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Original article

PASS-assisted design, synthesis and antioxidant evaluation of new butylated hydroxytoluene derivatives

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ABSTRACT

New multipotent antioxidants (MPAOs), namely 1,3,4-thiadiazoles and 1,2,4-triazoles bearing the well-known free radical scavenger butylated hydroxytoluene (BHT), were designed and synthesized using an acid-(base-) catalyzed intramolecular dehydrative cyclization reaction of the corresponding 1-acylthiosemicarbazides. The structure–activity relationship (SAR) of the designed antioxidants was performed along with the prediction of activity spectra for substances (PASS) training set. Experimental studies based on antioxidant activity using DPPH and lipid peroxidation assays verified the predictions obtained by the PASS-assisted design strategy. Compounds **4a**–**b**, **5a**–**b** and **6a**–**b** showed an inhibition of stable DPPH free radicals at a 10^{-4} M more than the well-known standard antioxidant BHT. Compounds with *p*-methoxy substituents (**4b**, **5b** and **6b**) were more active than *o*-methoxy substituents (**4a**, **5a** and **6a**). With an IC_{50} of 2.85 ± 1.09 μM, compound **6b** exhibited the most promising *in vitro* inhibition of lipid peroxidation, inhibiting Fe(2+)-induced lipid peroxidation of essential oils derived from the egg yolk-based lipid-rich medium by 86.4%. The parameters for the drug-likeness of these BHT derivatives were also evaluated according to Lipinski's 'rule-of-five'. All of the BHT derivatives were found to violate one of Lipinski's parameters (Log $P \geq 5$) even though they have been found to be soluble in protic solvents. The predictive TPSA and %ABS data allow for the conclusion that these compounds could have a good capacity for penetrating cell membranes. Therefore, these novel MPAOs containing lipophilic and hydrophilic groups can be proposed as potential antioxidants for tackling oxidative stress and lipid peroxidation processes.

1. Introduction

The presence of free radicals in biological materials was discovered approximately 50 years ago [1]. Reactive oxygen species (ROS) are considered to be responsible for many undesired processes such as aging [2], inflammation [3] and many others [4–8]. It is becoming increasingly certain that certain types of inflammatory tissue injury are mediated by reactive oxygen metabolites. These reactive radicals and oxidants may injure cells and tissue directly via oxidative degradation of essential cellular components as well as injure cells indirectly by altering the protease/antiprotease balance that normally exists within the tissue interstitium [9]. Natural and synthetic antioxidants may protect against oxidant-mediated inflammation and tissue damage by virtue of their ability to scavenge free radicals.

BHT is a well-known antioxidant utilized in a wide variety of products. It was patented in 1947 [10] and has been approved for use in foods and food packaging in low concentrations by the U.S. FDA since 1954 [11]. Currently, BHT is one of the most commonly used antioxidants in foods containing fats [12], petroleum products and rubber [13]. Due to these widespread applications, BHT and its derivatives have become attractive antioxidants or even co-antioxidant groups [14]. It is therefore no surprise that BHT has been modified to prepare a series of new antioxidants with new properties for both the polymer and pharmaceutical industries [15–17]. For instance, Parke-Davis has disclosed a new class of

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potent, selective and orally active COX-2 inhibitors composed of 2,6-di-tert-butyl phenol [18,19]. This encouraged researchers to prepare new BHT derivatives as potential dual inhibitors of COX-2 and 5-lipoxygenase [20]. Most recently, we have reported that four different series of BHT derivatives improved the survival of *Staphylococcus aureus*-infected nematodes due to their antioxidant activities [21].

Thiosemicarbazides have been reported to show anti-inflammatory [22], antibacterial [23], antimicrobial [24,25] and anti-toxoplasmagondii [25] activities. Derivatives of thiosemicarbazide bearing BHT moieties are rarely synthesized. Compounds containing a 1,3,4-thiadiazole nucleus have been reported to have a variety of biological activities, such as anti-inflammatory [26], antimicrobial [27], antitubercular [28], anticancer [29,30] and urease inhibition activities [31]. 1,2,4-Triazoles are an important class of five-membered heterocyclic compounds. 3-Substituted-1,2,4-triazole-5-thiones are known for their anti-inflammatory [26], selective COX-2 inhibition [32], antimycotic [33] and urease inhibition [31] activities.

In the present study, SAR and rational design strategies were used to combine multiple functions that include a radical-scavenging ability and diversified pharmacological activities in designing hybrid compounds with markedly enhanced radical-scavenging ability and anti-lipid peroxidation. These strategies were performed together and tested based on the SAR analysis of the PASS training subsets, drugs and non-drugs. Antioxidant activities predicted by the PASS program were experimentally verified by DPPH and TBARS (thiobarbituric acid reactive substance) assays. Furthermore, a computational study for prediction of absorption and distribution (ADMET) properties of the molecules under study was performed by determination of polar surface area (PSA), absorption (ABS) and Lipinski parameters. The acid-(base-) catalyzed intramolecular dehydrative cyclization reactions of acylthiosemicarbazide **4a**–**b** to the corresponding 1,3,4-thiadiazole **5a**–**b** and 1,2,4-triazole **6a**–**b** are described. The synthesized compounds have been characterized by IR, NMR and mass spectral analysis. The X-ray structures of **4a** and **6a** will be further discussed in this paper.

2. Results and discussion

2.1. Rational MPAO design

Rational antioxidant design has two strategies. The first strategy is to modify the existing antioxidants to improve their activity according to specific demand, which does not need the aid of theoretical computation. The other is to find novel structures by computer-aided methodologies. In recent years, a great deal of effort has been devoted to finding MPAOs in an attempt to combine radical-scavenging (and/or radical-generation-preventing) activity and enzyme-inhibiting potential into a single structure [34–36].

2.1.1. SAR and rational design of MPAO

First, our design strategy involved assembling the beneficial features of two or more antioxidants into one structure. These designed structures were then evaluated for their potential antioxidant activities through SAR using the PASS training set, which involved two subsets of drug and non-drug databases. This strategy was applied to improve the antioxidant activities and other physical properties of the well-known antioxidant (BHT) to create MPAOs with specific functional groups that are very important in the design and introduction of promising new antioxidant candidates.

2.1.1.1. Phenolic ring. It has been reported that electron-donating substituents, such as methyl and *tert*-butyl at the 2,4,6-positions, increase the primary antioxidant activity of phenols [37]. This is due to the lowering of the bond dissociation enthalpy (BDE) of the phenol O–H group [38] and the stabilization of the phenoxyl radical by inductive and hyperconjugative effects. Two di-*tert*-butyl groups at the *ortho* position provide enough steric hindrance to minimize undesirable reactions, such as pro-oxidation [39–42] (Fig. 1). It has been observed that two *tert*-butyl groups flanking the OH group are required to retain *in vivo* anti-inflammatory potency [15].

2.1.1.2. Thioether groups. Thioethers are classified as secondary antioxidants that can be used in combination with primary antioxidants during processing to improve the performance of the primary antioxidant [40]. Thioethers do not act as radical scavengers but instead undergo redox reactions with hydroperoxidants to form non-radical stable products [43,44]. Phenols containing *p*-thioether groups $-CH_2-S-R$ act as strong free radical scavengers [45]. Thioether bridges between phenol and heterocyclic rings could provide synergistic effects between different combinations of primary and secondary antioxidants in a given hyperstructure.

2.1.1.3. Amides and thioamides. The degree of conjugation in thioamides is considerably higher than that in amides. Both the amide and the thioamide functional groups withdraw electron density from the conjugated system, but the thioamide is a stronger π-electron attractor [46,47]; hence, thioamides are better antioxidants than amides [48].

2.1.1.4. Secondary aromatic amine group. Aromatic amines and their derivatives can easily transfer their amine hydrogen to aminyl radicals [49]. Phenolic or amine antioxidants are able to suppress two oxidative chains [50]. Interestingly, in 2002, Riccardo et al. [51] studied a mixture of α-TOH and a secondary aromatic amine and found that the aromatic amine can act as a co-antioxidant by reversibly recycling α-TOH.

2.1.1.5. Effect of the methoxy substituent position. Alkoxy groups linked at the *ortho* and/or *para* positions of phenols develop phenolic antioxidant activity for both naturally occurring compounds [52–56] and synthetic antioxidants [56]. Regarding the effect of the *m*-substituents, Tetsuto et al. [57] evaluated the antioxidant activities of different donating substituents on the *m*-substituted phenol and found that an *m*-substituent does not influence the antioxidant activity of a phenol. This is probably because an m-substituent does not make a significant contribution to the stability of free radical comparing observations of *ortho* and/or *para* positions [44]. Considering these results, the target compounds were designed to be have *ortho* and *para* methoxy substituents on the phenyl ring.

