Antioxidant activity of the new thiosulfinate derivative, *S*-benzyl phenylmethanethiosulfinate, from *Petiveria alliacea* L.

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The antioxidant effects of the new thiosulfinate derivative, *S*-benzyl phenylmethanethiosulfinate (BPT), against the oxidation of cumene and methyl linoleate (ML) in chlorobenzene were studied in detail using HPLC. The results showed that BPT provided effective inhibition with a well-defined induction period under these oxidation conditions, and it was found that the stoichiometric factor (n), the number of peroxyl radicals trapped by one antioxidant molecule, of BPT is about 2. We then undertook a thorough investigation aimed at elucidating the active structural site of BPT. Various model compounds, such as diphenyl disulfide, dibenzyl disulfide, *S*-phenyl benzenethiosulfinate and *S*-ethyl phenylmethanethiosulfinate, were used which provided evidence that the benzylic hydrogen of BPT is mainly associated with the peroxyl radical scavenging. Moreover, we measured the rate constant for the reaction of BPT with peroxyl radicals derived from cumene and ML in chlorobenzene, and based on these measurements, BPT reacts with these peroxyl radicals with a rate constant of $k_{inh} = 8.6 \times 10^3$ and 6.2×10^4 M^{-1} s^{-1}, respectively.

Introduction

It is generally known that garlic extract exhibits antioxidant activity against lipid peroxidation.[1–10] We reported that garlic extracts inhibit methyl linoleate (ML) oxidation in an acetonitrile solution, and the antioxidant activity is mostly due to the presence of allicin (**1f**) (Fig. 1), one of the main thiosulfinates in garlic.[11] More recently, we also showed that allicin (**1f**) inhibits the oxidation of cumene and ML in chlorobenzene, and this ability is characterized by a reaction with the peroxyl radical derived from cumene and ML with the rate constants (k_{inh}) 2.6×10^3 M^{-1} s^{-1} and 1.6×10^5 M^{-1} s^{-1}, respectively.[12] However, the antioxidant activity of allicin (**1f**) is weaker than that of α-tocopherol (α-toc). In addition, we elucidated that allicin (**1f**) antioxidant activity requires a combination of an allyl group and an –S(O)S- group, and that the antioxidant properties of allicin (**1f**) are due to the fact that the transfer of the allylic hydrogen of allicin (**1f**) causes scavenging of the chain-carrying peroxyl radicals of the substrates forming hydroperoxides.[12] Our present aim is to compare the antioxidant activity and antioxidative mechanism of allicin (**1f**) with those of other thiosulfinate derivatives.

Petiveria alliacea L. is widely distributed throughout the South and Central Americas, and has commonly been used in folk medicines.[13–15] It has been reported that various preparations made from these plants exhibit anti-inflammatory, antimicrobial, anticancer and stimulant effects. It is interesting to note that the root of this plant contains various thiosulfinate compounds, and *S*-benzyl phenylmethanethiosulfinate (BPT) (**1b**) is the most abundant thiosulfinate found in this extract.[15] BPT (**1b**) is believed to possess antibacterial and antifungal activities, however, as far as we know, reliable data on its antioxidant activities are lacking. This paper is the first report on kinetic and mechanistic investigations of the new thiosulfinate derivative, BPT (**1b**), as an antioxidant.

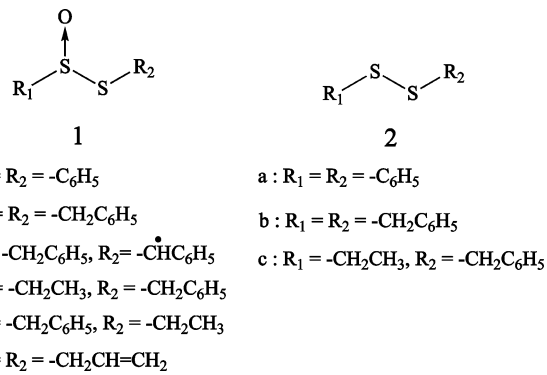

Fig. 1 Chemical structures of thiosulfinates (**1**) and related organosulfur compounds (**2**) used in this study.

