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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · OCTOBER 2003

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Antioxidant Activity of Bakuchiol: Experimental Evidences and Theoretical Treatments on the Possible Involvement of the Terpenoid Chain

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Received April 23, 2003

The protective activity of the plant-derived meroterpene, bakuchiol [1-(4-hydroxyphenyl)-3,7-dimethyl-3-vinyl-1,6-octadiene, **1**], against oxidative damages to lipids and proteins has been investigated and rationalized based on the scavenging activity of **1** against various oxidizing radicals ($Cl_3CO_2^{\bullet}$, linoleic acid peroxyl radicals, $LOO^{\bullet}$, DPPH radicals, $^{\bullet}OH$, and glutathiyl radicals). The rate constants of the scavenging reactions, transients formed in these reactions, and their mechanistic pathways have been probed using optical pulse radiolysis technique. Besides **1**, its methyl ether derivative **2** also could prevent lipid peroxidation in rat brain homogenate, indicating the probable participation of their terpenoid chains in scavenging $LOO^{\bullet}$. This was further corroborated from the pulse radiolytic studies on the reaction between the glutathiyl radicals and the compounds **1** and **2** as well as two other congeners, **3** and **4**, which showed transient absorptions at ∼300 nm attributable to some C-centered allylic radicals. On the basis of the strong signals at ∼300 nm with **1**−**3** as compared to compound **4**, formation of the allylic radical adjacent to the trisubstituted olefin function in **1**−**3** was envisaged. This was confirmed by quantum chemical calculations of the relative energies of the probable radical species derivable from **2** using Hartree−Fock and density functional theory along with self-consistent reaction field model. In the case of **1**, the allylic radical was found to be transformed into the phenoxyl radical at a later stage. All of these data revealed, for the first time, the importance of the terpenoid moiety of bakuchiol in controlling its antioxidant action via radical scavenging.

Introduction

There is a growing interest in the chemistry of antioxidants due to their proposed health benefits and potential in preventing various diseases. The majority of the efficient antioxidants reported so far are phenolics (*1*), which exhibit their activity via several mechanisms including scavenging of radicals and other reactive oxygen species (ROS) (*2a*,*b*). Of late, several theories have also been proposed to explain the antioxidant action of the phenolics (*3a*−*c*). Many known phenolic antioxidants contain alkyl chains, although their role in the antioxidant action has not been addressed. In principle, compounds possessing multiple double bonds and especially with active methylene groups can act as radical scavengers via addition to double bonds and/or abstraction of hydrogen atom from the allylic position as is the case with lipids. Indeed, the positive role of the β-diketone moiety in curcumin has been suggested for its antioxidant property (*4a*). Very recently, this was also substantiated by us while working on the antioxidant properties of both phenolic and nonphenolic β-diketone compounds (*4b*,*c*). Besides phenolics, antioxidant activities of other classes of compounds including terpenoids are also reported (*5a*,*b*) although the chemistry of their antioxidant action is not very clear. To this end, we have assessed the antioxidant activity of the meroterpene, bakuchiol [1-(4-hydroxyphenyl)-3,7-dimethyl-3-vinyl-1,6-octadiene, **1**] (*6*), an important constituent of the plant *Psoralea corylifolia*. *P. corylifolia* is an ancient medicinal plant of India and China and is still being cultivated for medicinal purposes in China today. It is abundant in various parts of India, and its seeds have enjoyed an honored place in Ayurvedic medicine, particularly for the treatment of inflammatory and skin diseases. In Chinese medicine, it has been used for centuries for the treatment of alopecia and vitiligo; also, systemic preparations have been used for enuresis, impotence, and frequent urination. Bakuchiol, one of the important constituents of *P. corylifolia* seeds, is also reported to possess antimicrobial, antiinflammatory, and lipid protective activities (*7a*−*c*). The present study on the antioxidant activity of bakuchiol may partly explain its medicinal attributes. We have envisaged that due to the presence of both a phenolic and a terpenoid moiety in bakuchiol, comparison of its antioxidant activity with that of its methyl ether derivative (Scheme 1) would enable us to quantify the radical scavenging activities of these structural motifs individually. With this aim, the present study has been undertaken and the observed results have been further supplemented by theoretical calculations.

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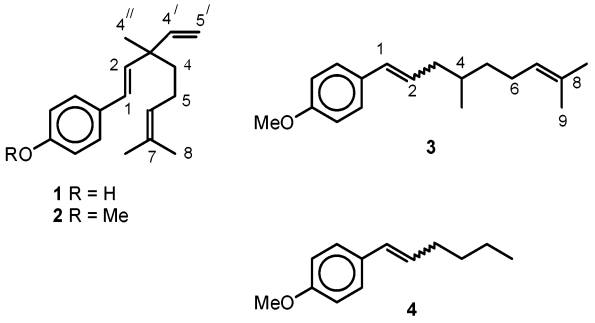
10.1021/tx034082r CCC: $25.00
Published on Web 08/08/2003

Scheme 1



Materials and Methods

Bakuchiol (**1**) was isolated (*6*) from the seeds of *P. corylifolia*, while O-methylbakuchiol (**2**) was prepared by methylation of **1** as previously described (*8*). Compounds **3** and **4** were synthesized by Wittig olefination of *para*-anisaldehyde with triphenylcitronellyl phosphonium bromide (for **3**) or triphenylhexyl phosphonium bromide (for **4**) by standard procedures. All compounds were characterized by ^{1}H NMR and IR spectroscopy as well as microanalyses (for **3** and **4**).

