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ORIGINAL PAPER

Free-Radical-Scavenging Effect of Carbazole Derivatives on DPPH and ABTS Radicals

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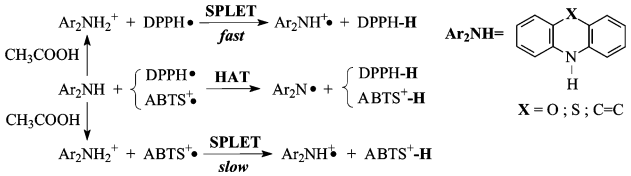
Abstract The major objective was to measure the trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) of carbazole derivatives (Ar_2NHs) by means of scavenging 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{+\cdot}$). The Ar_2NHs included phenoxazine (PozNH), phenothiazine (PtzNH), iminostilbene (IsbNH) and diphenylamine (DpaNH), and the TEAC of trolox, α-tocopherol (TocH), L-ascorbic acid (VC) and L-ascorbyl-6-laurate (VC-12) were measured as well. The TEAC results revealed that the ability to scavenge DPPH (PozNH > IsbNH ∼ PtzNH ∼ TroH ∼ TocH ∼ VC ∼ VC12), differed from the ability to scavenge $ABTS^{+}$ (PtzNH > IsbNH > PozNH > DpaNH ∼ TroH ∼ TocH ∼ VC ∼ VC12). CazNH did not react with DPPH and $ABTS^{+\cdot}$. Furthermore, the addition of acetic acid accelerated the reaction rate of Ar_2NH to scavenge DPPH, suggesting that a sequential proton loss electron transfer (SPLET) mechanism occurred with amine-type antioxidants during the trapping of DPPH. In contrast, the addition of acetic acid or pyridine reduced the reaction rate of Ar_2NH to scavenge $ABTS^{+\cdot}$, suggesting that the hydrogen atom transfer (HAT) mechanism is the basis for the reaction that is occurring.



Keywords ABTS · Antioxidant · Carbazole · Diphenylamine · DPPH · Iminostilbene · Phenothiazine · Phenoxazine

Abbreviations

$ABTS^{+\cdot}$	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation
Ar_2NH	Carbazole derivative
CazNH	Carbazole
DpaNH	Diphenylamine
DPPH	2,2′-Diphenyl-1-picrylhydrazyl
IsbNH	Iminostilbene
PozNH	Phenoxazine
PtzNH	Phenothiazine
TocH	α-Tocopherol
TroH	Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)
VC	L-Ascorbic acid
VC-12	L-Ascorbyl-6-laurate

Introduction

The correlation of certain diseases with lipid oxidation in vivo induced by free-radical formation has increased scientific interest in antioxidants that can protect against free-radical-induced damage by scavenging deleterious

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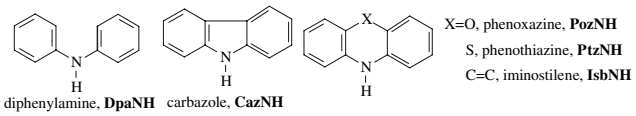
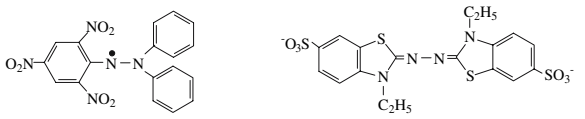
radicals [1]. Certain compounds containing N–H groups, especially the tricyclic aromatic amines (Ar_2NHs), such as carbazole (CazNH) and phenothiazine (PtzNH) have attracted recent research interest because of the neuroleptic and antihistaminic properties of Ar_2NHs are often used in medicinal chemistry [2–6]. PtzNH can inhibit the autoxidation of methyl linoleate, and phenoxazine (PozNH) can retard lipid peroxidation in the rat brain [7]. These facts suggest a relationship between the N–H bond in Ar_2NH and its radical-scavenging activities. The peroxidative kinetics of styrene induced by an alkoxyl radical and inhibited by Ar_2NHs has also been discussed in detail [2]. This information provided the motivation to evaluate the free-radical-scavenging activities of the following Ar_2NHs.



diphenylamine, **DpaNH** carbazole, **CazNH**

A convenient way to evaluate the antioxidative capacity is to react the antioxidant with 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and the free radical cation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{+\cdot}$) [8–13].