Experimental

Materials

Cumene, 2,2′-azobis(isobutyronitrile) (AIBN), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and chlorobenzene were obtained from Wako Pure Chemical Industries (Osaka, Japan). AIBN was recrystallized from methanol, and cumene was purified on a silica-gel column before use. ML was purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and purified on a silica-gel column before use. Dibenzyl disulfide (**2b**) and diphenyl disulfide (**2a**) were obtained from the Aldrich Chemical Co. (Milwaukee, WI, USA). α-Toc was purchased from the Kanto Chemical Co.

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(Tokyo, Japan) and used without purification. All other reagents were of the highest grade commercially available.

Synthesis of BPT (1b) from dibenzyl disulfide (2b)

BPT (**1b**) was chemically synthesized from dibenzyl disulfide (**2b**) using the method of Cruz-Villalon.[16] The synthesized BPT (**1b**) was purified by preparative HPLC (10 mL min^{-1} methanol + 30% H_2O) using a UV detector at 254 nm with an RP-C18 column (particle size 10 μm; 19 × 300 mm; Waters μBondapak™). BPT (**1b**) was identified by comparing its mass spectrum to that reported by Kubec *et al.*; MS *m/z* (relative intensity): 263 (MH^+, 20%), 139 ($C_6H_5CH_2SO^+$, 10).[15] A Micromass ZQ mass spectrometer (Waters) equipped with an atmospheric pressure chemical ionization (APCI) source was used in the positive ionization mode. The mass ionization conditions were as follows: desolvation temperature, 400 °C; source temperature, 120 °C; cone voltage, 9V; and desolvation gas flow, 160 L h^{-1}.

S-Phenyl benzenethiosulfinate (**1a**) and *S*-ethyl phenylmethanethiosulfinate (**1d**, **1e**) were also synthesized from diphenyl disulfide (**2a**) and benzyl ethyl disulfide (**2c**), respectively, in the same way as described above. However, the benzyl ethyl disulfide (**2c**) was synthesized using the method of Mugesh *et al.*[17] The purification and confirmation of these compounds were performed following the procedure given above.

Cumene and ML oxidation in chlorobenzene solution

In a typical experiment, either cumene (5.35 M) or ML (91 mM) was incubated in chlorobenzene in the presence of an appropriate amount of BPT (**1b**) at 30 °C in air. A solution of AIBN (23 mM for cumene and 40 mM for ML) in chlorobenzene was added to this reaction mixture.[18,19] The rates of the substrate oxidations were followed by measuring the peroxides generated from each substrate using reverse phase HPLC (0.3 mL min^{-1} methanol + 15% water, Shiseido CAPCELLPAK C_{18} 5 μm 3.0 × 150 mm column), and peaks were detected at 260 nm for cumene hydroperoxide (CHP) and at 234 nm for ML hydroperoxide (MLOOH). At the same time, the consumption of BPT (**1b**) was analyzed using a C_{18} HPLC with a UV detector at 254 nm.

Reactivity of BPT (1b) toward DPPH

DPPH (50 μM) and BPT (**1b**) (25 μM) were dissolved in chlorobenzene at 30 °C. The rate of the DPPH-scavenging reaction was monitored at 517 nm using a spectrophotometer. In the same way, DPPH (50 μM) and BPT (**1b**) (50 μM) were dissolved in methanol in the presence of $Mg(ClO_4)_2$ (0.2 M) and monitored at 516 nm using a spectrophotometer.[20] The consumption of BPT (**1b**) (0.5 mM or 1.0 mM) by reaction with DPPH (1.0 mM) was analyzed using an HPLC equipped with an RP-C18 column (0.3 mL min^{-1} methanol + 25% water, Shiseido CAPCELLPAK C_{18} 5 μm 3.0 × 150 mm column), and BPT (**1b**) was detected by its UV absorption at 254 nm.

