

## Antioxidant Activities of Two Novel Synthetic Methylbenzenediol Derivatives

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### Abstract

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2-(*tert*-Butyl)-5-methylbenzene-1,4-diol and 3-(*tert*-butyl)-5-methylbenzene-1,2-diol were synthesised by Friedel-Craft reaction of 2-methylbenzene-1,4-diol and 4-methylbenzene-1,2-diol, respectively, with tertiary butanol providing reasonable yields. The antioxidant activities of these two products, mother compounds and 2-(*tert*-butyl)benzene-1,4-diol were investigated and compared by means of 2,2-diphenyl-1-picrylhydrazyl radical and Rancimat at test; 3-(*tert*-butyl)-5-methylbenzene-1,2-diol is the most potent antioxidant tested by using Rancimat test experiment. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging abilities of 2-methylbenzene-1,4-diol, 4-methylbenzene-1,2-diol and 2-(*tert*-butyl)benzene-1,4-diol are almost equal and more than twice as strong as 2-(*tert*-butyl)-5-methylbenzene-1,4-diol and 3-(*tert*-butyl)-5-methylbenzene-1,2-diol. The antioxidant activities of the five compounds evaluated by Rancimat test mainly depend on their steric synergist effects between the two phenolic hydroxyl groups in their molecules. The antioxidant activities of the five compounds mainly depend on how many 2,2-diphenyl-1-picrylhydrazyl radicals can be scavenged by one mole of them in 2,2-diphenyl-1-picrylhydrazyl test. One mole of 2-methylbenzene-1,4-diol, 4-methylbenzene-1,2-diol and 2-(*tert*-butyl)benzene-1,4-diol can scavenge four moles of 2,2-diphenyl-1-picrylhydrazyl radicals, but one mole of 2-(*tert*-butyl)-5-methylbenzene-1,4-diol or 3-(*tert*-butyl)-5-methylbenzene-1,2-diol can only scavenge two mole 2,2-diphenyl-1-picrylhydrazyl radicals because 2,2-diphenyl-1-picrylhydrazyl radicals are very bulky.

**Keywords:** 2-(*tert*-butyl)-5-methylbenzene-1,4-diol; 3-(*tert*-butyl)-5-methylbenzene-1,2-diol; steric synergist effect; DPPH; Rancimat test

Autoxidation is the main process of fats, oils, and lipid-based food deterioration resulting in nutrients losses, off-flavour and harmful products formation (POKORNY *et al.* 2001). The addition of antioxidants is the most effective, convenient and economical way to retard lipids autoxidation (LI *et al.* 2006). The main antioxidants allowed in food and food industry are phenolic compounds. Thus a number of studies on natural and synthetic phenolic compounds attract researchers' attention.

Antioxidant activity can be studied by means of a variety of *in vitro* cell-free experimental protocols, such as the evaluation of the scavenging ability against relatively stable free radicals, for instance 2,2-diphenyl-1-picrylhydrazyl (DPPH) (BONDET *et al.* 1997; MENSOR *et al.* 2001; PHILIP 2004), superoxide anion radical, hydroxyl radical, and the abil-

ity to inhibit lipid peroxidation (WENG *et al.* 1992; JONATHAN *et al.* 2000).

2-(*tert*-Butyl)-5-methylbenzene-1,4-diol (TBMHQ) and 3-(*tert*-butyl)-5-methylbenzene-1,2-diol (TBHPC) belong both to phenolic compounds. They could be synthesised in the traditional way (MORGENSTERN *et al.* 1971; BELOSTOTSKAYA *et al.* 1972b) but in a low yield as the products were mixtures and had to be clarified. TBHPC has antioxidant activity (DIEPGEN *et al.* 2011) and the ability of chelating with metal (SUZUKI 1997). The antioxidant activity of TBHPC can be predicted by using Computer-assisted method (KHAIRULLINA *et al.* 2006). However, there are few literature date about the bioactivity of TBMHQ and structure-antioxidant activity relationship of five methylbenzenediol analogues by using different experiments *in vitro*. In our knowledge, we have never read any research paper

on the antioxidant activity of a diphenolic compound with a bulky alkyl group in *o*-position of one hydroxyl or on the comparison of the antioxidant activities of TBHPC and 2-(*tert*-butyl)benzene-1,4-diol (TBHQ). Also, for the first time is discussed here why TBHPC and TBMHQ are much weaker DPPH radical scavengers than TBHQ, 4-methylbenzene-1,2-diol (HPC), and 2-methylbenzene-1,4-diol (MHQ).