2,2'-diphenyl-1-picrylhydrazyl, **DPPH** 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate), **ABTS**

The interaction between certain antioxidants and $ABTS^{+\cdot}$ revealed the radical-scavenging ability of the hydroxyl groups attaching to the aromatic rings [14]. The interaction between the antioxidant and DPPH was attributed to the competition between two mechanisms, hydrogen atom transfer (HAT) and sequential proton loss electron transfer (SPLET) [15, 16]. The HAT and SPLET mechanisms was also attributed to the antioxidant α-tocopherol (TocH) [17]. Whether the SPLET and HAT mechanism found in hydroxyl-type antioxidants can be extended to amine-type antioxidants, and whether these mechanisms can be demonstrated in an $ABTS^{+\cdot}$ reaction system were the major concern of this work. In this study, the antioxidative properties of selected Ar_2NHs and some classical hydroxyl-type antioxidants (ArOHs) including L-ascorbic acid (VC) and trolox (TroH), and their lipophilic structural analogues involving TocH and L-ascorbyl-6-laurate (VC-12) [18, 19] were evaluated and compared for their efficacy in scavenging DPPH and $ABTS^{+\cdot}$. The reaction is illustrated by Eq. 1

$$Ar_2NH(ArOH) + DPPH^{\cdot}(ABTS^{+\cdot}) \rightarrow Ar_2N^{\cdot}(ArO^{\cdot}) + DPPH{-}\mathbf{H}(ABTS^{+}{-}\mathbf{H}). \quad (1)$$

Experimental Section

Carbazole and its derivatives, i.e., phenoxazine, phenothiazine, iminostilbene and diphenylamine, as well as trolox and α-tocopherol, were purchased from ACROS. The diammonium salt of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Fluka. L-Ascorbic acid (VC), ethanol, acetic acid and pyridine were from the Beijing Chemical Ltd Co., China, they were of analytical grade and used as received. L-Ascorbyl-6-laurate (VC-12) was synthesized according to the literature [18, 19].

The concentration of DPPH and $ABTS^{+\cdot}$ remaining after reaction with the antioxidants was determined according to previously published methods [8–17]. In brief, DPPH was dissolved in ethanol ([DPPH] ~100 μM) in sufficient concentration to produce a solution with an absorbance of ~1.00 at 517 nm (Abs_{ref}). The procedure to prepare the $ABTS^{+\cdot}$ stock solution was modified slightly. Sufficient amounts of the diammonium salts of ABTS and $K_2S_2O_8$ were dissolved in 2.00 mL water to achieve concentrations of 4.00 and 1.41 mM, respectively. This solution was kept in the dark for at least 16 h to form $ABTS^{+\cdot}$, then diluted to 100 mL with ethanol so that the solution had an absorbance or Abs_{ref} of ~0.70 at 734 nm. Various concentrations of Ar_2NHs were added to DPPH or $ABTS^{+\cdot}$ solution at ambient temperature to reach a stable absorbance (Abs_{detect}). Then, the percentage of DPPH (or $ABTS^{+\cdot}$) scavenged was calculated using Eq. 2

$$\text{Scavenging DPPH (or ABTS}^{+\cdot}\text{) (\%)} = (1 - Abs_{detect}/Abs_{ref}) \times 100. \quad (2)$$

The percentage of scavenged DPPH or $ABTS^{+\cdot}$ was plotted versus the concentration of antioxidants and the concentration of antioxidant required to obtain 50% inhibition (*50% inhibition concentration* or IC_{50}) was obtained from the graph.

Results and Discussion

Scavenging DPPH and $ABTS^{+\cdot}$ by ArOHs

The antioxidant capacity is described quantitatively by the concentration of antioxidant needed to scavenge 50% of either DPPH or $ABTS^{+\cdot}$ which is referred to as the IC_{50}. The IC_{50} or *50% inhibition concentration* (IC_{50}) was obtained from the graph of the percentage of scavenged radical versus the concentration of antioxidant [20, 21]. Figure 1 outlined the influence of the ArOH concentrations on the percentages of scavenged DPPH and $ABTS^{+\cdot}$. From

the dashed line in Fig. 1, the IC_{50} for TroH, TocH, VC and VC-12 can be determined (Table 1).

The Trolox equivalent antioxidant capacity (TEAC), the ratio between the IC_{50} of TroH and the IC_{50} for the antioxidant in question for scavenging the same radical, is often used to compare antioxidant capacities (Table 1) [22, 23]. A TEAC close to 1.00 indicated that the antioxidant had a scavenging activity for DPPH and $ABTS^{+\cdot}$ similar to that of TroH.