Analysis of MLOOH isomers

The oxidation of ML (91 mM) was performed in chlorobenzene with AIBN (40 mM) in the presence of BPT (**1b**) (50 μM) at 30 °C in air. Samples (0.5 mL) were withdrawn from the mixture at known intervals during the induction period and were immediately reduced to ML hydroxides (MLOH) by the addition of 1.0 mL of 2 mM triphenylphosphine in chlorobenzene to these samples. The analysis was performed using silica-gel HPLC (1.0 mL min^{-1} n-hexane + 1.0% 2-propanol, Supelco SUPELCOSIL LC-Si 5 μm 250 × 4.6 mm column) and peaks in MLOH were detected at 234 nm.[21]

Calculation of bond dissociation enthalpy (BDE) values

The calculation of the BDE values of BPT (**1b**) was performed by the Gaussian 03 program (Gaussian, Inc., Carnegie, PA, USA) as follows: geometrical optimization and determination of the vibrational frequencies were performed using HF/6-31+G(2d,p).[22] The single-point electronic energies were obtained using B3LYP/6-31+G(2d,p).

Results and discussion

Inhibitory effect of BPT (1b) against cumene and ML oxidation

The effects of BPT (**1b**) as an antioxidant against cumene and ML oxidation have been investigated in chlorobenzene, which has proven useful in quantitative assays of the chemical activities of antioxidants against lipid peroxidation. In fact, these experimental conditions are frequently employed as a simple means to determine the rate constants for peroxyl radical trapping by antioxidants.[23,24] We previously determined the rate constants k_{inh} for the reaction of allicin (**1f**) with peroxyl radicals.[12] Therefore, at this time, we decided to study the antioxidant activity of BPT (**1b**) under these conditions using HPLC.

Fig. 2 shows the increase in traces of CHP by BPT (**1b**)'s inhibition of the cumene oxidation initiated by AIBN. BPT (**1b**) displayed a very clear induction period during cumene oxidation. The induction period ended when the BPT (**1b**) had

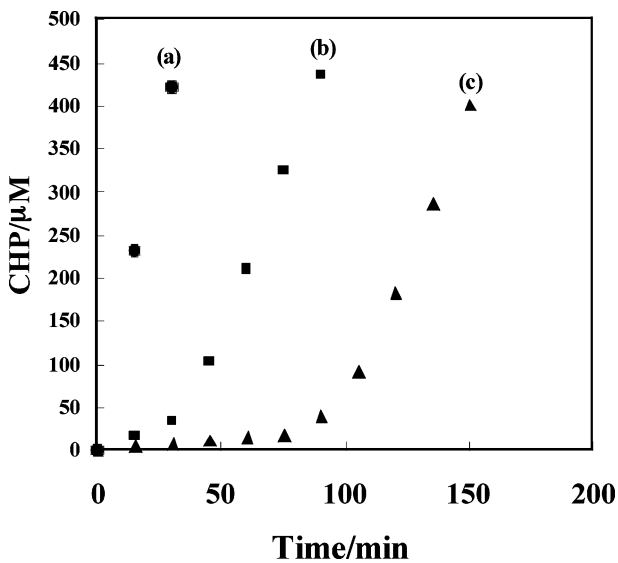

Fig. 2 Inhibitory effect of BPT (**1b**) on the oxidation of cumene induced by AIBN in chlorobenzene. Cumene (5.35 M) was oxidized at 30 °C in chlorobenzene in air with AIBN (23 mM) in the absence (a) and presence (b) of 20 μM, and (c) 30 μM BPT (**1b**). The CHP was measured by HPLC.

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been exhaustively consumed, and the rates of oxidation after the induction period were almost the same as those in the absence of BPT (**1b**). A very similar behavior was exhibited when BPT (**1b**) was added to the ML oxidation system (Fig. 3). In addition, we examined the effect of BPT (**1b**) as a hydroperoxide decomposer in chlorobenzene at 30 °C. However, it was found that BPT (**1b**) had no significant effect on the decomposition of CHP or MLOOH (data not shown).