Our study focused on the synthesis of TBMHQ and TBHPC by a new modified and convenient process providing reasonable yields. In addition, the structure-antioxidant activities of these two products and their analogues are also investigated by using Rancimat test and DPPH spectrophotometric assay.

## MATERIAL AND METHODS

**Material and chemicals.** Lard was rendered in the laboratory and stored in a deep freezer for use. MHQ and HPC were purchased from Sigma-Aldrich Trading Co, Ltd. (Shanghai, Beijing, China). TBHQ was purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Silica gel and other chemicals used in this experiment were all AR grade came and from the latter company.

**Spectra recording.** Electron impact ionisation mass (EI-Mass) spectra were recorded with an MS300 spectroscopic instrument (Beijing City East and West Electronic Technology Institute, Beijing, China). Ultraviolet (UV) spectra were recorded with a UV-2450 spectroscopic instrument (Shimadzu Corp, Kyoto, Japan). Methanol was used as the solvent and a quartz cell was used for the spectroscopic measurements. Infrared spectra were recorded by using an AVATAR370 FT-IR Instrument (Thermo Nicolet Corp., Madison, USA). Nuclear magnetic resonance (NMR) spectra were recorded with an AVANCE 500MHz (Bruker, Bern, Switzerland). Deuterated chloroform was used as the solvent. Tetramethylsilane (TMS) was used as the internal standard.

**Synthesis and purification of the compounds.** TBMHQ and TBHPC were prepared bases on the method as published by BELOSTOTSKAYA (1972a) with some improvement. MHQ (5 g, 40 mmol) and 2.21 ml phosphoric acid (98%) as catalyst were added to a 500 ml three-neck flask. The reaction mixture was heated on a hot plate using a magnetic stirrer and refluxed in 4.75 ml toluene at 110°C. Then a mixture of 4.62 ml tertiary butanol and 3.35 ml toluene was added dropwise into the reaction mixture. The molar ratio of the three substances [MHQ-tertiary butanol-phosphoric acid (98%)] was 1 : 1.2 : 0.95. The mixture was refluxed for 3 hours. After which it was

left cool to room temperature. The solid substance which appeared was collected by filtration and was subsequently washed with hot water (100 ml). After filtering and drying, pale pink powder was obtained, the yield of crystal being 80%. However, in the case of TBHPC, HPC was used as the mother compound. The molar ratio of the three substances was 1 : 3 : 0.95 and the mixture of the products that required careful separation by column chromatograph gave the objective compound, TBHPC. The yield of crystal was 20%. The column (500 × 40 mm) contained 75 g silica gel. The elution system was petroleum ether-chloroform-acetonitrile (6 : 4 : 0.6, 530 ml). The products of the above two reactions were identified by thin-layer chromatography (TLC). Petroleum ether-chloroform-acetonitrile (6 : 4 : 0.6) was used as TLC developing solvent.  $R_f$  values were recorded at the same time. The plates were prepared by coating 0.5 mm silica gel (F254) onto 200 × 200 mm glass plates. The plates were activated at 110°C in the oven for 1.5 hours.

**Antioxidant activity evaluated by Rancimat test.** The antioxidant activity of methylbenzenediol analogues at different concentrations in lard was determined by Rancimat (Metrohm, Shanghai, China) based on the method published by GUO (2005). The air flow rate was controlled at 20 l/h, the temperature was controlled at 100°C and lard was used as the substrate. Lard (3 ± 0.02 g) and different levels of antioxidants were added to each sample. Each sample was prepared in duplicate.