The analysis described above led to a proposed model of action of MPAOs based solely on SAR. Compounds **4a**–**b** (Fig. 1) were

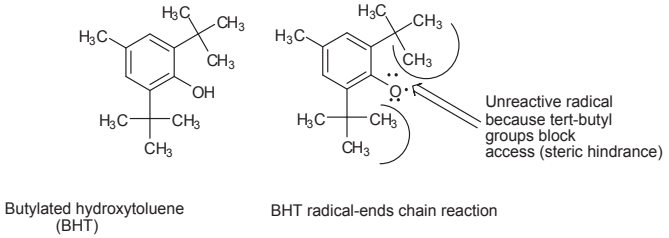


Fig. 1. Steric hindrance effects on stabilization of phenolic antioxidants.

designed to form an MPAO in a single structure by linking the well-known antioxidant BHT at the 4-position via a thioether bridge, which provides a linkage between an amide, thiourea (which is classified as a free radical scavenger [58] and secondary antioxidant [59]) and a secondary amine. Compounds **5a**–**b** (Fig. 1) were designed to contain secondary aromatic amines, which act as inhibitors of the radical-chain oxidation. Compounds **6a**–**b** (Fig. 1), which have N*H* ionizable protons and a thiourea system in the triazole ring, may have enhanced antioxidant activity as well as solubility and biological properties.

2.2. PASS – predication and assistant design

PASS prediction tools are constructed using 20,000 principal compounds and approximately 4000 types of biological activities at the molecular level, that providing an estimated profile of compound's action in biological space, including pharmacological effects, mechanisms of action, toxic and adverse effects, interaction with metabolic enzymes and transporters, influence on gene expression, etc. Such profiles can be used to recognize the most probable targets, interaction with which might be a reason of compound's toxicity [60]. PASS mean accuracy exceeds 90% in leave-one-out cross-validation [61–64].

Based on the prediction results, PASS is successfully applied in the pharmacological field, where a dozen of predictions were afterwards confirmed by the experiment [60]. For example, PASS is successfully applied in the pharmacological field, new anti-leishmanial agents were found among the benzothiazoles and their corresponding anthranilic acid derivatives [65], 7-substituted 9-chloro and 9-amino-2-methoxyacridines [66] and beta-carboline alkaloids [67]. PASS could be used for successfully prediction of adverse and toxic effects [60]. Thus, the present PASS approach can be very useful in designing drug molecules according to their properties. It would save unnecessary waste of chemicals as well as time [68]. The prediction results are presented as a list of activities with an appropriate Pa and Pi ratio. Pa and Pi are the estimates of probability for the compound to be active and inactive, respectively. Pa and Pi values are independent, and their values vary from 0 to 1. PASS result of prediction is valuable at planning of the experiment, but one should take into account some additional factors: Particular interest to some kinds of activity, desirable novelty of a substance, available facilities for experimental testing. Actually, each choice is always the compromise between the desirable novelty of studied substance and risk to obtain the negative result in testing. The more is Pa value, the less is the probability of false positives in the set of compounds selected for biological testing. For example, if one selects for testing only compounds for which a particular activity is predicted with Pa $\geq$ 0.9, the expected probability to find inactive compounds in the selected set is very low, but about 90% of active compounds are missed. If only compounds with Pa $\geq$ 0.8 are chosen, the probability to find inactive compounds is also low, but about 80% of active compounds are missed etc. By default, in PASS Pa = Pi value is chosen as a threshold, therefore, all compounds with Pa > Pi are suggested to be active. Another criterion for selection is the compounds' novelty. If Pa value is high, sometimes one may find close analogs of known biologically active substances among the tested compounds. For example, if Pa > 0.7 the chance to find the activity in experiment is high, but in some cases the compound may occur to be the close analog of known pharmaceutical agents. If 0.5 < Pa < 0.7 the chance to find the activity in experiment is less, but the compound is not so similar to known pharmaceutical agents. If Pa < 0.5 the chance to find the activity in experiment is even more less, but if it will be confirmed, more than 50% chances that this structure has not been reported with this activity and might a valuable lead compound.

Obviously, the PASS approach has some important limitations. PASS is not able to predict the activity spectrum for essentially new compounds that have no identifier in the training set. The accuracy of the PASS predictions is significantly higher than random speculations. It can be applied to the activities for which the training set will include no less than 5 active compounds per activity. PASS predicts both drugs and nondrugs actions simultaneously. Thus, only experiments can clarify the intrinsic activity of a compound, but it probably has an affinity to an appropriate receptor (enzyme).

In this study, to accelerate the search for potent new MPAOs, computer-aided drug discovery program PASS was used to predict the cognition-enhancing action for BHT derivatives from chemical series. The potential biological effects of the designed compounds were predicted based on SAR analysis of the PASS training set. Therefore, before we started the synthesis planning process, we used the PASS program to validate whether using the SAR strategy to design the compounds resulted in designs that agreed with the SAR values of the PASS database training set. It is also to evaluate the level of similarity of the designed compound to the known pharmaceutical agents. PASS training set is compiled from many sources, including publications, patents, databases, private communications, etc., Therefore, PASS predicted different synonyms of antioxidants such as lipid peroxidase inhibition, antioxidant and free radical scavenging. A portion of the predicted biological activity spectra for the synthesized compounds and BHT are given in Table 1. Probable activities generated by PASS were validated by experimental bioassay. Only compounds that were predicted by PASS to have predicted antioxidant, free radical scavenging and lipid peroxidase activities were experimentally verified by DPPH and TBARS assays.

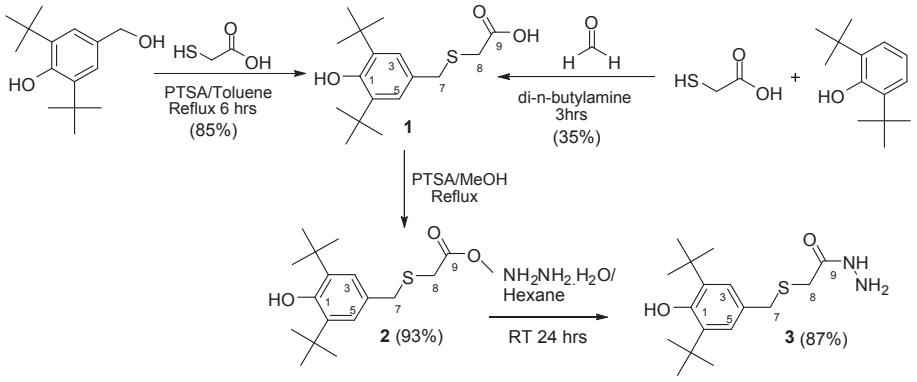
2.3. Chemistry

The preparation of the target compounds is outlined in the following Schemes 1–3. 2-(3,5-di-*tert*-butyl-4-hydroxybenzylthio) acetic acid **1** was prepared by the reaction between 3,5-di-tert-butyl-4-hydroxybenzyl alcohol with thioglycolic acid in the presence of PTSA. This new method gave compound **1** in very good yield (85%), while the established solvent-free procedure [69] gave low yield (35%) through the reaction between 2,6-di-*tert*-butyl-phenol with formaldehyde and thioglycolic acid in the presence of di-*n*-

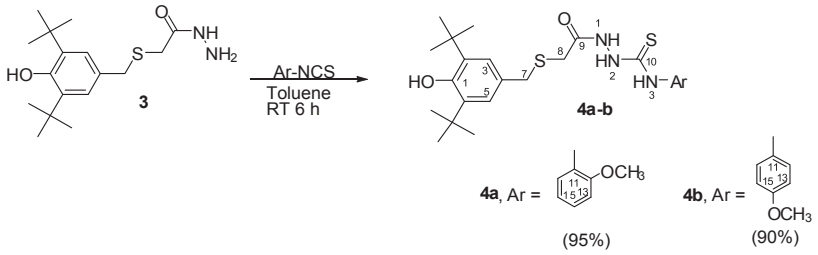
Table 1
Part of the predicted biological activity spectra of the synthesized compounds **1**, **4**–**6** and BHT on the basis of PASS prediction software.