Scavenging DPPH and $ABTS^{+\cdot}$ by Ar_2NHs

As seen in Fig. 2, all the Ar_2NHs, except DpaNH and CazNH, can scavenge DPPH effectively in ethanol (panel A), a mixture of ethanol and acetic acid (panel B), or in pyridine (panel C).

The TEAC values shown in Table 1 indicate that the antioxidant capacities of PtzNH (0.90) and IsbNH (1.01) are approximately equal to TroH. The TEAC value for PozNH (1.74) indicated that PozNH is much more effective at trapping DPPH, than PtzNH and IsbNH. Hence, the relative antioxidant capacities were PozNH > IsbNH ~ PtzNH ~TroH for scavenging DPPH.

Figure 3 shows the relationship between the percentage of scavenged $ABTS^{+\cdot}$ and the concentration of Ar_2NHs. The TEAC values for PozNH, PtzNH and IsbNH (>1.00) shown in Table 1 indicate that the antioxidant capacities of Ar_2NHs were greater than for TroH. In particular, the antioxidant activity for DpaNH (TEAC = 0.93) was less than TroH upon reaction with $ABTS^{+\cdot}$. Therefore, the relative antioxidant activities are PtzNH > IsbNH > PozNH > DpaNH ~TroH for the reaction with $ABTS^{+\cdot}$, which is different than the rates for scavenging DPPH.

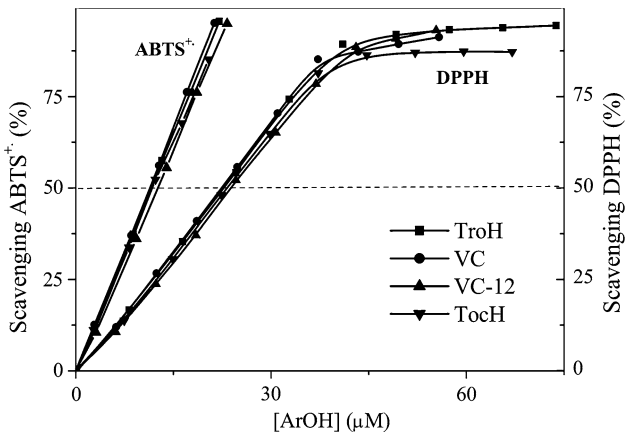


Fig. 1 The determination of IC_{50} of ArOHs, in which the *left lines* indicated the scavenging ABTS+., whereas the *right curves* exhibited the scavenging DPPH

Table 1 The IC_{50} and trolox equivalent antioxidant capacity (TEAC) of Ar_2NHs and ArOHs in scavenging DPPH and $ABTS^{+\cdot}$

Antioxidant	IC_{50} (μM)		
	In C_2H_5OH	In 130 mM CH_3COOH/C_2H_5OH solution	In 130 mM pyridine/C_2H_5OH solution
In scavenging DPPH			
TroH	22.82 (1.00)		
TocH	23.30 (0.98)		
VC	22.35 (1.02)		
VC-12	23.66 (0.96)		
PozNH	13.14 (1.74)	14.06 (1.62)	16.24 (1.41)
PtzNH	25.48 (0.90)	7.57 (3.01)	19.30 (1.18)
IsbNH	22.55 (1.01)	16.98 (1.34)	18.57 (1.23)
DpaNH	–	–	–
CazNH	–	–	–
In scavenging $ABTS^{+\cdot}$			
TroH	11.61 (1.00)		
TocH	11.61 (1.00)		
VC	11.39 (1.02)		
VC-12	12.63 (0.92)		
PozNH	8.88 (1.31)	6.56 (1.77)	4.52 (2.57)
PtzNH	5.72 (2.03)	4.34 (2.68)	3.88 (2.99)
IsbNH	6.50 (1.79)	7.64 (1.52)	8.76 (1.33)
DpaNH	12.47 (0.93)	–	8.49 (1.37)
CazNH	–	–	–

Data in parentheses were the TEAC = (IC_{50} of TroH)/(IC_{50} of other antioxidants)