**Antioxidant activity evaluated by DPPH spectrophotometric assay.** The antioxidant activities of TBMHQ, TBHPC, MHQ, HPC, and TBHQ were also measured in terms of hydrogen-donating or radical-scavenging ability using the DPPH method (JONATHAN *et al.* 2000; LI *et al.* 2006). All spectrophotometric measurements were performed with a UV-2012 PC spectrophotometer (Mr Nick instrument Co., Ltd., Shanghai, Beijing, China). The 50% effective concentration ( $EC_{50}$ ) values, defined as the concentration of the substrate that causes 50% loss of DPPH activity, were calculated by linear regression of plots, where the abscissa represented the concentration of the tested sample and the ordinate the average percentage of scavenging capacity from three replications.

**Statistical analysis.** Statistical significance between multiple does groups was determined by using the analysis of variance (ANOVA) followed by Duncan multiple comparisons test ( $P < 0.05$ ).

## RESULTS AND DISCUSSION

TBMHQ and TBHPC were synthesised from methylbenzenediols and tertiary butanol. In the case of

Table 1. Spectroscopic data for TBMHQ and TBHPC

Compound	<sup>1</sup> HNMR(500 MHz) TMS as int. standard	<sup>13</sup> CNMR(125 MHz) TMS as int. standard	EIMS ( <i>m/z</i> )	IR(cm <sup>-1</sup> ) (KBr)	UV(nm) (EtOH)
TBMHQ	1.399(s,9H)	15.120	180(46.4)	3476.58	205
	2.211(s,3H)	29.601	165(99.9)	3340.07	292
	4.349(s,1H)	34.257	137(56.6)	1524.42	
	4.457(s,1H)	114.125	123(3.9)	1412.65	
	6.484(s,1H)	118.893	95(1.3)	1207.06	
	6.738(s,1H)	121.650	77(5.7)	1152.96	
		134.876	64(4.2)	1002.03	
		147.132	51(4.5)	887.05	
		147.712		849.97	
				797.27	
TBHPC	1.420(s,9H)	21.022	180(47.8)	3314.87	222
	2.260(s,3H)	29.595	165(99.9)	1597.25	281
	5.007(s,1H)	34.540	137(54.6)	1484.35	
	5.464(s,1H)	113.682	119(12.7)	1308.57	
	6.578(s,1H)	119.754	91(8.3)	1214.88	
	6.693(s,1H)	128.581	77(6.3)	1029.90	
		136.337	65(4.5)	976.24	
		140.851	51(7)	918.53	
		142.722		847.05	

TBMHQ, the molar ratio of the three substances [MHQ-tertiary butanol-phosphoric acid (98%)] was 1:1.2:0.95. As to TBHPC, the molar ratio of the three substances (HPC-tertiary butanol-phosphoric acid [98%]) was 1:3:0.95. The mixture of products was separated by column chromatography. The elution system was petroleum ether-chloroform-acetonitrile (6:4:3500.6, 530 ml). The yields were about 80% for TBMHQ and 20% for TBHPC.

The  $R_f$  values of MHQ, HPC, TBMHQ, TBHPC, and TBHQ were 0.044, 0.078, 0.288, 0.311, 0.155, respectively. It means that the polarity of the five compounds was reduced in the order: MHQ > HPC > TBHQ > TBMHQ > TBHPC.

The spectroscopic data for TBMHQ and TBHPC are listed in Table 1. All the UV, IR, EIMS, <sup>1</sup>H, and <sup>13</sup>C NMR spectral data obtained in the laboratory confirm the structures of the TBMHQ and TBHPC which are shown in Figures 2 and 3, respectively.

**Antioxidant activity by Rancimat test.** The antioxidant activities of five methylbenzenediol analogues were tested on the Rancimat at 100°C. To interpret the effects of the compound structures on the antioxidant activities, the protection factors ( $P_f$ ) were calculated according to Equation 1.

$$P_f = \frac{IP_{(\text{induction periods of lard added antioxidant})}}{IP_{(\text{induction period of lard})}} \quad (1)$$