Mode of biological activity	1		4a		4b		5a		5b		6a		6b		BHT	
	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi
Lipid peroxidase inhibitor	0.652	0.006	0.489	0.018	0.487	0.018	0.510	0.015	0.535	0.013	0.651	0.006	0.673	0.005	0.843	0.003
Antioxidant	0.712	0.004	0.437	0.025	0.466	0.022	0.471	0.021	0.512	0.017	0.573	0.011	0.589	0.010	0.845	0.003
Free radical scavenger	0.807	0.004	0.617	0.020	0.640	0.016	0.606	0.022	–	–	0.552	0.031	0.590	0.025	0.797	0.004
Prostaglandin E2 antagonist	–	–	0.351	0.010	0.357	0.008	–	–	–	–	0.229	0.104	0.380	0.039	–	–
Lipoxygenase inhibitor	–	–	0.296	0.027	0.309	0.025	0.377	0.014	–	–	0.308	0.025	0.345	0.019	–	–

Pa—probability 'to be active'; *Pi*—probability 'to be inactive'.



Scheme 1. Synthesis of acid hydrazide **3**.



Scheme 2. Synthesis of acylthiosemicarbazides **4a**–**b**.

butylamine (Scheme 1). This carboxylic acid was then esterified to give the corresponding methyl ester **2** in very good yield (93%). This ester was then converted almost quantitatively to the acid hydrazide **3** after treatment with hydrazine hydrate in the presence of hexane as a solvent at room temperature (Scheme 1).

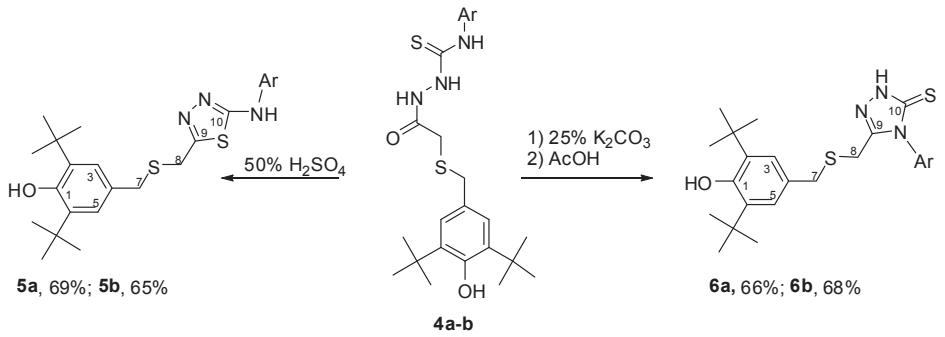
Hydrazide **3** was treated with arylisothiocyanates to give the corresponding acylthiosemicarbazides **4a**–**b** in very good yield (Scheme 2).

Compounds **4a**–**b** under acidic or basic conditions gave thiadiazoles **5a**–**b** or triazoles **6a**–**b**, respectively (Scheme 3).

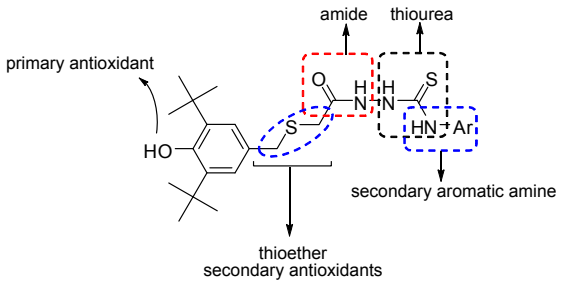
The structures of the synthesized compounds were confirmed on the basis of their physical and spectral data. The structures of compounds **4a** and **6a** were further confirmed by X-ray crystallography.

The IR spectra of all synthesized compounds showed strong absorption at 3615-3655 cm^{-1}, attributed to free ν (Ar–O–H). Acylthiosemicarbazides **4a**–**b** showed NH stretching bands between 3211 and 3293 cm^{-1}, a C=O stretching band at 1655-1713 cm^{-1}, and did not show a ν(S–H) band at 2570, while the presence of a C=S stretching band at 1247-1251 cm^{-1} indicated that **4a**–**b** exist in the thione form in the solid-state [70,71]. Compounds **6a**–**b** showed NH stretching bands at 3019-3088 cm^{-1} and ν(C=S) at 1253-1258 cm^{-1} due to the thione form. This result is in agreement with X-ray analysis showing that compounds **4a** and **6a** exist in the thione form in the solid-state.

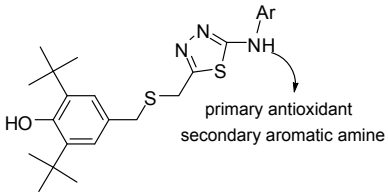
Interestingly, the ^{1}H NMR spectrum of compound **6a**, recorded in $CDCl_3$, showed that each CH_2 of the thioether system clearly resonated as an AX system, with two separate doublets at 3.18–3.49 and 3.56–3.63 ppm (Fig. 2) due to –C***H***$_2$-triazole and BHT-C***H***$_2$- with coupling constants of 16 and 12 Hz, respectively. In contrast, each CH_2 of **6b** clearly resonated as an A_2 system, showing two singlets at 3.34 and 3.64 ppm (Fig. 3) that can be attributed to –C***H***$_2$-triazole and BHT-C***H***$_2$, respectively. This indicates a germinal coupling, suggesting that the protons of each CH_2 of **6a** are magnetically non-equivalent due to restricted rotation about the C–N (Fig. 4, I, II). The ^{2}J germinal coupling constant of the methylene group neighboring the triazole ring is larger than that of the



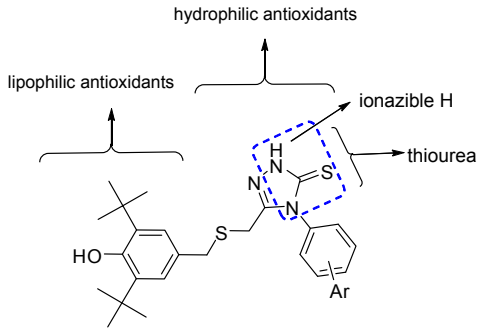
Scheme 3. Synthesis of thiadiazoles **5a**–**b** and triazoles **6a**–**b**.



4a-b



5a-b



6a-b

Fig. 2. SAR analysis of 1-acylthiosemicarbazide (**4a**–**b**), 1,3,4-thiadiazoles (**5a**–**b**) and 1,2,4-triazoles (**6a**–**b**) ionazible.

other methylene due to the HCH angle. In general, ^{2}J germinal coupling constants increase as the H–C–H angle α decreases [72].

Compounds **4a**–**b** showed a singlet peak at 7.2–8.3 ppm due to the N***H***-3 attached to the phenyl group, while the other two singlets at 7.9–8.3 ppm and 8.3–9.7 ppm were attributed to the N*H*'s of the hydrazido group. Both appeared as broad bands, which supports the formation of intramolecular hydrogen bonding [70,73].

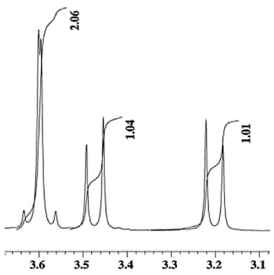


Fig. 3. Geminal H–H coupling ($^{2}J_{HH}$) of each CH_2 group of **6a**.

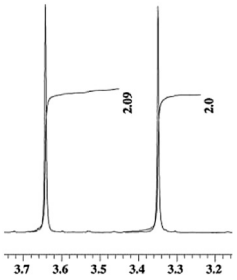


Fig. 4. Uncoupled protons with normal integrate of each CH_2 group of **6b**.

2.4. Single crystal X-ray crystallography of compounds ***4a*** and ***6a***

The crystal structure of molecule **4a** is depicted in Fig. 5 and the selected bond lengths and angles are given in Table 2. In the crystal, the molecule exists in its thione form. The two methylene carbon atoms, C15 and C16, subtend an angle of 100.14 (7)° at the S1 atom. Pairs of the molecule are connected via N1–H1 … O_2 hydrogen bonding in a bifurcated system to form centro-symmetric dimers. The hydroxyl group is shielded by the two di-*tert*-butyl residues and is therefore not involved in any hydrogen bonding.

Fig. 6 is an ORTEP diagram showing the structure of compound **6a**. The two methylene C atoms subtend an angle of 99.34(9)° at the S1 atom (C16 S1C15). The *o*-methoxy substituent is approximately coplanar with the aromatic ring as is usual in the absence of steric crowding with dehydral angle C25 O2 C24C23 0.2(3)°. The 1,2,4-triazole and o-anisol rings are, of course, nearly perpendicular to each other, making a dihedral angle of C18 N1 C19C24–90.9(7)° (Table 3). Similar to what was observed in the structure of **4a**, the hydroxyl group is not involved in any hydrogen bonding, as it is shielded by the two sterically hindered *tert*-butyl groups.