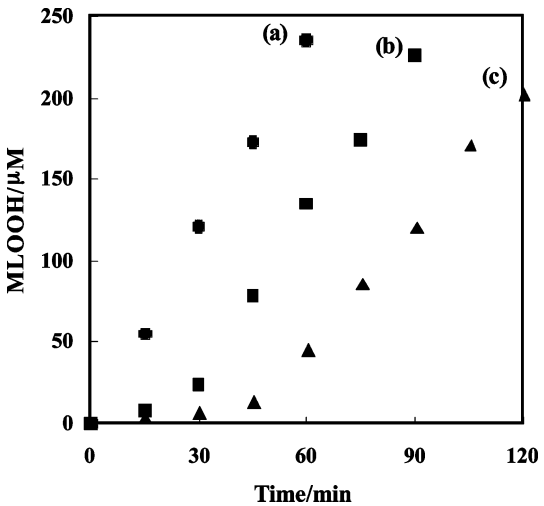

Fig. 3 Inhibitory effect of BPT (**1b**) on the oxidation of ML induced by AIBN in chlorobenzene. ML (91 mM) was oxidized at 30 °C in chlorobenzene in air with AIBN (40 mM) in the absence (a) and presence (b) of 30 μM, and (c) 50 μM BPT (**1b**). The MLOOH was measured by HPLC.

Structure–antioxidant activity relationship of BPT (1b)

To elucidate the active structural site of the BPT (**1b**) as an antioxidant, we studied the relationship between the structure and antioxidant activity of BPT (**1b**) against cumene oxidation in chlorobenzene at 30 °C.

The results indicated that the diphenyl disulfide (**2a**), dibenzyl disulfide (**2b**) and *S*-phenyl benzenethiosulfinate (**1a**) had no significant antioxidant activity, though BPT (**1b**) has fairly strong activity at the same concentration (data not shown). Similarly, as shown in Fig. 4, $C_6H_5CH_2S(O)SCH_2CH_3$ (**1e**) was much less efficient in suppressing cumene oxidation. However, $CH_3CH_2S(O)SCH_2C_6H_5$ (**1d**) was more effective in inhibiting the oxidation of cumene than **1e**. It is evident from these results that the $–S(O)S–CH_2C_6H_5$ portion of BPT (**1b**) is an essential site for its antioxidant activities.

Antioxidant mechanism of BPT (1b)

The ratios of MLOOH isomers are known to be remarkably different during ML oxidation in the presence of hydrogen-donating antioxidants.[21] That is, the amount of *cis*, *trans*-MLOOH increases and the *trans*, *trans*-MLOOH decreases with increasing concentrations of hydrogen-donating antioxidants, which results in a decrease in the *cis*, *trans* to *trans*, *trans* (*c*, *t* : *t*, *t*) ratio of the formed MLOOH. Therefore, we analyzed the ratios of the MLOOH isomers in the presence of BPT (**1b**) to confirm

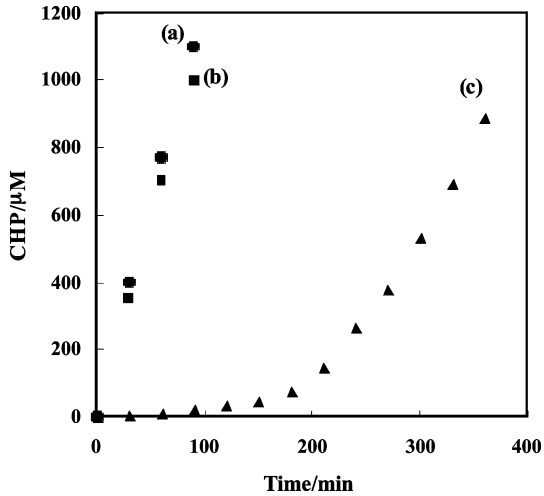

Fig. 4 Inhibitory effects of **1d** and **1e** on the oxidation of cumene induced by AIBN in chlorobenzene. Cumene (5.35 M) was oxidized at 30 °C in chlorobenzene in air with AIBN (23 μM) in the absence (a) and presence (b) of 30 μM **1e**, and (c) 30 μM **1d**.

the contribution of BPT (**1b**)'s hydrogen atoms to its antioxidant activity.