GSH (Aldrich, U.S.A.) was used as received. Ethanol from E. MERCK and DPPH and thiobarbituric acid (Sigma, U.S.A.) were used as received. All other chemicals were of AR grade. The solvent used was freshly prepared deionized Nanopure water (conductivity < 0.06 μS cm^{-1}) obtained from a Barnstead nanopure cartridge filtration system (Barnstead Corp., Boston, MA).

Lipid Peroxidation Assay. Rat brain homogenate (RBH), prepared from the brains of freshly killed Wistar rats, was subjected to Fe^{2+}-induced lipid peroxidation as described previously (*9*) with minor modifications. In brief, the total reaction mixture (1.0 mL) contained Tris-HCl buffer (12.5×10^{-2} mol dm^{-3}, pH 7.4) and brain homogenate (0.5 mg of protein/mL), with or without the test compounds. The reaction was triggered by addition of ferrous ammonium sulfate (2×10^{-5} mol dm^{-3}) and vitamin C (2×10^{-4} mol dm^{-3}) followed by incubation at 37 °C for 30 min. The reaction was terminated by addition of TBA−TCA−HCl solution (2 mL, 0.37% TBA, 2.8% TCA, 0.25 mol dm^{-3} HCl) and boiling of the mixture at 100 °C for 10 min. The extent of lipid peroxidation was measured spectrophotometrically by recording the absorbance at 532 nm.

DPPH Assay. The DPPH scavenging assay was carried out (*10*) by monitoring the absorbance of an ethanolic solution of DPPH (1×10^{-4} mol dm^{-3}) at 517 nm in the presence and absence of the test compounds. The concentration ($IC_{0.200}$) of the test compound at which the absorbance decreases by 0.200 of a unit during a 30 min observation was taken as the free radical scavenging potency. The IC_{50} value was also calculated from the results of the same experiment.

Cyclic Voltammetry. The experiment was carried out with a cyclic voltammeter (Acochemie Autolab, model PGSTAT 20) using a three electrode system viz. Ag/AgCl as the reference electrode, a glassy carbon electrode as the working electrode, and a platinum wire as a counter electrode. The cell contained 10 mL of the sample solution and 0.1 mol dm^{-3} KCl. Cyclic voltammetry tracings were recorded from −0.25 to 1.2 V at a scan rate of 50 mV/s.

Pulse Radiolysis. The pulse radiolysis system using 7 MeV electrons was described earlier (*11*). The dosimetry was carried out using an air-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} KSCN assuming $G\epsilon$ for $(SCN)_2^{\bullet-} = 23\,889$ dm^3 mol^{-1} cm^{-1} per 100 eV at 500 nm (*12*). The kinetic spectrophotometric detection system covered the wavelength range from 250 to 800 nm. Alkaline pH was obtained by adding NaOH only. High purity (>99.9%) N_2O, from BOC India Pvt. Ltd., was used as per requirement. The following reactions occured after irradiation in the reaction medium used.

$$H_2O \rightsquigarrow e_{aq}^-, H^\bullet, {}^\bullet OH, H_2, H_2O_2 \quad (1)$$

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^\bullet OH + OH^- + N_2 \quad (2)$$

$${}^\bullet OH \overset{pK_a = 11.8}{\rightleftharpoons} O^{\bullet-} \quad (3)$$

$${}^\bullet OH + N_3^- \rightarrow OH^- + N_3^\bullet \quad (4)$$

$$H^\bullet/{}^\bullet OH + C_2H_5OH \rightarrow {}^\bullet C_2H_4OH + H_2/H_2O \quad (5)$$

$${}^\bullet C_2H_4OH + RSH \rightarrow RS^\bullet + C_2H_5OH \quad (6)$$

The bimolecular rate constants were calculated by plotting the pseudo-first-order rates of formation of the transients against the concerned solute concentrations. The uncertainty in the measurement in bimolecular rate constant was ±10%.

Quantum Chemical Calculations. Ab initio molecular orbital techniques have been used to investigate the structure and energetics of **2** as well as additional possible radical species (obtained by H-atom abstraction from it). The ground state global minimum geometries of **2** and all of the possible radical species have been fully optimized (without any symmetry constraint) at the Hartree−Fock (HF) level of theory with a 6-31G(d,p) basis set. The relevant atom-in-molecule electron populations as well as the spin populations were obtained using a Mulliken population analysis scheme for obtaining the net charge and spin at the atomic sites. All of the calculations in this work have been performed using the GAMESS (*13*) electronic structure program.