2.5. Molecular properties and drug-likeness

2.5.1. Lipophilicity

α-TOH is a fat-soluble antioxidant [74], and the distribution of α-TOH within the membrane has been shown to alter its antioxidant potency [75]. Generally, lipophilic antioxidants demonstrated more potent scavenging properties than hydrophilic antioxidants. The Partition Coefficient is a measure of how well a substance partitions between a lipid and water. It is therefore important to determine the physiochemical properties associated with a compound's antioxidant activity. The log *P* measurement is a useful parameter for understanding the behavior of antioxidant molecules. Log *P* was calculated using the computed log *P* values (where *P* is the partition coefficient of the molecule in the water–octanol system) by using ADMET predictive software, as shown in Table 4. Compounds having log $P \geq 5$ were considered to have a higher lipophilicity and higher permeation across biological membranes but lower aqueous solubility [76]. The log *P* values of the designed compounds showed moderate lipophilic properties with log *P* values between 6.217 and 6.72, while the natural lipophilic antioxidant α-TOH had a log *P* value of 10.44 and the hydrophilic antioxidant ascorbic acid (AA) had a log *P* value of −1.7.

2.5.2. Calculation of drug-likeness properties

Drug-likeness can be deduced as a delicate balance of various structural features that determine whether a particular molecule is similar to known drugs, generally meaning "molecules which contain functional groups and/or have physical properties consistent with most of the known drugs". These properties are as follows: absorption, distribution, metabolism, and excretion from the

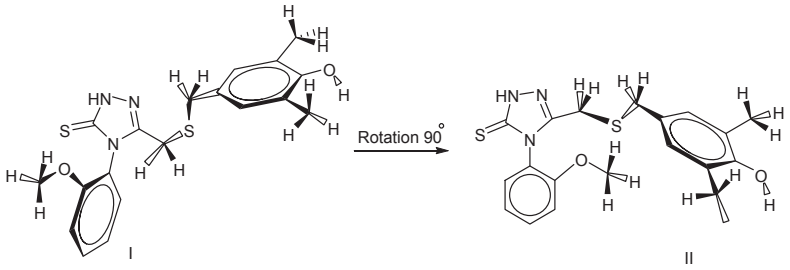


Fig. 5. Restricted internal rotation about aryl C–N bonds in an aryl substituted triazole.

Table 2
Selected bond lengths (Å), bond and torsion angles (deg) for **4a**.

S2–C18 (C=S)	1.6769(14)	C16 S1C15	100.14(7)	C15–O3–C24–C23	0.6(2)
O2–C17 (C=O)	1.3824(16)	N1–N2–C18	120.32(12)	C18N3C19C20	14.7(2)
C1–O1	1.3824(16)	C18–N3–C19	128.22(13)		
S1–C15	1.8211(14)				
S1–C16	1.8051(14)				
N1–C18	1.3557(18)				
N3–C18	1.3513(18)				
N3–C19	1.4173(18)				

Table 3
Selected bond lengths (Å), bond and torsion angles (deg) for **6a**.

C16–S1	1.820(2)	C16 S1C15	99.34(9)	C25–O2–C24–C23	0.2(3)
C16–S1	1820(2)	C24O2C25	116.26(18)	C18N1C19C24	90.9(7)
S1–C15	1.825(2)			C17N1C19C24	85.3(2)
O1–C1	1.378(2)				
N1–C19	1.429(2)				

in human body like a drug. Lipinski [77] used these molecular properties in formulating his Rule of Five. The rule states that most molecules with good membrane permeability have log $P \leq 5$, molecular weight ≤500, number of hydrogen bond acceptors ≤10 and number of hydrogen bond donors ≤5.

2.5.3. *Violations of Lipinski's rule of five*

It is important to note that there are many violations of this rule among existing drugs and vice versa. Therefore, qualifying according to the "rule of five" does not guarantee that a molecule is "drug-like" [78]. Polar surface area (PSA) is recognized as a good indicator of drug absorbance in the intestines, penetration of Caco-2 monolayers and the ability to cross the blood brain barrier [78]. The mentioned parameters were calculated for the BHT derivatives obtained in this analysis, and the results are depicted in Table 4. From the data obtained, one can notice that the synthesized compounds possess an adequate number of proton acceptor and proton donor groups to ensure efficient interaction with the hydrogen bonding groups of the receptors. Hydrogen-bonding capacity has also been identified as an important parameter for describing drug permeability [79]. All of the BHT derivatives were found to violate one of the Lipinski's parameters (log P (cLog P) > 5), though they were found to be soluble in protic solvents. The magnitude of absorption is expressed by the percentage of absorption, which was calculated using the following equation: % ABS = 109−0.345 × PSA [80]. According to their predictive low topological polar surface area (TPSA) (PSA values are considerably less than 90 A^2) and high %ABS data, it seems that these types of antioxidants could have a good capacity for penetrating cell membranes [81].

2.6. *In vitro antioxidant activities*

In the present study, the antioxidant activities of seven BHT derivatives were carried out by DPPH and TBARS, two well-known *in vitro* antioxidant assays. The effects of antioxidants in the DPPH-

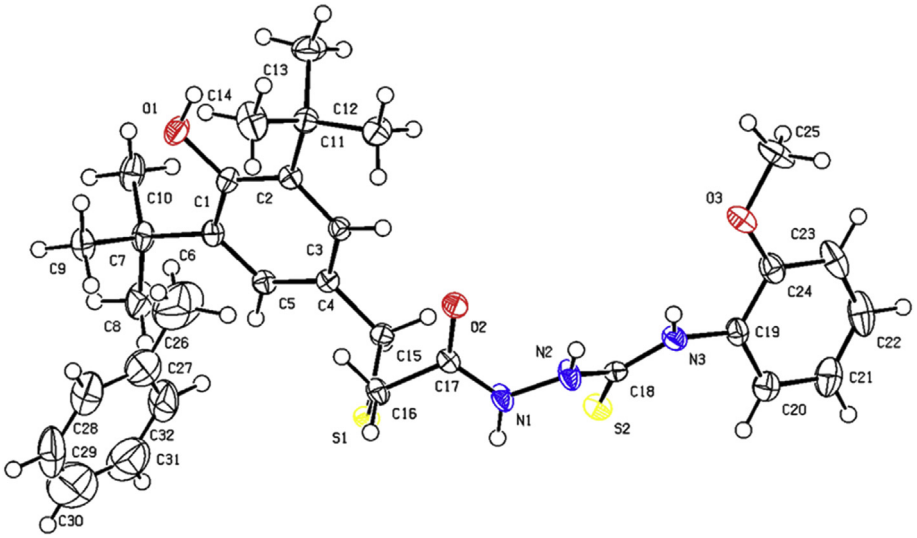


Fig. 6. ORTEP drawing of **4a**.

Table 4
Lipinski's rule of five main parameters.

Compound	Violation of rule of	HBA	HBD	Log *P*	MW	NROTB	%ABS	PSA A^2
4a	1	5	4	6.217	489.694	11	79.51	85.476
4b	1	5	4	6.217	489.694	11	79.51	85.476
5a	1	6	2	6.721	471.678	9	86.30	65.077
5b	1	6	2	6.721	471.678	9	86.30	65.077
6a	1	5	2	6.621	471.678	8	89.12	57.615
6b	1	5	2	6.621	471.678	8	89.12	57.615
BHT	0	1	1	4.875	220.35	2	100	20.815
α–TOH[a]	–	–	–	10.44	430.71	–	98.73	29.745
AA[a]	–	–	–	−1.70	176.12	–	71.23	109.492

[a] α-TOH and AA are outside the 'rule of 5' [77]; Violation of Rule of 5 (≤1); HBA–hydrogen bond acceptor (≤10); HBD–hydrogen bond donor (≤5), Log *P* (≤5); MW (≤500), NROTB (≤10); %ABS PSA–polar surface area *A*2 ≤ 90.

radical-scavenging test reflect the hydrogen-donating capacity of a compound. In its radical form, DPPH• absorbs at 570 nm. The radical form of DPPH• is scavenged by an antioxidant through the donation of a hydrogen to form a stable DPPH molecule, resulting in a color change from purple to yellow and a decrease in absorbance [82,83]. The thiobarbituric acid reactive substances (TBARS) assay was chosen for screening and monitoring lipid peroxidation. The basis of the TBARS assay is the spectrophotometric absorbance of a pink color complex at 532–535 nm, which is formed by the interaction of thiobarbituric acid and the oxidation products of unsaturated lipids [84,85].