BPT (**1b**) was added to the ML oxidation induced by AIBN in chlorobenzene, and then the MLOOH isomers formed during oxidation were reduced by triphenylphosphine to their corresponding alcohols, which were analyzed by HPLC. As shown in Fig. 5, the

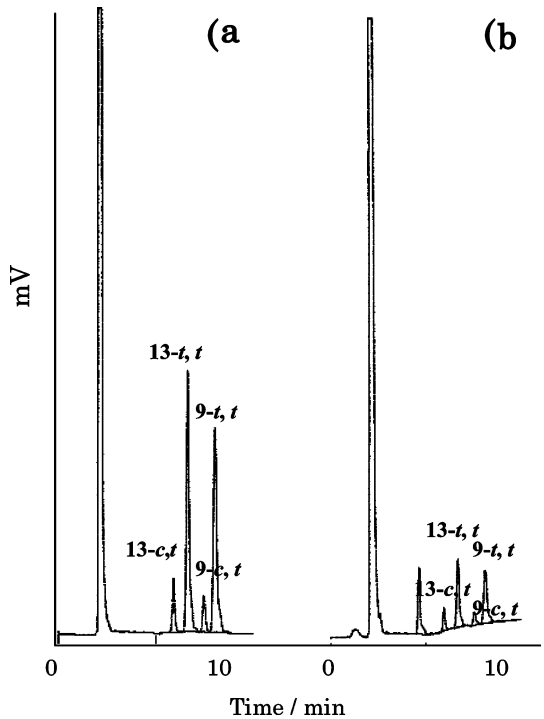

Fig. 5 Distribution of geometric isomers of the MLOH. ML (91 mM) was oxidized at 30 °C in chlorobenzene in air with AIBN (40 mM) in the absence (a) and presence (b) of 50 μM BPT (**1b**). Sample (0.5 ml) was withdrawn from the mixture after 30 min and was reduced to MLOH by adding triphenylphosphine to the sample. The MLOH was analyzed by HPLC.

$c, t : t, t$ ratio of the MLOOH formed during the uninhibited AIBN-initiated oxidation of ML in chlorobenzene remained constant at 0.16 from 5 to 30 min. However, the addition of 50 μM BPT (**1b**) to this reaction mixture induced a $c, t : t, t$ ratio of about 0.23 over the course of the induction period. This fact suggests that the hydrogen atom of BPT (**1b**) may at least contribute to determining its antioxidant activity.

Furthermore, we have also extended the investigation to the radical-scavenging mechanism of BPT (**1b**). It is well known that there are two mechanisms for the radical-scavenging reactions of antioxidants: a one-step hydrogen atom transfer and an electron transfer followed by a proton transfer.[25] In addition, the electron-transfer mechanism is known to be accelerated in the presence of Mg^{2+}.[20] We then examined the effect of Mg^{2+} on the radical-scavenging rate of BPT (**1b**) in methanol according to the method of Nakanishi *et al.*[20] As shown in Fig. 6, the rate of the DPPH-scavenging reaction with BPT (**1b**) was not affected by the addition of Mg^{2+}. This shows that the radical-scavenging reactions of BPT (**1b**) proceed *via* a one-step hydrogen atom transfer.

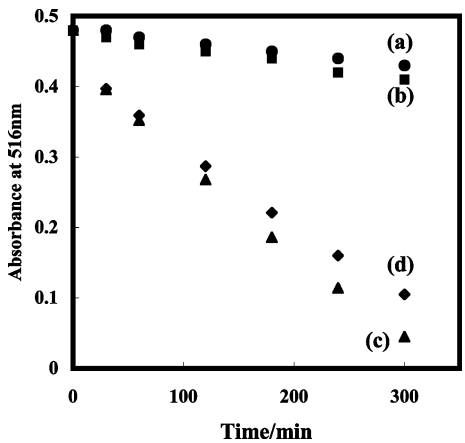

Fig. 6 Reactivity of BPT (**1b**) toward DPPH in the presence of Mg^{2+} in methanol. DPPH (50 μM) was reduced at 30 °C in methanol under air in the absence (a) and presence (b) of 0.2 M $Mg(ClO_4)_2$, (c) 50 μM BPT (**1b**), and (d) 0.2 M $Mg(ClO_4)_2$ and 50 μM BPT (**1b**).