– Indicated that no reaction took place

The Mechanism of Ar_2NHs to Scavenge DPPH and $ABTS^{+\cdot}$

The interaction between an antioxidant and $ABTS^{+\cdot}$ is an indication of the ability of the hydroxyl functional group in the AO to react with or scavenge free radicals [14]. The N–H bond in Ar_2NHs can also react with $ABTS^{+\cdot}$. In addition, the trapping of $ABTS^{+\cdot}$ by hydroxyl-type antioxidants can be regarded as a HAT from O–H to $ABTS^{+\cdot}$. Therefore, the reaction mechanism is best explained by the HAT mechanism, where the reaction rate, k^{HAT}, is related to the Abraham's parameters (α_2^H and β_2^H) as shown as Eq. 3, in which k^0 stands for the reaction rate for the reaction that would occur in nonpolar media [17]

$$\log k^{HAT} = \log k^0 - 8.3\,\alpha_2^H\,\beta_2^H. \quad (3)$$

The α_2^H and β_2^H parameters reflect the hydrogen-bond-donating and hydrogen-bond-accepting ability of a solvent. Although only a few α_2^H and β_2^H values have been determined, the positive values of α_2^H and β_2^H in Table 2 indicate the rate of the reaction between $ABTS^{+\cdot}$ and an antioxidant

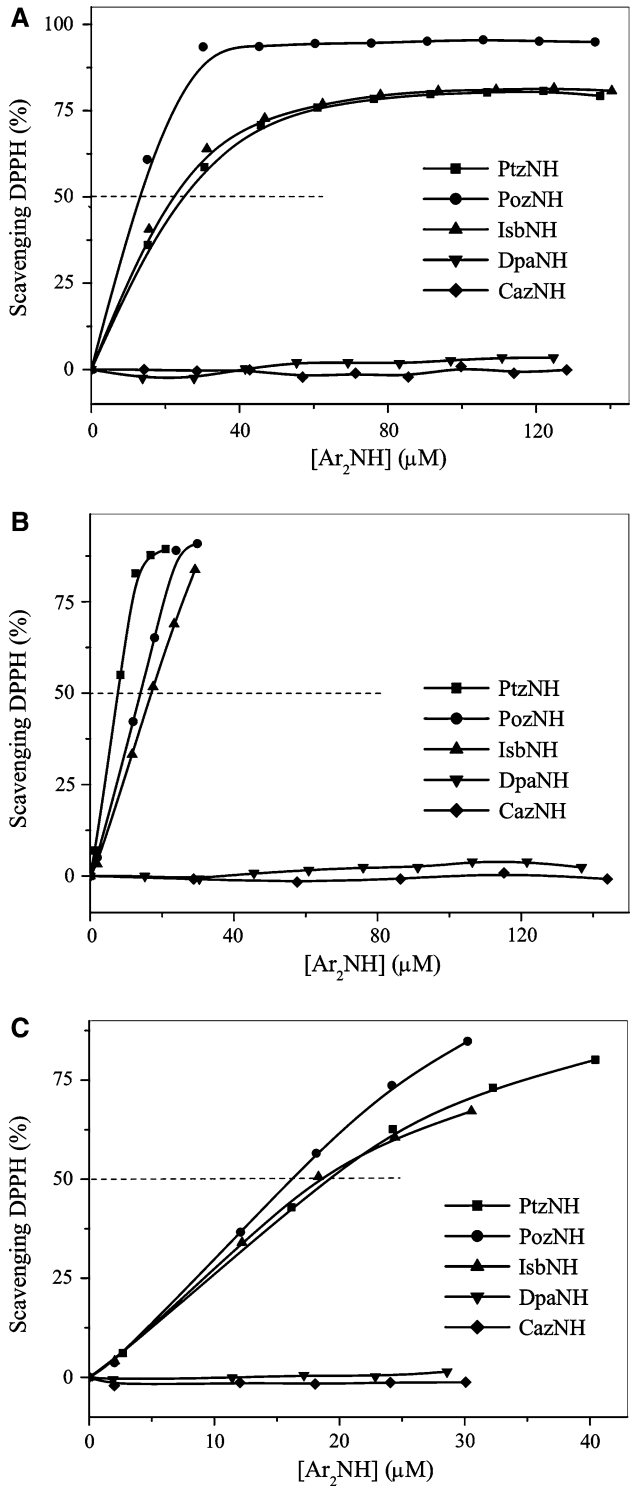


Fig. 2 The determination of IC_{50} of Ar_2NHs in scavenging DPPH, in which the reaction media was C_2H_5OH (**a**), 130 mM CH_3COOH/C_2H_5OH solution (**b**), and 130 mM pyridine/C_2H_5OH solution (**c**), respectively