Results and Discussions

Oxidation of lipids and oxidation of proteins are two independent events in cellular systems and lead to mutagenesis, loss of membrane integrity, enzyme deactivation, etc., which are implicated in various pathogenic conditions in human (*14a*−*d*). Consequently, we chose these biomacromolecules as the susceptible target for oxidation and studied the efficacy of **1** and **2** in protecting them against oxidative damage. For studying the antilipid peroxidation activities of **1** and **2**, RBH was chosen as the biological lipid model and the efficacy of the test compounds in preventing Fenton-mediated damages was assayed. In addition, the scavenging activity of **1** and **2** against two biologically relevant chemical peroxide models, $Cl_3CO_2^\bullet$ and the linoleic acid peroxyl radical, was also studied using the pulse radiolysis technique. Likewise, using GSH as the protein prototype, the protecting activity of **1** and **2** against its oxidation and its possible repair was also assessed.

The redox properties are crucial for better understanding of the electron transfer processes, and cyclic voltametry is an established instrumental tool for the evaluation of antioxidant capacity of a test compound (*15*). Hence, we initially measured the oxidation potential of **1** by cyclic voltammetry and in fact found two values, 0.34 and 0.74 V, at pH 10 (Figure S1 in the Supporting Information). In view of its structure and pH condition, we assign the peak at lower potential value to be responsible for the phenolic and the higher one to be due to the methylenic moiety. The low oxidation potential values for **1** suggested it to be an efficient antioxidant.

Inhibition of RBH Lipid Peroxidation. In unstimulated control experiments in the RBH assay, little thiobarbituric acid reactive substrates (TBARS) were formed ($abs_{532} = 0.045 \pm 0.001$). However, addition of Fe^{2+} and vitamin C triggered the lipid peroxidation

leading to an increase in TBARS concentration (abs_{532} = 0.321 ± 0.007). Control experiments showed that **1** itself did not interfere with the TBARS measurement, as its addition to a terminated reaction mixture did not change TBARS absorption at 532 nm. However, **1** inhibited microsomal lipid peroxidation in a concentration-dependent manner showing 74.7% protection at a concentration of 10 μmol dm^{-3}. In view of its solubility in both lipid and water (at higher pH), **1** is expected to be distributed in both of these phases. This may account for its low IC_{50} = 6.1 ± 0.2 μmol dm^{-3} value against lipid peroxidation. This value was better than that of the positive control α-tocopherol (IC_{50} = 8.5 ± 0.4 μmol dm^{-3}) under similar experimental conditions. Compound **1** did not chelate Fe^{2+} or Fe^{3+} as was evident from the absorption spectroscopic study carried out with **1** (100 μmol dm^{-3}) and Fe^{2+} or Fe^{3+} (50−200 μmol dm^{-3}). Thus, the protective effect of **1** against lipid peroxidation must be due to radical scavenging only. To substantiate this, the scavenging activity of **1** against the stable N-centered DPPH radical was also measured (*10*), as the assay results are known to correlate with the degree of protection of lipid peroxidation activities of these test compounds. In this case also, **1** showed a concentration-dependent scavenging of DPPH radical with an $IC_{0.200}$ value of 10.2 ± 0.4 μmol dm^{-3}. The calculated IC_{50} value was 16.8 ± 2.2 μmol dm^{-3}.

Surprisingly, the methyl ether **2** of bakuchiol also prevented Fenton-mediated peroxidation of RBH, with an IC_{50} value of 50.2 ± 5.3 μmol dm^{-3}. The higher inhibitory concentration of **2** as compared to that of **1** was expected considering the lack of the easily oxidizable phenol group, i.e., in the former. The activity of **2** can possibly be explained by abstraction of its allylic hydrogen by the lipid peroxyl radical (LOO•) or addition of the radical to one of its olefinic functionalities. The first possibility seemed more apparent as compound **2** scavenged even DPPH radical, albeit at a much higher concentration (30% scavenging at 1.4 ± 0.2 mmol dm^{-3}). This was more evident from the subsequent studies as later part.

Pulse Radiolysis Study. 1. Reaction with Cl_3CO_2• Radical. The reaction of **1** with Cl_3CO_2• radical, generated by pulse radiolysis, was carried out in aqueous *tert*-butyl alcohol medium. Despite its higher reduction potential value (*16*), Cl_3CO_2• radical is extensively used as a representative peroxyl radical (*17*) to explain important biophysical phenomena (*4b*, *9*, *18*) in antioxidant chemistry. This chosen medium is known to produce *tert*-butyl radical by the reaction of the generated •OH radicals with the alcohol. However, the radical is known to be unreactive at least within the time scale of our measurement, thereby providing an opportunity to study the reaction of **1** with Cl_3CO_2• radical exclusively.