2.6.1. *In vitro DPPH radical scavenging activity*

Most compounds tested significantly inhibited DPPH radical levels compared to the standard antioxidants (AA and BHT) used in the study (Table 5). As observed in this table, thiosemicarbazide derivatives **4a**–**b** exhibited strong scavenging effects on the DPPH stable radical, with respective IC_{50} values of 52.03 ± 1.27 and 50.93 ± 1.47 μM. These values were lower than the positive controls in the study, BHT and AA, indicating that **4a**–**b** have good radical scavenging activities.

Recently, we noted a few papers in which thiosemicarbazides and related compounds have been evaluated for their ability to scavenge free radicals and found no evidence for their antioxidant activities or mechanisms [86–89]. Canan and coworkers [90] have reported that 1-acylthiosemicarbazides are more effective as free radical scavengers than triazoles and thiadiazoles. Consequently, the higher antioxidant activity of acylthiosemicarbazides **4a**–**b** could be attributed to two factors: First, the aryl radicals generated by thermal decomposition have been reported to react with compounds containing the S=C-NHR group to give S-substituted isothiosemicarbazides (Scheme 4); and second, when *R* = Ar, better yield was obtained due to hydrogen abstraction by the aryl radical from the substituted thioamide group [90,91].

Thus, the scavenging potential of the DPPH free radical reaction could occur as proposed in Scheme 5 (**A**).

Further evidence for the proposed mechanism came from a report that described the antioxidant activities of aromatic amine derivatives [49,92] and five-membered heterocyclic amines [93]. Similar to phenolic derivatives, aromatic amines form an important class of antioxidants [94]. They are also excellent H-donors [49,95] and can easily transfer their amine hydrogen to peroxyl radicals [50]. Thus, the reactions of aromatic amines of thiosemicarbazides (**4a**–**b**) with free radicals led to hydrogen abstraction from the N–***H*** bond, neutralizing DPPH• by delocalizing the nitrogen electron pair over the aromatic system and the thione group to form a stable aminyl radical.

When the N–H BDE value is low, depending on the nature of the substituent [38], the scavenging of DPPH• by aryl thiourea is proposed to go through pathway **B** (Scheme 5) in which hydrogen radicals are extracted from the N–H of thiosemicarbazides. This pathway (**B**) showed that aryl thiourea in thiosemicarbazides is able to suppress two DPPH• radicals. Thus, DPPH was inhibited by 75% (**4a**), 76% (**4b**), 71% (AA) and 25% (BHT) (Table 5).

Further support for our proposed mechanism (Scheme 5, **A** and **B**) was obtained from the following results: phenethyl-5-bromopyridyl thiourea have thiol and thione forms, and the thiol has been shown to have antioxidative activity. Moreover, thiols and thiones found in S-alkylated derivatives (Fig. 7) have been found and evaluated, and all phenethyl-5-bromopyridyl thiourea compounds exhibited antioxidant activity (Fig. 7, I). Meanwhile, S-alkylation virtually eliminated the antioxidant activity (Fig. 7, I), indicating that an unalkylated thiourea group is critical for antioxidant activity. This result suggests that the thiol group (I) is responsible for the antioxidant activity due to it is favorable electron-donating characteristics [96]. Similar to thiol group, the reaction of aromatic amine of thiourea with free radicals led to hydrogen abstraction from the N–H bond to form a stable aminyl radical by delocalizing the nitrogen electron pair over the aromatic system.

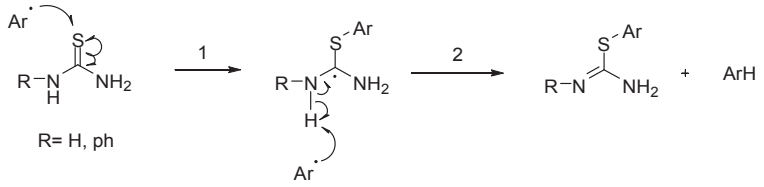
Compounds **4** and **5** have secondary aromatic amines. Similarly, compounds **4** and **6** have thione groups that play a significant role in reducing DPPH. Thus, the antioxidant activities of thiadiazoles **5** and traiazoles **6** could be attributed to the same effects mentioned above (compounds **4a**–**b**).

2.6.1.1. Substituent effects on radical scavenging ability. Table 5 shows the *p*-methoxy substituents to be more active than *o*-methoxy substituents. This result is in agreement with the literature in which phenol (or aniline) has been described to have radical scavenging activity that decreases with substitution on the *o*-position due to hydrogen bonding that can form between OH and NH

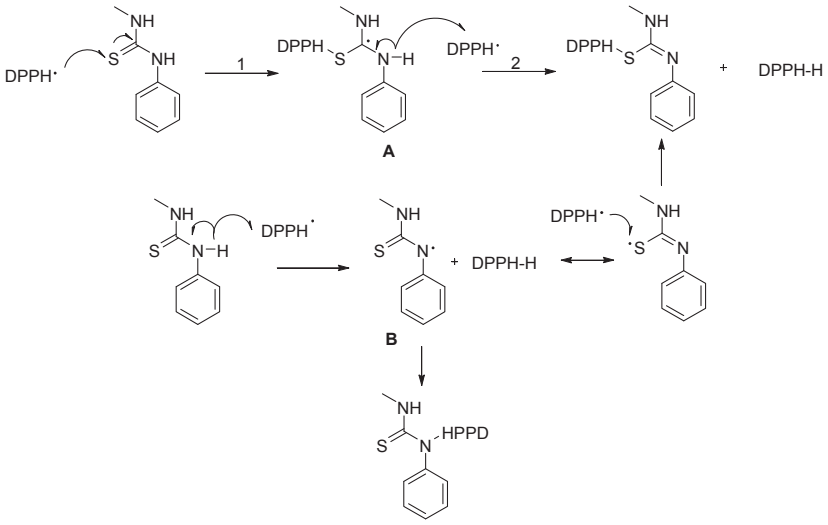
Table 5
IC_{50} values and maximum inhibition of activity at 100 μM of the DPPH radical scavenging and lipid peroxidation inhibition assays.

Compounds	IC_{50}[a] values (μM) ± S.E.M[b] and max. Inhibition % ± S.E.M	
	DPPH radical scavenging	Lipid peroxidation inhibition
1	96.73 ± 1.87 (51.250.82)	38.84 ± 1.54 (73.99 ± 1.30)
4a	52.03 ± 1.27 (75.42 ± 0.47)	60.53 ± 1.8 (94.75 ± 1.27)
4b	50.93 ± 1.47 (76.76 ± 0.74)	30.10 ± 4.07 (96.58 ± 1.45)
5a	90.77 ± 0.98 (54.69 ± 0.58)	13.17 ± 1.90 (89.18 ± 0.51)
5b	76.80 ± 0.62 (59.29 ± 0.10)	7.98 ± 1.51(89.84 ± 1.50)
6a	>100 (27.83 ± 10)	38.87 ± 2.12 (76.47 ± 0.95)
6b	92.69 ± 1.86 (52.94 ± 0.90)	2.85 ± 1.09 (86.43 ± 1.23)
BHT	>100[c] (25.23 ± 0.17)	36.67 ± 1.78 (79.45 ± 1.27)
AA	67.77 ± 0.17 (71.39 ± 1.61)	–
α–TOH	–	5.63 ± 1.09 (84.69 ± 1.23)

[a] IC_{50}: 50% effective concentration.
[b] S.E.M: *standard error* of the mean.
[c] Did *not reach 50%* inhibition.



Scheme 4. Proposed mechanism of S-arylisothiouronium base formation [91].



Scheme 5. Proposed scavenging of DPPH• by aryl thiourea.

(Fig. 8) [37]. The increase in this activity depends on the position of the substituent (para > ortho > meta) [97].

The antioxidant activity of methoxy substituents is known to increase the antioxidant activity of a mono-phenol [98]. However, the antioxidant activity of phenols reflects the differences not only in the degree of hydroxylation but also in the position of the hydroxyl groups and neighboring substituents such as methoxy groups [82]. Thus, the order of the antioxidant properties of phenol and o-, p- and m-methoxyphenols using a DPPH reagent is p-OMe > o-OMe > m-OMe > phenol [52,99].