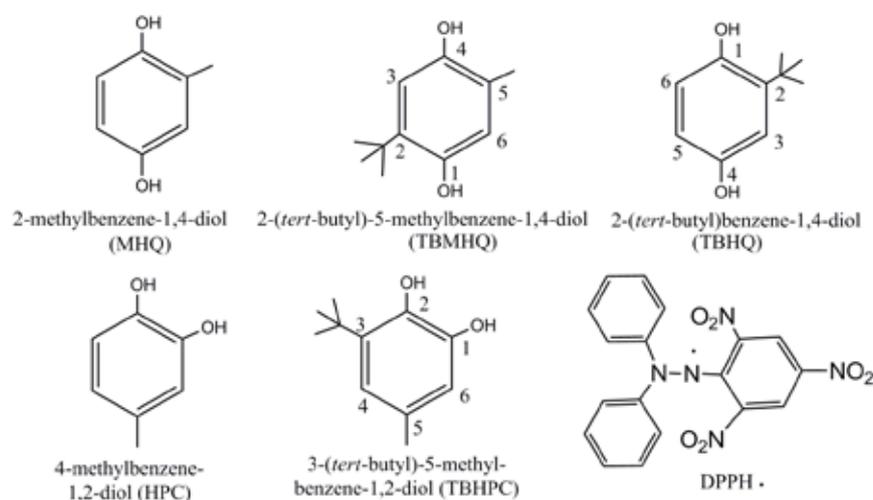


Figure 1. Chemical structures of five methylbenzenediol analogues used as antioxidants and DPPH radical

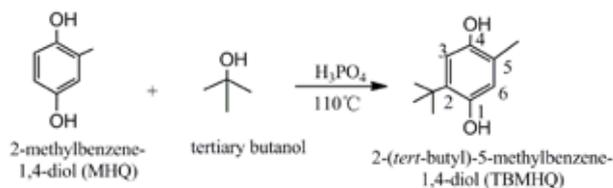


Figure 2. The synthesis of TRMHQ

The compounds used in the experiment on antioxidant activity are shown in Figure 1.  $P_f$  values are shown in Table 2. A higher value of  $P_f$  means a stronger antioxidant activity of the sample. If  $P_f < 1$ , the sample has pro-oxidant activity; if  $P_f = 1$ , the sample has no antioxidant activity; if  $2 > P_f > 1$ , the sample has some antioxidant activity; if  $3 > P_f > 2$ , the sample has an obvious antioxidant activity and if  $P_f > 3$ , the sample has a strong antioxidant activity (WANG *et al.* 2000).

The results in Table 2 show that all of the five compounds have very strong, but very different levels of antioxidant activity. It is interesting that TBHPC, which possesses a *tert*-butyl group in position 3 and 1,2-dihydroxyl presents the strongest antioxidant activity. The *tert*-butyl group which attaches to the *o*-position of 2-position of TBHPC (Figure 1) is a very bulky group with great steric hindrance, and shields its neighbored (2-position) hydroxyl group firmly (Figures 1 and 4). Thus, this hydroxyl group reacts with difficulty with the active radicals because of the shielding effect, but it has a greater tendency to provide the hydrogen atom because of the crowded environment. This means that the 2-position hydroxyl group donates the hydrogen atom to active radicals more slowly than the 1-position hydroxyl group, but

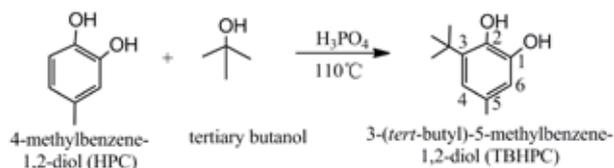


Figure 3. The synthesis of TBHPC

its free radical formed is more stable. However the 1-position hydroxyl group can react fast with active free radicals and its free radical formed is less stable than the 2-position hydroxyl group. Consequently, the two hydroxyl groups have very strong steric synergy. Because of the hydrogen bond between phenolic oxyl radical and hydroxyl group, the less stable free radical can easily convert to more stable one. This synergistic mechanism can be expressed very clearly by Figure 4 (GORDON 1990).