Bakuchiol reacted with Cl_3CO_2• producing a transient absorption spectrum with a maximum at 350 nm and a broad absorption at 400−475 nm (Figure 1). Both of the absorption peaks had similar formation constants, indicating them to be for the same transient. This was ascribed to the one electron oxidized phenoxyl radical (*19a*,*b*) of bakuchiol only.

2. Reaction with Linoleic Acid Peroxyl Radical. Linoleic acid is an important lipid component, and prevention of its peroxidation is also an excellent criterion for an efficient antioxidant (*20*). In the present study, it was observed that at pH > 10, **1** could scavenge the linoleic acid peroxyl radical generated by pulse radiolysis. Under the experimental conditions (pH 11), even when the linoleate ions exist as negatively charged micelles, their reaction with **1** produced the same phenoxyl transient absorbing at 360 nm. The kinetic trace at 360 nm (inset of Figure 1) shows the formation of the phenoxyl radical in the first part and its decay in the second part.

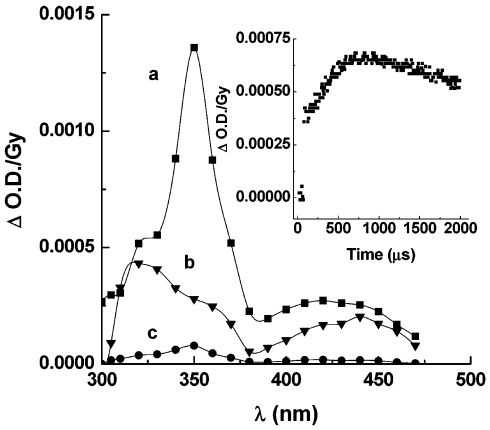


Figure 1. Transient absorption spectrum obtained from air-saturated aqueous solution containing bakuchiol (9.4 × 10^{-5} mol dm^{-3}), *tert*-butyl alcohol (2 mol dm^{-3}), and CCl_4 (1 × 10^{-2} mol dm^{-3}) at pH 10.9; (a) 50, (b) 200, and (c) 850 μs after the electron pulse. Inset: Kinetic trace recorded at 360 nm for N_2O:O_2 (4:1)-saturated solution containing linoleic acid (2 × 10^{-2} mol dm^{-3}) and bakuchiol (2 × 10^{-4} mol dm^{-3}) at pH 11.0, after the electron pulse.

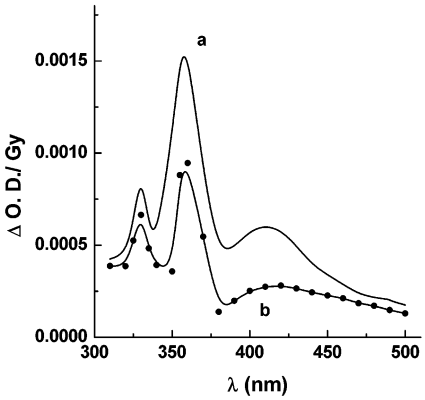


Figure 2. Transient absorption spectrum obtained from N_2O-saturated aqueous solution containing bakuchiol (1 × 10^{-4} mol dm^{-3}) and NaN_3 (1 × 10^{-2} mol dm^{-3}) at pH 10.0; (a) 60 and (b) 500 μs after the electron pulse.

3. Oxidation Reactions with N_3• Radical. The transient absorption peak at 350 nm and the broad band at the 400−450 nm wavelength region obtained by the reaction of **1** and the peroxyl radicals have been ascribed to the corresponding phenoxyl radical, on the basis of previous papers (*19a*,*b*). However, given that the organic peroxyl radials can react by several possible mechanisms, such as hydrogen abstraction, addition to unsaturated bonds, and oxidation (*21a*−*c*), it was imperative to confirm this unambiguously. The azide radical is known to react selectively with organic molecules to give their one electron oxidized radical species. Hence, the reaction of **1** with the azide radical was studied. The reaction was facile, again producing the same transient with absorption maxima at 360 and 410 nm (Figure 2). The bimolecular rate constants for the formation of the peaks at

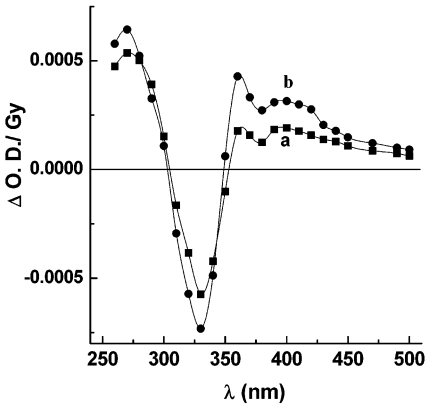


Figure 3. Transient absorption spectrum obtained from N_2O-saturated aqueous solution containing bakuchiol (1×10^{-4} mol dm^{-3}) at pH 10.0; (a) 4 ms and (b) 45 μs after the electron pulse.

pH 11 were the same and found to be 1.3×10^9 dm^3 mol^{-1} s^{-1}. As in the case with the peroxyl radicals, no bleaching in absorption due to the depletion of the parent compound was observed down to 300 nm, showing that the transient absorption was stronger than that of the parent throughout the wavelength region.