Amorati et al. [14] found that an H-bond stabilizes phenols such that the energy needed to abstract the hydrogen atom from the hydroxyl group is larger than in non-H-bonded phenols. Similarly, the NH group of compounds **4a** and **5a** exhibited hydrogen bonding

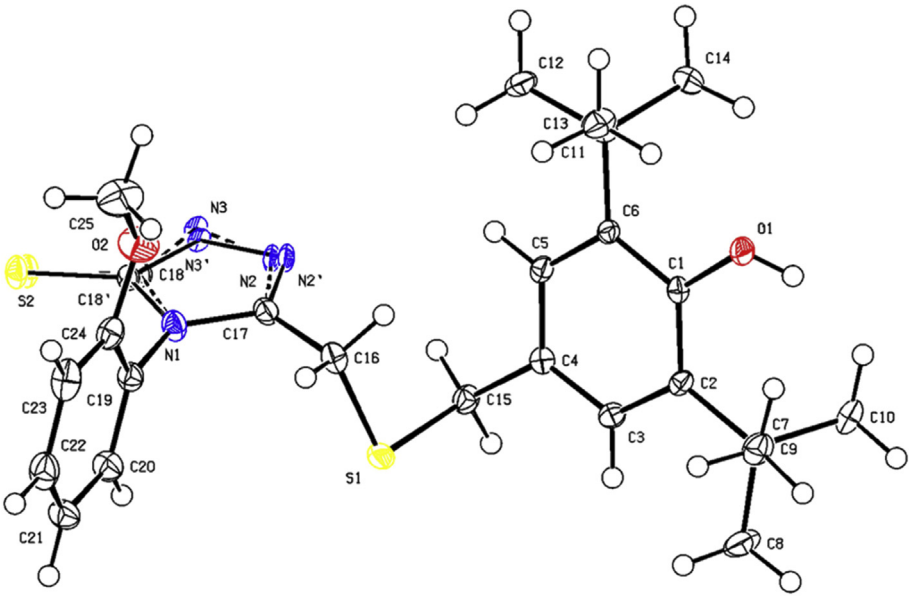


Fig. 7. ORTEP drawing of **6a**.

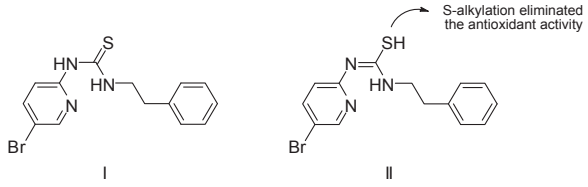


Fig. 8. Thiol-thione tautomerism and S-alkylation in phenethyl-5-bromo-pyridyl thiourea.

with the oxygen of the *o*-methoxy group (Fig. 8). This is due to the formation of a bridge between the N–H and the oxygen atom of *o*-methoxy (N–H–O), which could increase the BDE by stabilizing the amine form and sterically hindering the approach of free radicals to the N–H group. This was contrary to *p*-methoxy, found in compounds **4b** and **5b**.

There is clear evidence that the stereoelectronic effects of *p*-methoxy stabilize the aryloxyl or arylaminiyl radicals through the *p*-type lone-pair orbital on the *para* heteroatom with respect to the aromatic plane [100,101]. This observation was corroborated by the X-ray structure of compounds **4a** and **6a**, where there is clear evidence that the *o*-methoxy groups lie in the same plane as the benzene ring. Thus, the effect of the direct link between *o*-, *p*-, and *m*-methoxy substituents and phenols or aromatic amines has been evaluated, while those that are indirectly linked have not been evaluated. We attempted to further understand why compound **6b** was more active than **6a**. We anticipated that the stereoelectronic effects of the methoxyl group played an important role in determining the effectiveness of a phenolic antioxidant. Compound **6a** has been characterized by X-ray diffraction in the solid state and by ^{1}H NMR in $CDCl_3$. In the crystal structure of **6a**, the methoxy group was co-planar to the benzene ring such that the molecule was at its lowest energy level (Fig. 6). It is known that a *p*-methoxy group attached to a benzene ring prefers a planar conformation [102]. The maximum electron-donating effect occurred when the O–C bond of the methoxy group, which is co-planar with the benzene ring, overlaps the lone pair of the π-symmetry [103,104]. It is clear that the ^{1}H NMR spectrum of compound **6a** (*o*-methoxy) exhibited restricted internal rotation about the aryl C–N bond in the aryl-substituted triazole ring. The steric barriers to aryl group rotation in compound **6a** are expected to be high as there would be severe crowding between the o-methoxy phenyl and the triazole ring, particularly at the 3- and 5-positions of the triazole ring.

In contrast to compound **6a**, compound **6b** did not exhibit restricted internal rotation due to the *p*-methoxy group. The steric crowding in **6a** forced a large dihedral angle between the methoxy group and the ring, twisting the methoxy group out of the plane (Fig. 4). Thus, the electronic density effect was reduced. The absence of crowding in **6b** led to a lower dihedral angle and lowering of the electron density effect. Thus, restricted rotation could be a reason behind the changes in the position of the methoxy group. As a result the oxygen *p*-type lone-pair orbital overlapped less with the SOMO of the radical when $\theta > 0^\circ$. The maximum stabilization of the radical occurred when $\theta = 0^\circ$ and was at a minimum when $\theta = 90^\circ$, as shown in Fig. 9 [100,101,104].

2.6.2. *In vitro lipid peroxidation*

Peroxidation of lipids has been shown to be a cumulative effect of ROS, which disturbs the assembly of the membrane. This disturbance causes changes in fluidity and permeability as well as alterations in ion transport and inhibition of metabolic processes [105]. α-TOH has been demonstrated to be a potent inhibitor of lipid peroxidation in cellular membranes, preventing a one-electron oxidation from forming a tocopheryl radical that promotes the

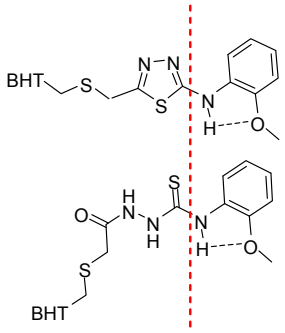


Fig. 9. Intramolecular hydrogen bond formation in compounds **4b** and **5b**.

propagation of a lipid peroxidation chain reaction [106]. α-TOH is located in membranes, while AA is located in aqueous phases due to it is low lipid solubility [11,107]. However, it is worth noting that hydrophilic scavengers of oxygen radicals located in the aqueous region cannot scavenge radicals within the lipid region of membranes. Therefore, α-TOH could be a suitable control in lipid peroxidation assays (Fig. 10).

The TBARS assay was used to measure the formation of essential oils from lipid peroxide in the lipid-rich media provided by egg yolk homogenate. A review of antioxidant assays showed the most important parameters for increasing or decreasing the inhibition of free radicals to be the following: a multiphase medium (such as an emulsion) [108], steric inaccessibility [109], and a BDE of free radicals such as BDE(ROO–H) ≈ 88 [110]. With these parameters, we expected to observe different inhibition activities between DPPH and lipid peroxidation assays.

Contrary to the DPPH results, compound **4a** exhibited the lowest lipid peroxidation activity, yielding the highest IC_{50} value of 60.53 ± 1.80 μM. Compound **4b** was observed to have a higher lipid peroxidation activity than BHT but had a low IC_{50} value of 30.10 ± 4.07 μM.

Thiadiazoles **5a** and **5b** exhibited the strongest free radical scavenging activity, reducing power and anti-lipid peroxidative activity compared with two commercial antioxidants, namely α-TOH and BHT (Table 5). This seems to suggest that thiadiazoles bearing the BHT moiety can easily donate electrons and hydrogens.