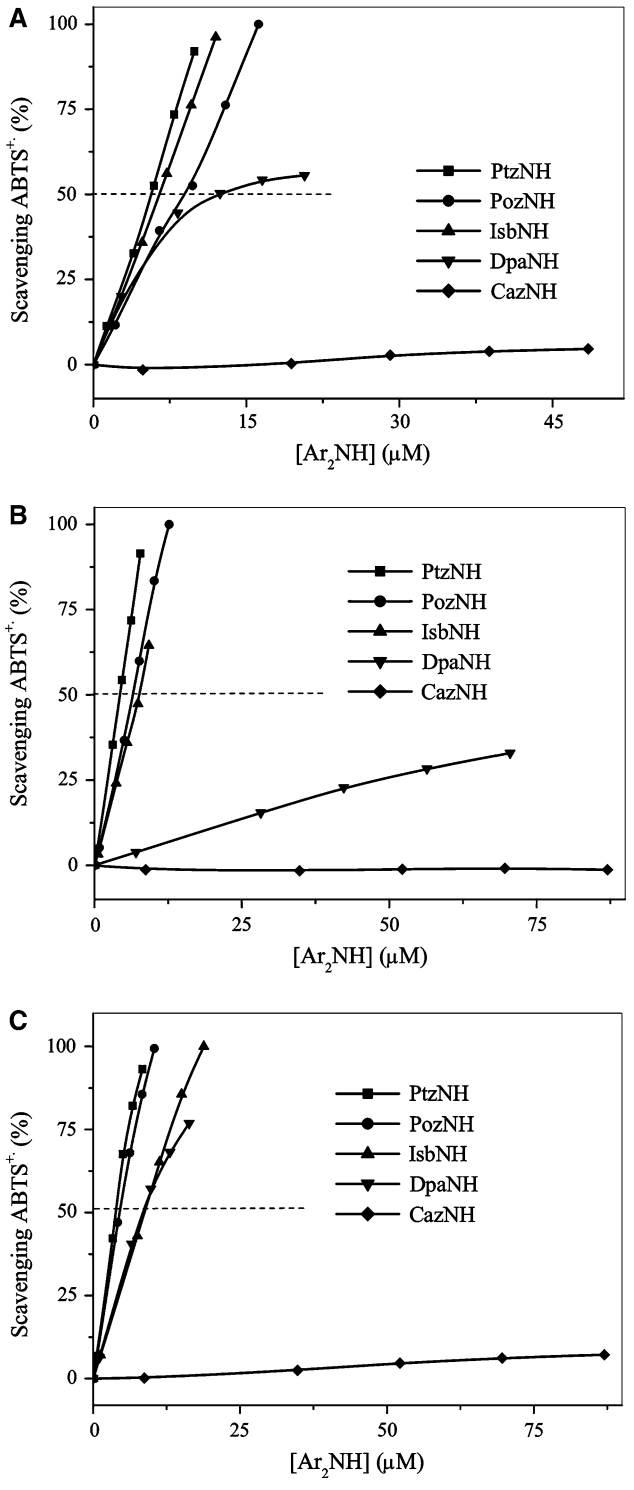


Fig. 3 The determination of IC_{50} of Ar_2NHs in scavenging $ABTS^{+\cdot}$, in which the reaction media was C_2H_5OH (**a**), 130 mM CH_3COOH/C_2H_5OH solution (**b**), and 130 mM pyridine/ C_2H_5OH solution (**c**), respectively

will be affected by either hydrogen-bond-donating solvents or hydrogen-bond-accepting solvents, if the reaction follows the HAT mechanism [24, 25].

The reaction between $ABTS^{+\cdot}$ and Ar_2NHs was done in an acetic acid/ethanol or pyridine/ethanol solution, respectively, and the times to reach equilibrium were measured and these values are listed in Table 3.