4. Oxidation Reaction with OH• Radical. Among the various ROS, •OH radical is the most reactive oxidant and is the most predominant ROS generated endogenously during aerobic metabolism. Effective scavenging of this radical is important from the health perspective. Consequently, the reactivity of **1** with •OH radical was studied at pH values of 10 and 11.1 using pulse radiolysis. In this case also, formation of the transient with absorption maxima at 360 and 410 nm with the bimolecular formation rate constant of 3.6×10^9 dm^3 mol^{-1} s^{-1} was observed at pH 10 (Figure 3). Even though at pH 11.1 approximately 17% of hydroxyl radicals exist in the deprotonated form ($O^{\bullet-}$) and may react in a different way, there was no significant change in the transient absorption spectra at pH values of 10 and 11.1.

As compared to the reaction with the azide radicals, in this case, the transient absorbencies were much lower. This may be explained as follows. The azide radicals can react with **1** via only one pathway, which leads to the phenoxide radical. However, the hydroxyl radical is known to react by both addition and one electron oxidation. Possibly, besides one electron oxidation, the hydroxyl radicals also form an adduct with **1**, leading to a significant lowering in the phenoxide radical concentration. Moreover, the transient absorption due to the adduct formation might have a lower absorption coefficient below 350 nm. Both of these factors contribute to the bleaching signal at the 300−350 nm (Figure 3) wavelength region.

Being a 1,2-diarylethylene, the compound *trans*-resveratrol has structural similarity with **1**, which is an arylalkylethylene. Very recently, the reaction of the hydroxyl radical with resveratrol has been reported (*22*) to generate the respective phenoxide ions of the rings A and B via the initially formed adducts. In the present case, however, such an evolution in the transient spectra was not noticed suggesting that the •OH adduct of **1** does not lead to the formation of its one electron oxidized radical.

5. Reaction with the Glutathiyl Radical. Thiols are present in living systems, which, in addition to participating in cellular redox processes (reaction 7), also take part in scavenging free radicals (reaction 8) (*23a,b*). The antioxidant action of the thiols is attributed to the latter reaction.

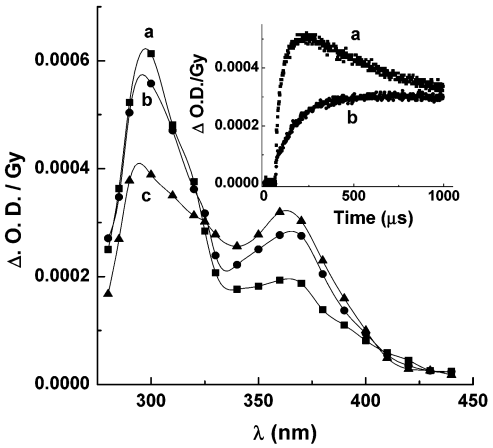


Figure 4. Transient absorption spectrum obtained from N_2O-saturated aqueous solution containing bakuchiol (1.77×10^{-4} mol dm^{-3}), ethanol (50% v/v), and GSH (1×10^{-3} mol dm^{-3}) at pH 5.4; (a) 100, (b) 250, and (c) 700 μs after the electron pulse. Inset: Kinetic traces under the same conditions at (a) 310 and (b) 370 nm.

Table 1. Formation and Decay Rate Constants for the Radicals Produced in Reactions of Bakuchiol (1), Methoxybakuchiol (2), and the Analogue (3) with Different Radicals

radical	pH	λ_{max} (nm)	k_f (dm^3 mol^{-1} s^{-1}) [λ]	k_d (s^{-1}) [λ]
			1	
•OH	10	360, ∼410	3.6×10^9 [360]	2.3×10^3 [360]
$^{\bullet}N_3$	11	360, ∼410	1.3×10^9 [360]	1.0×10^3 [360]
GS•	5.4	300, 360	9.0×10^7 [300]	1.8×10^3 [300]
$CCl_3O_2^{\bullet}$	10.9	350, ∼420	2.8×10^8 [350]	3.0×10^3 [350]
			2	
GS•	5.4	300	8×10^7 [300]	2.55×10^3 [300]
			3	
GS•	5.4	300	1.2×10^8 [300]	2.6×10^3 [300]

$$RSH \rightarrow RS^{\bullet} + e^- + H^+ \quad (7)$$

$$R_1CH_2^{\bullet} + RSH \rightarrow R_1CH_3 + RS^{\bullet} \quad (8)$$

Both of the reactions generate thiyl radicals (RS•), which, in turn, can act as reactive oxidants (*23a*) as they are able to initiate lipid peroxidation by abstracting the bis-allylic hydrogen from polyunsaturated fatty acids (*24a,b*). Furthermore, the thiyl radicals can also add to the lipid double bond leading to efficient cis/trans isomerization of mono- and polyunsaturated fatty acids (*25a−d*). Hence, their repair is necessary for both their storage as efficient antioxidants and their protection of lipids from thiyl radical-induced denaturation. To this end, the reaction of **1** with the thiyl radicals derived from the cellular antioxidant GSH was followed by pulse radiolysis. The reaction led to a transient spectrum with an absorption maximum at 300 nm and a shoulder at 370 nm (Figure 4). Table 1 lists the bimolecular rate constant for the reaction between GS• radical with **1** along with those for the reactions between various radicals and other test compounds including **1**.