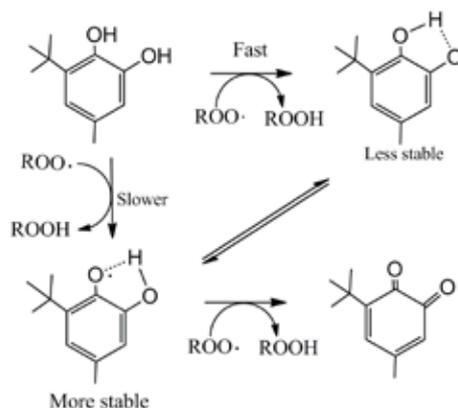


Figure 4. Mechanism of antioxidant synergy between two group with different steric hindrance of TBHPC

Table 2. The induction periods (IP, h)<sup>a</sup> and the protection factors ( $P_f$ )<sup>b</sup> of the lard containing different compounds at different concentration by Rancimet test

Compounds	TBMHQ	TBHPC	MHQ	HPC	TBHQ
<b>0.01%</b>					
IP (h)	6.74 ± 0.005	44.76 ± 0.005	12.51 ± 0.033	10.77 ± 0.045	21.15 ± 0.026
$P_f$	1.45 ± 0.005	9.64 ± 0.013	2.69 ± 0.033	2.322 ± 0.044	4.55 ± 0.026
<b>0.02%</b>					
IP (h)	9.08 ± 0.020	52.68 ± 0.007	16.125 ± 0.016	13.93 ± 0.027	31.16 ± 0.005
$P_f$	1.96 ± 0.02	11.34 ± 0.007	3.47 ± 0.017	3.0 ± 0.027	6.71 ± 0.026
<b>0.04%</b>					
IP (h)	10.98 ± 0.02	60.52 ± 0.008	24.52 <sup>*</sup>	15.64 ± 0.009	46.88 ± 0.072
$P_f$	2.36 ± 0.02	13.03 ± 0.005	5.28 <sup>*</sup>	3.34 ± 0.003	10.09 ± 0.031
<b>0.08%</b>					
IP (h)	30.35 ± 0.001	77.12 ± 0.033	46.30 ± 0.002	39.33 ± 0.004	60.31 ± 0.015
$P_f$	6.53 ± 0.001	16.60 ± 0.005	9.97 ± 0.002	8.47 ± 0.008	12.99 ± 0.013

3.00 ± 0.02 g lard was added; IP of lard is 4.64 ± 0.013 h under 100°C; air flow rate was 20 l/h; <sup>a</sup>IP results are duplicates; <sup>b</sup> $P_f$  = the IP of lard with antioxidant/the IP of control lard; values were expressed as mean ± relative deviation; <sup>\*</sup>the results are single

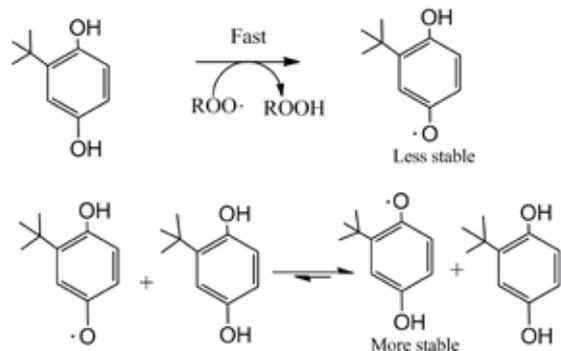


Figure 5. Mechanism of antioxidant synergy between two phenolic hydroxyl groups with different steric hindrance of TBHQ

It is also very interesting that the antioxidant activity of TBMHQ is not only weaker than that of TBHQ, but even its mother compound, MHQ. This is because TBHQ has the strongest steric synergy between two phenolic hydroxyl groups (Figure 5), MHQ having a weaker one and TBMHQ the weakest (Figure 1).

Another question is why TBHPC has much stronger antioxidant activity (0.01%,  $P_f = 9.64$ ) than TBHQ ( $P_f = 4.55$ ) although both of them have a similar strong steric synergy? This is mainly because the less stable TBHPC free radical can convert to more stable TBHPC free radical intramolecularly (Figure 4) and the less stable TBHQ free radical can convert to the more stable TBHQ free radical intermolecularly (Figure 5). Obviously, the intramolecular conversion shown in Figure 4 is much easier than that shown in Figure 5 (POKORNY *et al.* 2001).