The triazole compound **6a** demonstrated moderate lipid peroxidation activity due to intramolecular hydrogen bonding at the *ortho* position (Fig. 8). Table 5 shows that *p*-methoxy substituents were more active than *o*-methoxy substituents. This result is similar to our findings with the DPPH assay, which can be attributed to the stereoelectronic effect [100], intramolecular hydrogen bonding [37] and presumably intramolecular lipophilicity effects [11]. In support of our result, an antioxidant has to be highly active at a low concentration on the surface of the fat or oil phase. α-TOH and BHT are known to be strongly lipophilic antioxidants due to

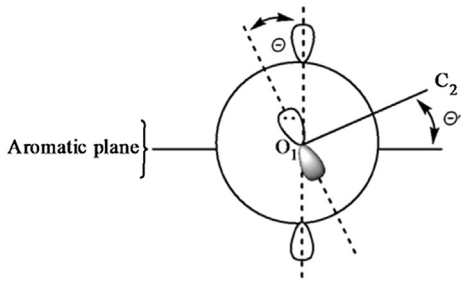


Fig. 10. Interpreting the stereoelectronic effect of the dihedral angle.

their long hydrocarbon chains and di-*tert*-butyl groups that are similar to fatty acid tails and therefore able to reach a higher level of bioavailability [111]. Presumably, the significantly greater value of lipid peroxidation could be attributed to the balance between the hydrophilicity of the polar moieties and the lipophilicity of the hydrocarbon moieties. Thus, the stronger inhibitory activity of compound **6b** (IC_{50} 2.85 ± 1.09 μM; inhibition = 86.43%) could be attributed to both hydrophilic and lipophilic effects on emulsified oils [112,113]. In the emulsified medium used in the TBARS assay, the non-polar free radical scavengers accumulated in the lipid phase and at the oil-water interface, where interactions between hydroperoxides at the droplet surface and pro-oxidants in the aqueous phase occur. Thus, the log*P* measurement (where *P* is the partition coefficient of the molecule in the water–octanol system) shown in Table 4 is a useful parameter for understanding the behavior of antioxidant molecules. In calculating log *P*, we used computed log *P* values predicted with ADMET software. Calculations for **5b** and **6b** showed **6b** (log *P* = 6.62) to be slight more polar than **5b** (log *P* = 6.72) due to the thiol–thione tautomerism effect. This suggested that compound **6b** may have hydrophilic and lipophilic groups that may act as amphiphilic antioxidants in one molecule rather than requiring the separate use of two antioxidants. Thus, the inhibition of lipid peroxidation by **6b** suggested that this compound could be a possible candidate with promising antioxidant activity.

3. Conclusion

The PASS-assisted design strategy for improving the antioxidant activity of BHT has been successfully applied. The results of PASS indicated that the most probable activities are lipid peroxidation inhibition, antioxidant, scavenging of free radicals and anti-inflammatory effects. This strategy prevents the unnecessary waste of chemicals and saves time, thus allowing the present approach to be very useful in designing drug molecules according to their required properties without undesirable side effects. This makes the use of PASS-assisted design generally important. Using PASS, we improved the free radical scavenging capacity of BHT inhibition (25%) by more than two-fold in most compounds. The DPPH and lipid peroxidation assays of the tested compounds showed that *para* substituents possessed higher antioxidant activities than *ortho* substituents. The ^{1}H NMR spectrum helped us to understand the relationship between restricted rotation of *o*-methoxy substituents and the effect of the electronic density, which could increase or decrease antioxidant activities. The synthesized compounds **4**, **5** and **6** satisfied Lipinski's RO5 and ADMET properties. RO5 and ADMET predictions can be important initial steps toward the development of novel pharmaceuticals in the fight against free radicals. Compounds **5** and **6** have the exact same molecular weight with different polarity and antioxidant activity, and therefore, this wide range of properties could help us to solve problems in the pharmacological sciences. Compounds **5b** and **6b** may be possible candidates for PASS-ADMET-assisted design strategies. These interesting findings encouraged us to consider the BHT moiety as a building block for new synthetic antioxidant projects.

4. Experimental section

4.1. General

All materials and solvents were obtained from Sigma–Aldrich. Melting points were determined on a MEL-TEMP II melting point instrument. IR spectra were recorded on a Perkin–Elmer RX1 FT-IR spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz FT-NMR using $CDCl_3$ or DMSO-d_6 as a solvent and tetramethylsilane as an internal standard. The abbreviations *s* = singlet, *d* = doublet, *t* = triplet, *q* = quadruplet, *m* = multiplet and bs = broad signal were used throughout. HR-mass spectra (ESI) were obtained with a MAT 95 xl-T mass spectrometer at 70 ev. UV–visible spectra were recorded on a UV-1650PC model UV–visible spectrophotometer.

The Supplementary Data section reports the synthesis and physicochemical characterization of S-(3,5-di-tert-butyl-4-hydroxybenzyl)thioglycolic acid (**1**), methyl-S-(3,5-di-tert-butyl-4-hydroxybenzyl)thioglycolate (**2**), and S-(3,5-di-tert-butyl-4-hydroxybenzyl)thioglycolic acid hydrazide (**3**) and the synthesis of 2-(2-(3, 5-di-tert-butyl-4-hydroxybenzylthio)acetyl)-N-(substituted phenyl) hydrazinecarbothioamide (**4a**–**b**), 2,6-di-tert-butyl-4-(((5-(substituted phenylamino)-1,3,4-thiadiazol-2-yl) methylthio)methyl)phenol (**5a**–**b**) and 3-((3,5-Di-tert-butyl-4-hydroxybenzylthio)methyl)-4-(substitutedphenyl)-1H-1,2,4-triazole-5(4H)-thione (**6a**–**b**). The general synthetic procedures and one example of a compound are given below.

4.2. General procedure for the synthesis of 2-(2-(3, 5-di-tert-butyl-4-hydroxybenzylthio)acetyl)-N-(substituted phenyl) hydrazinecarbothioamide (*4*)

To a solution of S-(3,5-di-*tert*-butyl-4-hydroxybenzyl)thioglycolic acid hydrazide (**3**; 0.45 g, 1.39 mmol), dry toluene or benzene (5 ml) was added isothiocyanate (1.39 mmol), and the reaction mixture was stirred under nitrogen gas for 2 h at rt. The precipitate was collected by filtration, washed with boiled hexane, dried at rt, and recrystallized using the appropriate solvent.

4.2.1. 2-(2-(3,5-Di-tert-butyl-4-hydroxybenzylthio)acetyl)-N-(2-methoxyphenyl)hydrazine carbothioamide (*4a*)

2-(2-(3,5-Di-tert-butyl-4-hydroxybenzylthio)acetyl)-N-(2-methoxyphenyl)hydrazine carbothioamide (**4a**) was recrystallized from toluene to give 0.64 g of a colorless crystal (95%). Mp 119–121 °C. IR (KBr pellet), cm^{-1}: ν = 3615 (free OH), 3211–3293 (NH), 2880–2952 (C–H of t-Bu and $-OCH_3$), 1655 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz), δ, ppm: 1.40 (s, 18H, 2 × t-Bu), 3.22 (s, 2H, H-8), 3.80 (s, 3H, $-OCH_3$), 3.82 (s, 2H, H-7), 5.19 (s, 1H, OH), 6.91 (d, 1H, H-16, ^{3}J = 8 Hz), 6.98 (*t*, 1H, H-15, ^{3}J = 8, ^{3}J = 8 Hz), 7.10 (s, 2H, H-3, H-5), 7.16 (dt, 1H, H-14, ^{3}J = 8.8, ^{3}J = 8, ^{4}J = 2.4 Hz), 7.92 (d, 1H, H-13, ^{3}J = 6.4 Hz), 8.32 (s, 1H, NH-3), 9.26 (b, 1H, NH-2), 9.84 (b, 1H, NH-1). ^{13}C NMR ($CDCl_3$, 100 MHz), δ, ppm: 30.31 (6C, 2 × $-C(\mathbf{C}H_3)_3$), 33.76 (1C, C-8), 34.42 (2C, 2 × $-\mathbf{C}(CH_3)_3$), 37.71 (1C, C-7), 55.90 (1C, $-O\mathbf{C}H_3$), 111.42 (1C, C-16), 120.96 (1C, C-15), 124.07 (1C, C-13), 125.39 (1C, C-4), 125.99 (2C,C-3, C-5), 126.11 (1C, C-11), 129.13 (1C, C-14), 136.40 (2C, C-2, C-6), 151.26 (1C, C-12), 153.34 (1C, C-1), 165.22 (1C, C-9), 177.21 (1C, 10). HREIMS m/z 489.2108 $[M]^+$ (calcd for $C_{25}H_{35}O_3N_3S_2$ 489.2120).

4.3. General procedure for the synthesis of 2,6-di-tert-butyl-4-(((5-(substituted phenylamino)-1,3,4-thiadiazol- 2-yl)methylthio) methyl)phenol (*5*)

Thiosemicarbazide (**4a**–**b**) (0.50 mmol) was added gradually under stirring to cold sulfuric acid (50%, 5 ml) in 10 min. The reaction mixture was heated for 20 min at 100 °C. It was then poured over crushed ice under stirring. After 1 h, the precipitate was filtered, washed with distilled water, dried at rt, and recrystallized using the appropriate solvent.