To further explore the contribution of the BPT (**1b**) hydrogen atom, we calculated the BDE values of the benzylic C–H bond of BPT (**1b**) using Gaussian 03. Because the one-step hydrogen atom transfer mechanism is governed by BDE to a large extent,[26] we focused our attention on the BDE values of the benzylic C–H bond of BPT (**1b**). As a result, the BDE value of the benzylic C–H bond of C_6H_5–CH_2–S(O)$SCH_2C_6H_5$ (**1b**) was 93.0 kcal mol^{-1}, and that of $C_6H_5CH_2S(O)S$–CH_2–C_6H_5 (**1b**) was 88.9 kcal mol^{-1}. This result shows that the benzylic hydrogen atom of $C_6H_5CH_2S(O)S$–CH_2–C_6H_5 (**1b**) could contribute to its antioxidant activities, consistent with the observation that $CH_3CH_2S(O)SCH_2C_6H_5$ (**1d**) was capable of more effectively inhibiting cumene oxidation than $C_6H_5CH_2S(O)SCH_2CH_3$ (**1e**).

Determination of stoichiometric factor (*n*)

In order to determine the rate constants for the scavenging of peroxyl radicals by BPT (**1b**), we measured the stoichiometric factor *n*, the number of peroxyl radicals trapped by one molecule of BPT (**1b**), by measuring its reactivity with DPPH. DPPH has often been used in estimating of the reactivity of antioxidants with radicals and can also be used to estimate antioxidant stoichiometries.[27] Therefore, we measured the rate of reduction of DPPH by BPT (**1b**) and the amount of unreacted BPT (**1b**) remaining after the BPT (**1b**) was mixed with equimolar quantities of DPPH in chlorobenzene. As shown in Fig. 7, it was found that 50% of the BPT (**1b**) remained, despite the fact that DPPH was completely reduced by the BPT (**1b**). This result clearly shows that the *n* for BPT (**1b**) is almost 2.0 in chlorobenzene at 30 °C.

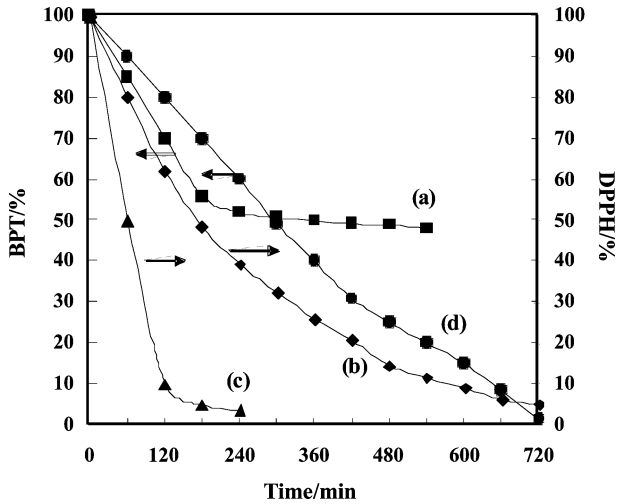

Fig. 7 Consumption of BPT (**1b**) and DPPH by reaction of BPT (**1b**) with DPPH in chlorobenzene. DPPH (1.0 mM) was reduced at 30 °C in chlorobenzene in air in the presence of (a) and (c) 1.0 mM, and (b) and (d) 0.5 mM BPT (**1b**). BPT (**1b**) and DPPH were measured by HPLC.

It is obvious from the results of the Gaussian 03 calculation that the S–S bond of BPT (**1b**) was significantly weaker after the benzylic hydrogen atom of BPT (**1b**) was abstracted by radicals. That is, the BDE value of the S–S bond of BPT (**1b**) was calculated to be 34.8 kcal mol^{-1}, while that of the corresponding radical (**1c**) obtained by the abstraction of a benzylic hydrogen atom was only slightly above 0 kcal mol^{-1}. This means that BPT (**1b**) may have decomposed after the benzylic hydrogen atom of BPT (**1b**) was abstracted by radicals. It is suggested that the products arising from the decomposition of BPT (**1b**) by loss of a benzylic hydrogen atom would contribute to determining the *n* value. In fact, we detected some products using LC-MS after the reaction of BPT (**1b**) with DPPH. However, their structures have not yet been characterized. The structure of the products formed from BPT (**1b**) during the reaction with DPPH needs to be further examined in detail.