It is noteworthy that the antioxidant activity of MHQ is noticeably stronger than that of HPC. This is because the two hydroxyl groups in MHQ have some steric synergistic effect, but those in HPC do

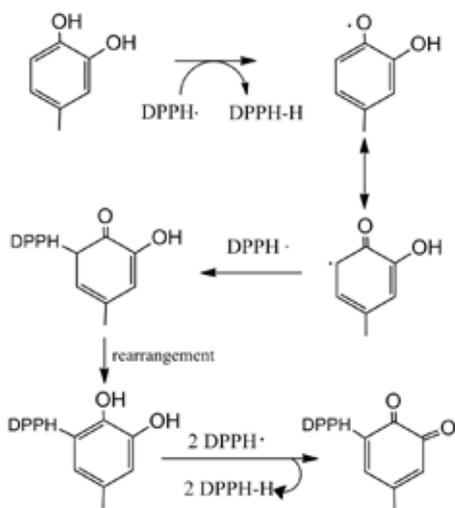


Figure 6. Mechanism of scavenging DPPH radical of HPC

Table 3. The  $EC_{50}$  value of different antioxidants as determines in the DPPH system

Sample	$EC_{50}$ (mg/ml)
TBMHQ	$0.039 \pm 0.003^b$
TBHPC	$0.044 \pm 0.002^a$
MHQ	$0.018 \pm 0.000^c$
HPC	$0.016 \pm 0.004^d$
TBHQ	$0.017 \pm 0.001^{cd}$

A value obtained from regression line with 95% of confidence level;  $EC_{50}$  is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%; values were expressed as mean  $\pm$  SD,  $n = 3$ ; mean with different characters are significantly different by using ANOVA followed by Duncan multiple comparison test ( $P < 0.05$ )

not have any. In another words, thermodynamic effect plays an important role in Rancimat test.

To sum up, the antioxidant activities of the five methylbenzenediol analogues by Rancimat test decrease in the following order: TBHPC  $\gg$  TBHQ  $>$  MHQ  $>$  HPC  $>$  TBMHQ.

**Antioxidant activity by DPPH scavenging activity assay.** DPPH radical scavenging ability of different antioxidants, MHQ, HPC, TBMHQ, TBHPC, and TBHQ was determined at 517 nm. Table 3 shows that the radical scavenging abilities of HPC ( $EC_{50} = 0.016$  mg/ml), TBHQ ( $EC_{50} = 0.017$  mg/ml), and MHQ ( $EC_{50} = 0.018$  mg/ml) are almost equal. They are more than twice as strong as those of TBHPC ( $EC_{50} = 0.044$  mg/ml) and TBMHQ ( $EC_{50} = 0.039$  mg/ml). This finding is very different from the results of Rancimat test. Figure 1 shows that DPPH radical is very bulky. It can combine only with MHQ, HPC, and TBHQ free radicals (Figure 6) but not with TBHPC and TBMHQ free radicals because their steric hindrance limits their combination as Figure 7 clearly explains (BRAND-WILLIAMS *et al.* 1995).

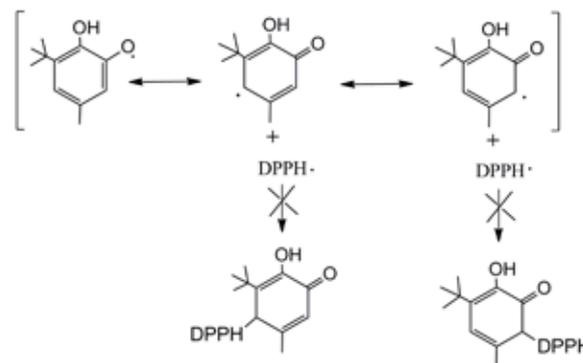


Figure 7. Mechanism of scavenging DPPH radical of TBHPC

## CONCLUSION

The authors used a convenient method to synthesise pure TBMHQ and TBHPC in reasonable yields. In the Rancimat test (Table 2) TBHPC exhibited a stronger antioxidant activity than the commonly used synthetic antioxidant TBHQ because TBHPC can convert to more stable TBHPC free radical intramolecularly in contrast to TBHQ. Therefore, TBHPC may be used as a novel synthesised antioxidant in edible oils and fats. However, the radical scavenging abilities of HPC, TBHQ, and MHQ are almost equal. They more than twice as strong as those of TBHPC and TBMHQ. The reason may reside in the steric hindrance of TBHPC and TBMHQ presenting an opposite effect.

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