It is known that at a pH > 7, the thiyl radicals can associate with the parent anion to furnish the $RSSR^{\bullet-}$

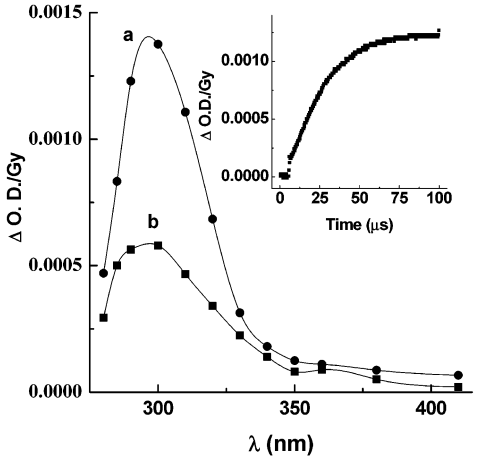


Figure 5. Transient absorption spectrum obtained from N_2O-saturated aqueous solution containing methoxybakuchiol (3.4 $\times$ 10^{-3} mol dm^{-3}), ethanol (50% v/v), and GSH (1 $\times$ 10^{-3} mol dm^{-3}) at pH 5.4; (a) 100 and (b) 800 μs after the electron pulse. Inset: Kinetic trace at 300 nm under identical conditions.

radical according to the following equilibrium (*26a*,*b*)

$$RS^{\bullet} + RS^{-} \rightleftharpoons RSSR^{\bullet -} \qquad (9)$$

However, under the present experimental conditions (pH < 6), we are justified in ignoring the contribution from the $RSSR^{\bullet -}$ radical. To confirm this, a blank experiment was also carried out without **1** at pH 5.4 using the same dose rate. As expected, no signal for $RSSR^{\bullet -}$ was detected under the experimental conditions.

Studying the dynamics of the reaction between **1** and $GS^{\bullet}$ revealed that with the progress of time, while the absorption at 300 nm decreased, the intensity of the absorption at 370 nm (inset of Figure 4) increased and became a peak at around 700 μs (Figure 4). The transient absorption at around 370 nm can be ascribed to the phenoxyl radical produced by the reaction of $GS^{\bullet}$ with **1**. However, the possibility of the absorption peak at 300 nm to be an adduct can be eliminated, based on the observation that the $^{\bullet}OH$ adduct of **1** did not lead to the formation of any other radical. In contrast, the present study revealed the evolution of the phenoxyl radical from the transient absorbing at 300 nm. Hence, we envisaged that the designated transient might be due to a radical, which is derived possibly by abstraction of one of its allylic hydrogen atoms of the alkyl chain.

Further confirmation of the generation of the allylic radical in **1** was confirmed by studying the reaction of $GS^{\bullet}$ with the protected phenol derivative **2** prepared (*8*) from compound **1**. As anticipated, **2** produced only one transient absorption at 300 nm with increased intensity (Figure 5), as only one reaction pathway was possible in this case. This confirmed our previous suggestion of the formation of a C-centered radical preferably at the C-5 position of its terpenoid chain. The absence of transient absorption over 350 nm also supported that the absorption peak at 370 nm in the case of **1** was due to the phenoxyl radical only. The formation kinetic traces at 300 nm observed in the reaction of **1** and **2** (inset of Figure 5) were similar, and these were formed with almost the same rate constant (Table 1). This phenomenon has important consequences especially in cellular systems. It revealed that **1** not only scavenged $GS^{\bullet}$ radicals but might also repair it by abstracting a hydrogen atom from its terpenoid chain. This repair phenomenon is very attractive especially with respect to its biological activity.

Table 2. Relative Energy Values[a] (kcal/mol) of the Different Radical Species Calculated Using HF and DFT/SCRF Methods

radical center	ΔE_{HF}	$\Delta E_{DFT/SCRF}$
C1	16.2	21.8
C2	16.5	22.9
C4′	16.1	22.7
C5′	20.3	27.7
C4	7.4	14.6
C5	0.0	0.0
C6	15.7	22.5

[a] The relative energies are with reference to the C5 radical energy, which has been set to 0.0 for convenience.