Determination of rate constants (k_{inh}) for the scavenging reaction of BPT (1b) with peroxyl radicals

We determined the rate constants for the scavenging of peroxyl radicals, k_{inh}, by BPT (**1b**) for comparison with those of another antioxidant. We first experimentally measured the τ and R_{inh}, the length of the induction period, and the rate of hydroperoxide formation during the course of the induction periods of the cumene

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Table 1 Summary of antioxidant activity of BPT (**1b**), allicin (**1f**) and α-toc

	n	k_p/M^{-1} s^{-1}	R_i/M s^{-1}	R_{inh}/M s^{-1}	τ/s	k_{inh}/M^{-1} s^{-1}
Cumene oxidation system						
30 μM BPT (**1b**)	2.0			4.6×10^{-9}	5766	8.6×10^{3}
25 μM allicin (**1f**)	1.0	0.18	2.5×10^{-9}	3.7×10^{-8}	2788	2.6×10^{3}
25 μM α-toc	2.0			3.9×10^{-10}	20286	1.2×10^{5}
ML oxidation system						
50 μM BPT (**1b**)	2.0			4.8×10^{-9}	2895	6.2×10^{4}
50 μM allicin (**1f**)	1.0	62	5.3×10^{-9}	3.6×10^{-9}	2659	1.6×10^{5}
50 μM α-toc	2.0			2.7×10^{-10}	18948	1.1×10^{6}

and ML oxidations induced by BPT (**1b**). We then determined the rate of radical chain initiation (R_i) by the inhibitor method, using α-toc as the reference antioxidant: $R_i = 2[\text{α-toc}]/\tau$. The k_{inh} values were determined by a previously used method, following eqn (1),[28,29]

$$R_{inh} = \frac{d[\text{ROOH}]}{dt} = \frac{k_p[\text{RH}]R_i}{nk_{inh}[\text{IH}]} \quad (1)$$

where ROOH, RH, IH and k_p are the substrate hydroperoxide, substrate, antioxidant and the rate constant for the chain propagation, respectively. The k_p values used for cumene and ML in chlorobenzene at 30 °C were 0.18 M^{-1} s^{-1} and 62 M^{-1} s^{-1}, respectively.[30,31] The k_{inh} can be determined by introducing each value into eqn (1). The k_{inh}, n, R_{inh} and τ *versus* the cumene and ML oxidations in chlorobenzene at 30 °C are summarized in Table 1 for comparison to those of allicin (**1f**) and α-toc. The k_{inh} values gave the rate constants for BPT (**1b**) with the peroxyl radicals derived from cumene and ML of 8.6×10^3 M^{-1} s^{-1} and 6.2×10^4 M^{-1} s^{-1}. This is about three times that of allicin (**1f**) and one-fourteenth that of α-toc in the cumene oxidation system and about half that of allicin (**1f**) and one-seventeenth that of α-toc in the ML system.

Based on the above results, BPT (**1b**) is chemically less reactive toward the peroxyl radical than α-toc, but it was found that BPT (**1b**) reacts with peroxyl radicals derived from cumene about 3 times faster than allicin (**1f**). In the ML oxidation system, BPT (**1b**) has half the antioxidant activity of allicin (**1f**). However, this k_{inh} indicates the value for one peroxyl radical trapped by one molecule of BPT (**1b**). As described above, one molecule of BPT (**1b**) can scavenge two molecules of peroxyl radicals. In addition, we obtained valuable data about the mechanism of the thiosulfinate derivative as an antioxidant, that is, the benzyl group would be more effective as an antioxidant of the thiosulfinate derivatives than the allyl group, and one molecule of BPT (**1b**) can scavenge two molecules of radical.

It is generally believed that the oxidative modification of biological molecules leads to tissue damage and eventually to various diseases.[32,33] The action of BPT (**1b**) as a radical-scavenging antioxidant, first discovered in this study, could contribute to a decrease in the development of these diseases.

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