4.3.1. 2,6-Di-tert-butyl-4-(((5-(2-methoxyphenylamino)-1,3,4-thiadiazol-2-yl) methylthio)methyl)phenol (**5a**)

2,6-Di-tert-butyl-4-(((5-(2-methoxyphenylamino)-1,3,4-thiadiazol-2-yl) methylthio)methyl)phenol (**5a**) was recrystallized from MeOH 4:1H_2O to give 0.17 g of a white solid (72%). Mp 113–115 °C. IR (KBr pellet), cm^{-1}: ν = 3614 (free OH), 3210–3293 (NH), 2881–2953 (C–H of t-Bu and $-OCH_3$), 1655, 1604 (2C=N). ^{1}H NMR ($CDCl_3$, 400 MHz), δ, ppm: 1.42 (s, 18H, 2 × t-Bu), 3.69 (s, 2H, H-7), 3.89 (s, 2H, H-8), 3.90 (s, 3H, $-OCH_3$), 5.14 (s, 1H, OH), 6.89–6.91 (dd, 1H, H-16, ^3J = 7.2, ^4J = 1.2 Hz), 6.97–7.05 (m, 2H, H-14, H-15) 7.07 (s, 2H, H-3, H-5), 7.11–7.17 (b, 1H, NH), 7.78–7.80 (dd, 1H, H-13, ^3J = 8.8, ^4J = 1.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz), δ, ppm: 30.08 (1C, C-8), 30.36 (6C, 2 × $-C(\mathbf{C}H_3)_3$), 34.42 (2C, 2 × $-\mathbf{C}(CH_3)_3$), 36.58 (1C, C-7), 55.87 (1C, $-O\mathbf{C}H_3$), 110.43 (1C, C-16), 116.60 (1C, C-13), 121.25 (1C, C-15), 123.02 (1C, C-14), 125.99 (2C, C-3, C-5), 127.61 (1C, C-4), 129.53 (1C, C-11), 136.14 (2C, C-2, C-6), 147.89 (1C, C-12), 153.14 (1C, C-1), 160.13 (1C, C-9), 165.92 (1C, C-10). HREIMS m/z 471.2034 $[M]^+$ (calcd for $C_{25}H_{33}O_2N_3S_2$ 471.2014).

4.4. General procedure for the synthesis of 3-((3,5-di-tert-butyl-4-hydroxybenzylthio)methyl)-4-(substituted phenyl)-1H-1,2,4-triazole-5(4H)-thione (**6**)

A mixture of thiosemicarbazide (**4a**–**b**; 0.50 mmol) and potassium carbonate (25%, 5 ml) was stirred for 18 h. Then, 250 ml water was added with stirring for 1 h. The solution was adjusted to pH (5–6) with diluted hydrochloric acid and was kept aside for 1 h. A white precipitate was filtered, washed with water, dried and recrystallized using the appropriate solvent.

4.4.1. 2,6-Di-tert-butyl-4-(((5-mercapto-4-(2-methoxyphenyl)-4H-1,2,4-triazol-3-yl)methylthio)methyl)phenol (**6a**)

2,6-Di-tert-butyl-4-(((5-mercapto-4-(2-methoxyphenyl)-4H-1,2,4-triazol-3-yl)methylthio)methyl)phenol (**6a**) was recrystallized from toluene 3:1 hexane to give 0.15 g of a colorless crystal (66%). Mp 150–152 °C. IR (KBr pellet), cm^{-1}: ν = 3589 (free OH), 3039, 3101 (NH), 2870–2957 (C–H of t-Bu), 1603 (C=N), 1256 (C=S). ^{1}H NMR ($CDCl_3$, 400 MHz), δ, ppm: 1.41 (s, 18H, 2 × t-Bu), 3.18, 3.22 (d, $1H_a$, H-8, $^2J_{HaHb\text{-geminal}}$ = 16 Hz), 3.45, 3.49 (d, $1H_b$, H-8, $^2J_{HbHa\text{-geminal}}$ = 16 Hz), 3.56, 3.59 (d, $1H_a$, H-7, $^2J_{HaHb\text{-geminal}}$ = 12 Hz), 3.60, 3.63 (d, $1H_b$, H-7, $^2J_{HbHa\text{-geminal}}$ = 12 Hz), 3.79 (s, 3H, $-OCH_3$), 5.16 (s, 1H, OH), 7.06–7.07 (m, 3H, H-3, H-5 and 1H of H-16), 7.11 (*t*, 1H, H-15, ^3J = 8, ^3J = 8 Hz), 7.36 (d, 1H, H-14, ^3J = 8 Hz), 7.49 (m, 1H, H-13), 11.93 (s, 1H, NH). ^{13}C NMR ($CDCl_3$, 100 MHz): δ, ppm: 24.92 (1C, C-8), 30.37 (6C, 2 × $-C(\mathbf{C}H_3)_3$), 34.41 (2C, 2 × $-C(CH_3)_3$), 36.31 (1C, C-7), 56.06 (1C, $-O\mathbf{C}H_3$), 112.60 (1C, C-16), 121.29 (1C, C-15), 121.55 (1C, C-4), 126.04 (2C, C-3, C-5), 127.29 (1C, C-11), 130.57 (1C, C-14), 131.95 (1C, C-13), 136.06 (2C, C-2, C-6), 151.07 (1C, C-9), 153.12 (1C, **C**-1), 154.69 (1C, C-12), 169.21 (1C, C-10). HREIMS m/z 471.2011 $[M]^+$ (calcd for $C_{25}H_{33}O_2N_3S_2$ 471.2014).

4.5. X-ray crystallography

Diffraction data were measured using a Bruker SMART Apex II CCD area-detector diffractometer (graphite-monochromated Mo K radiation, = 0.71073 Å). The orientation matrix, unit cell refinement and data reduction were all handled by the Apex2 software (SAINT integration, SADABS absorption correction) [114]. The structures were solved using direct methods in the program SHELXS-97 [115] and were refined by the full matrix least-squares method on *F2* with SHELXL-97. Drawings of the molecules were produced with XSEED [116].

4.6. Antioxidant assay

4.6.1. Materials and methods

4.6.1.1. DPPH free radical scavenging assay. The DPPH radical scavenging assay was carried out according to the literature [117] with some modifications. Briefly, 1.0 ml of DPPH solution (200 μM in DMSO) was added to a range of various sample concentrations (100, 10, 1, 0.1 and 0.01 μM). Then, 22.03–47.76 mg (1×10^{-4} M) of the test compound was dissolved in 1.0 ml DMSO (100%) as a stock solution. This stock solution was then diluted to a range of final extraction concentrations of 100, 10, 1, 0.1 and 0.01 μM. As a negative control, the same DPPH concentration in DMSO without sample was used. Each assay was carried out in triplicate. The mixture was then incubated in the dark for 60 min at room temperature. Absorbance at 570 nm for each sample was then measured. AA was used as a positive control. The free radical scavenging activity of the compounds was calculated as a percentage of radical inhibition using the following formula:

$$\text{Percentage of Inhibition}(\%) = [(A_c - A_s)/A_c] \times 100,$$

where A_s = Absorbance of the compounds/positive control and A_c = Absorbance of control (DPPH solution and DMSO). To determine the concentration required to achieve 50% inhibition (IC50) of the DPPH radical, the percentage of DPPH inhibition for each compound was plotted against the extract concentration.

4.6.1.2. Lipid peroxidation inhibition assay. The lipid peroxidation inhibition assay was carried out according to the reported method with some modifications [118]. Fowl egg yolk composed mainly of phospholipids, proteins and triacylglycerol, was used as an alternative to rat liver microsomes and linoleic acid. The reactive mixture for the induction of lipid peroxidation included 1.0 ml egg yolk emulsified with phosphate buffer saline (0.1 M, pH 7.4) to a final concentration of 12.5 g/l and 200 μl of 3000 μM $FeSO_4$. Next, 22.03–47.76 mg (1×10^{-4} M) of test compound was dissolved in 1.0 ml DMSO (100%) as a stock solution. This stock solution was then diluted to a range of final extraction concentrations of 100, 10, 1, 0.1, 0.01 and 0.001 μM. Each assay was carried out in triplicate. The mixture was incubated at 37 °C for 1 h and was then treated with 0.5 ml freshly prepared TCA (15%) and 1.0 ml of TBA (1%). The reaction mixtures were incubated in boiling water for 10 min. Upon cooling, the mixtures were centrifuged at 3500 rpm for 10 min. The formation of TBARS was measured by removing 100 μl of supernatant and measuring the absorbance at 532 nm, using α-TOH as a positive control. The percentage of inhibition was calculated from the following equation:

$$\%\ \text{inhibition} = [A_s/A_c] \times 100,$$

where A_s = Absorbance with compound and A_c = Absorbance of control. To determine the concentration required to achieve 50% inhibition (IC_{50}) of phospholipid oxidation in egg yolk, the percentage of lipid peroxidation inhibition was plotted against the extract concentration.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.10.001.

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