Quantum Chemical Calculations. So far, the sites of the reaction of $GS^{\bullet}$ with **1** and **2** were tentatively assigned based on conventional wisdom and literature support. For its rationalization, we calculated the relative energies of the probable radical species obtained after H-atom abstraction from **2**, which are presented in Table 2. The data in Table 2 reveal that the radical at C-5 is the most stable among all of the positions of the alkyl chain. The energy difference between this and the next most stable radical (C-4) was 7.4 kcal/mol. Single point energies were also calculated after incorporation of electron correlation effect through density functional theory (DFT) using the hybrid B3LYP (*27a*−*c*) exchange correlation function and also taking into account the solvent effect through the self-consistent reaction field model (SCRF) (*27d*,*e*), and the results are also presented in Table 2. Qualitatively, both the gas phase HF results and the solvent phase DFT (DFT/SCRF) results agreed with each other. However, the latter calculation predicted a better stability of the C-5 radical as compared to the second most stable radical (C-4) by 14.6 kcal/mol.

For further characterization of the radical, we have plotted the highest occupied molecular orbital (containing the unpaired electron) obtained using the HF method as well as the DFT/SCRF method. The nature of the plot (Figure 6) clearly demonstrated that the radical spin density is mostly delocalized over two carbon atoms, viz. C-5 and C-7. According to HF results, spin densities were about 80% on C-5 and 20% on C-7, while the DFT/SCRF method predicted these to be ∼60 and 40%, respectively. The optimized geometry of the most stable radical and the atomic, electron, and spin populations are given in the Supporting Information (Tables S-I and S-II).

Experimental Verification for the Site of Alkyl Radical Formed in the Terpenoid Chain: Reaction of Glutathiyl Radical with Bakuchiol Congeners. Given the important role of the alkyl chain in compounds **1** and **2** in scavenging biologically relevant thiyl radicals, it was of interest to study the effect of the nature of the olefin function on the scavenging property. Hence, we synthesized compounds **3** and **4** as the two simpler congeners of **2** and studied their reactions with $GS^{\bullet}$. Compounds **3** and **4** were synthesized as *E*/*Z* mixtures by Wittig reaction of *p*-anisaldehyde with the suitable triphenylphosphonium bromides as the olefin geometry in **3** and **4** is unlikely to play any significant role in their radical scavenging activities. As compared to **2**, which had three different types of olefinic function, compound **3** had both disubstituted and trisubstituted double bonds,

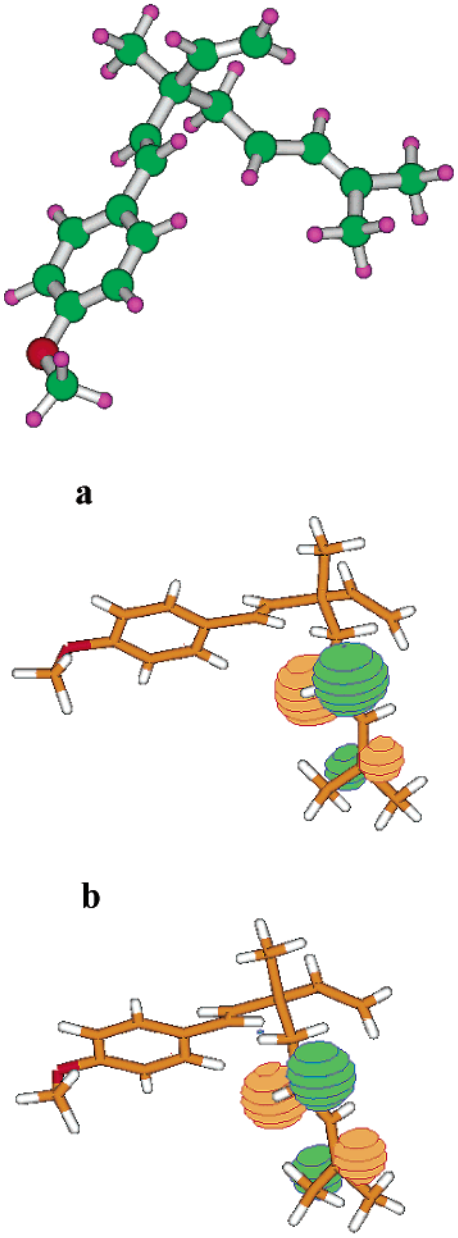


Figure 6. Optimized structure of the most stable radical (C6) and the plots of the highest singly occupied molecular orbital: (a) HF method and (b) DFT/SCRF method. See text for details.

while **4** had only one disubstituted double bond, placed at a similar position as in **2**. Thus, their reaction profiles with GS• would provide important information on the role of the nature of the olefin functions.

