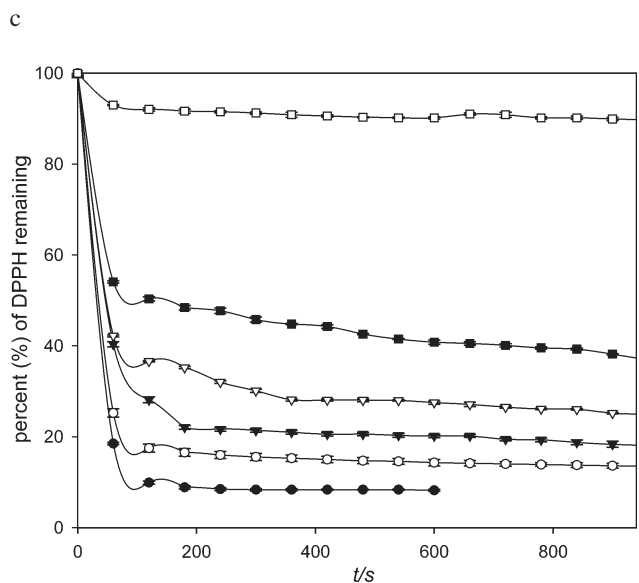
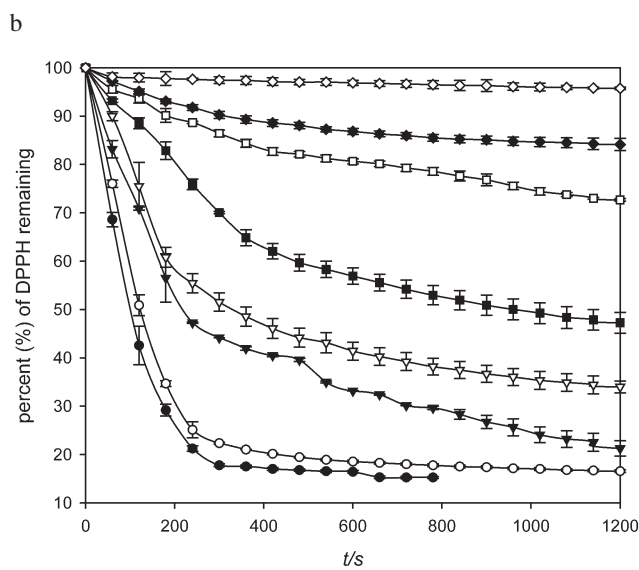
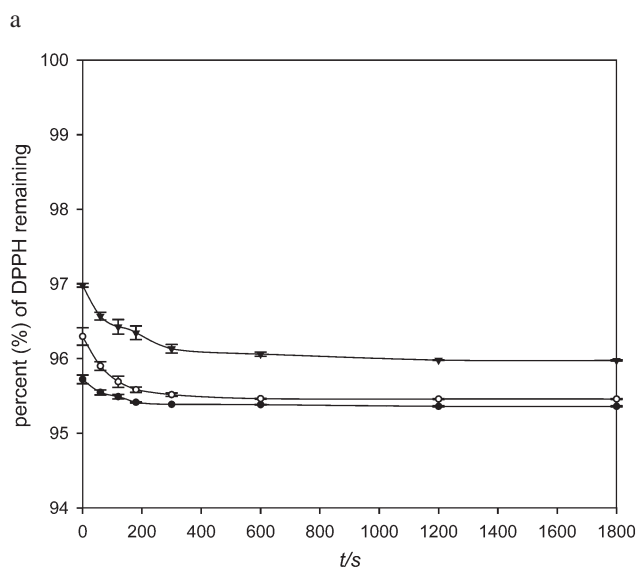


Fig. 3 Reaction curves between 6×10^{-5} M DPPH* solution and different concentrations of (a) **4** at molar ratios (●) 10.683, (○) 1.068, (▼) 0.427, (b) **2** at molar ratios (●) 42.74, (○) 21.37, (▼) 4.273, (▽) 2.135, (■) 0.427, (□) 0.042 and (c) **3** at molar ratios (●) 42.74, (○) 21.35, (▼) 4.273, (▽) 2.135, (■) 1.067, (□) 0.427, (◆) 0.213, (◇) 0.042.

Fig. 4 Reaction curves obtained between 6×10^{-5} M DPPH* and different concentrations of (a) α -tocopherol at molar ratios (●) 0.040, (○) 0.080, (▼) 0.201, (▽) 0.402, (■) 0.804, (b) resorcinol at molar ratios (●) 138.60, (○) 69.30, (▼) 27.72, (▽) 13.86, (■) 2.77 and (c) BHT at molar ratios (●) 1.572, (○) 0.786, (▼) 0.393, (▽) 0.157, (■) 0.078.

Table 1 DPPH scavenging activities of reference antioxidants and resorcinarene derivatives (1–4)

Compounds	Number of labile hydrogen ^a	IC ₅₀ ^b	Stoichiometric value	Number of reduced DPPH (σ) ^c	Ref.
1	8	0.067±0.006	0.134	7.46	^d
2	8	0.130±0.004	0.260	3.84	^d
3	4	0.250±0.002	0.500	2.00	^d
4^e	0	—	—	—	^d
<i>d</i> -α-tocopherol	1	0.220±0.004	0.440	2.27	17
resorcinol	2	0.740±0.003	1.480	0.67	18
BHT	1	0.190±0.009	0.380	2.63	10

^a Available for donation on hydroxyl groups as per the chemical structure.

^b Values are means ± standard deviation of three experiments.

^c Inverse of reaction stoichiometry.

^d Present work.

^e Resorcinarene derivative **4** could not inhibit 50% of the reaction under experimental conditions.

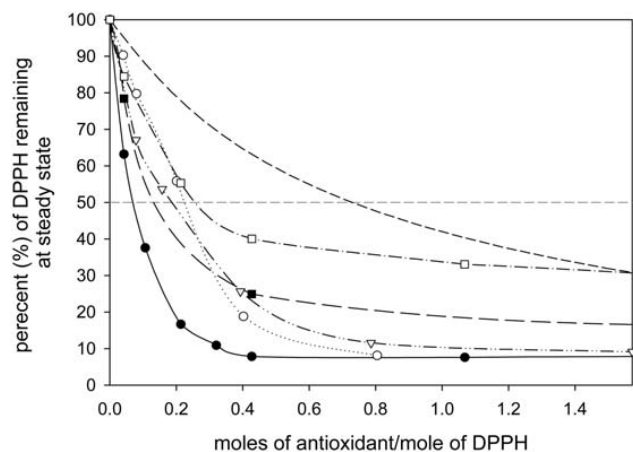


Fig. 5 The disappearance of DPPH* as function of number of moles of antioxidant/mole of DPPH (—●—) **1**, (---○---) *d*-α-tocopherol, (---▼---) resorcinol, (---□---) BHT, (—■—) **2**, (---□---) **3**, (-----) 50% level specification.

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