The transient absorption spectra (Figure 7) obtained in the reaction between GS• and **3** revealed only one absorption maximum at ~300 nm as was the case with **2**. The spectral and kinetic characteristics (see Table 1) matched well with those obtained with **2**. In the case of **4**, however, the intensity of the absorption peak at ~300 nm was significantly lower than that obtained with **3** as is revealed from the inset of Figure 7, which depicts the formation traces at 300 nm for compounds **3** and **4**. From this, it can be concluded that the allylic hydrogens (at C-5 for **1** and **2** and at C-6 for **3**) in the alkyl chains of the test compounds, **1**−**3** are most labile and susceptible for abstraction. Although the allylic radical at C-3 derivable from **3** (also from **4**) appears more resonance-

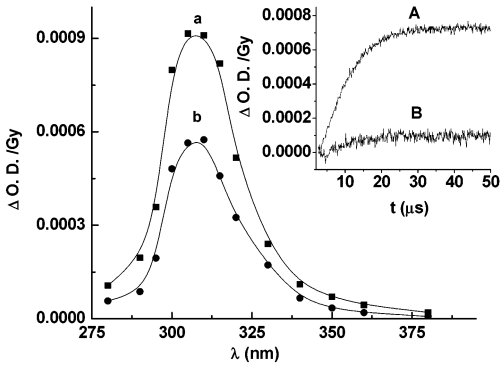


Figure 7. Transient absorption spectra obtained from a N_2O-saturated ethanolic aqueous (1:1 v/v) solution containing 3.0×10^{-3} mol dm^{-3} compound **3** and 1×10^{-3} mol dm^{-3} GSH at pH 6; (a) 100 and (b) 800 μs after the electron pulse. Inset: Absorbance vs time plot for the transient obtained at 300 nm. (A) For compound **3** (two double bonds); (B) for compound **4** (only one double bond) under identical conditions as that of compound **3** including concentration.

stabilized than that at C-6, this would be at the expense of the loss of aromaticity. In comparison, the allylic radical adjacent to the trisubstituted olefin, which may be formed only with **1**−**3** (and not **4**) would be more stable as one of its resonance structures is a tertiary radical at C-7. This accounts for its preferential formation over the alternative allylic radical (for **3**). In the case of compound **4**, the allylic radical adjacent to the trisubstituted olefin function is not possible; hence, the absorption peak (~300 nm) due to the allylic radical is an order of magnitude weaker than that in the case of compound **3**. The alkyl radical thus formed in compounds such as **1** can generate the phenoxyl radical by intramolecular electron transfer as proposed earlier in the case of curcumin (*28*).

So far, the reaction between GS• and compounds **1**−**4** has been explained considering the hydrogen abstraction mechanism only. The reaction can also proceed via addition of the GS• radical to the benzylic double bond of the compounds furnishing the radical at C-1. The resultant benzylic radical will also be stabilized by resonance and may have the transient absorption maximum at 280−300 nm. However, in that case, the transient absorption for the radical should have similar intensity with all of the compounds **1**−**4**, especially as the reactions were conducted with similar concentrations of the substrates. The effect of concentration, which might change the equilibrium constant for the reaction, and in turn, affect the intensity of the transient absorption, appears to be nonexistent. Thus, the formation of the benzylic radical (via addition reaction) cannot account for the observed low intensity of the transient with compound **4**, suggesting that the transient radical derived in these reactions is formed primarily via the abstraction of the allylic H-atoms of the compounds. Furthermore, between compounds **3** and **4**, the only difference was an additional trisubstituted olefin moiety in the former, which is also present in compounds **1** and **2**. Considering the superior reactivities of compounds **1**−**3** vis à vis that of compound **4**, the major contribution of the trisubstituted olefin moiety in scavenging the GS• radical is apparent. Thus, overall, the H-abstraction mechanism appears to be the deciding factor in the reactions between GS• and compounds **1**−**4**; the contribution of the addition reaction, if at all, might be minor.

Concluding Remarks

While summing up, biochemical assays, optical pulse radiolysis technique, quantum chemical calculations, and synthesis of required congeners have been employed to understand the antioxidant mechanisms of bakuchiol. In such a molecular system, the terpenoid chain may lead to enhancement of its antioxidant efficiency due to the presence of the easily abstractable hydrogen atom adjacent to its trisubstituted olefin group. The contribution of its other double bonds in the antioxidant property appears to be insignificant. Recently, the antioxidant activities of phenols have been correlated (*3a*) with the bond dissociation enthalpy (BDE) of the phenolic O−H bond. Compound **1** can be considered to possess two antioxidant pharmacophores viz. the phenolic group and the terpenoid substituent. As an electron-donating group, the terpenoid substituent of **1** is anticipated to reduce the BDE of its phenolic moiety, thereby enhancing its antioxidant activity. Thus, besides the phenolic group, the alkenic side chain, which generally helps the molecule to be solubilized in the lipid phase, may also play a crucial role in the antioxidant action of bakuchiol and other related compounds.

Supporting Information Available: Synthesis of compounds **3** and **4**; experimental data for compounds **3**, **4**, **6**, and **7**; tables of Cartesian coordinates and atomic spin calculations; figure of cyclic voltammetry study; and numbering scheme. This material is available free of charge via the Internet at http://pubs.acs.org.

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