Table 2 The α_2^H and β_2^H parameters of CazNH, DpaNH, acetic acid, pyridine and ethanol

Compound	α_2^H	β_2^H
CazNH	0.469	
DpaNH	0.324	
Acetic acid	0.550	
Pyridine	0	0.625
Ethanol	0.367	

Cited from ref. [24, 25]

Table 3 The periods to reach equilibrium after mixing Ar_2NHs with DPPH and $ABTS^{+\cdot}$

Antioxidant	The period to reach equilibrium (hours)		
	In C_2H_5OH	In 130 mM CH_3COOH/C_2H_5OH solution	In 130 mM pyridine/C_2H_5OH solution
In scavenging DPPH			
PozNH	34	8.0	29
PtzNH	30	7.0	25
IsbNH	30	7.0	29
In scavenging $ABTS^{+\cdot}$			
PozNH	4.5	10.5	3.5
PtzNH	2.5	3.5	3.25
IsbNH	2.5	3.0	3.25
DpaNH	5.0	–	3.5

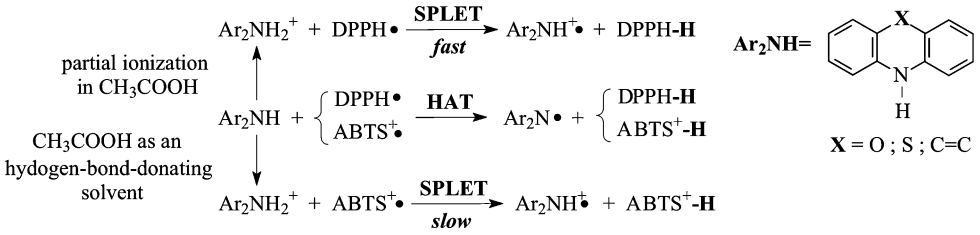
The equilibrium of mixing TroH, TocH, VC and VC-12 with either DPPH or $ABTS^{+\cdot}$ was reached immediately. CazNH and DpaNH cannot react with DPPH, and CazNH cannot react with $ABTS^{+\cdot}$ either

The data in Table 3 indicate that the addition of acetic acid and pyridine increased the time required to reach equilibrium, indicating that the reaction rates for the reaction of $ABTS^{+\cdot}$ with Ar_2NHs were reduced by the addition of either acetic acid or pyridine. In particular, the presence of acetic acid increased the time required for PozNH to reach equilibrium from 4.5 to 10.5 hours. This was because that both the acetic acid and the Ar_2NHs functioned as hydrogen-bond-donors, and the positive α_2^H values of the acetic acid decreased the k^{HAT}. As for pyridine, a hydrogen-bond-acceptor, the positive value for β_2^H also decreased the k^{HAT}. The decrease of the reaction rate as a result of the presence of these solvents indicated that the reaction of Ar_2NH to scavenge $ABTS^{+\cdot}$ followed the HAT mechanism.

In addition to the HAT mechanism, the SPLET mechanism has also been used to explain why the reaction between DPPH and hydroxyl-type antioxidant was inhibited by the presence of acetic acid. The presence of acetic acid suppresses the ionization of hydroxyl group, and this inhibits proton transfer to DPPH [17]. The SPLET mechanism suggests that the partial ionization of the antioxidant will enhance proton transfer to DPPH. The time required to reach equilibrium for the reaction between DPPH and Ar_2NHs was determined and that time is listed in Table 3. The time required to reach equilibrium for the reaction between DPPH and Ar_2NHs is decreased substantially by the addition of acetic acid. Acetic acid did not increase the ionization of ArOH groups, but it did increase the ionization of the alkaline antioxidants, Ar_2NHs. The ionization of Ar_2NHs accelerated the reaction of DPPH with Ar_2NHs. Since pyridine, which is not acidic, does not ionize Ar_2NHs, the addition of pyridine did not affect the time required to achieve equilibrium. Thus, the mechanism of the interaction between Ar_2NHs and DPPH is thought to be based on the SPLET mechanism. The mechanism for the reaction between DPPH or $ABTS^{+\cdot}$ and Ar_2NHs is shown in Scheme 1.

The reactions between Ar_2NHs and two radical species followed the HAT mechanism in the neutral solvent. The addition of acetic acid accelerated the reaction between Ar_2NHs and DPPH via partial ionization of Ar_2NHs, but the acetic acid reduced the rate of the reaction between Ar_2NHs and $ABTS^{+\cdot}$. Nevertheless, the addition of acetic acid and pyridine can also affect the TEAC of Ar_2NHs. As seen in Table 1, the TEAC values for PtzNH and IsbNH for trapping DPPH increased to some extent. In particular, TEAC of PtzNH increased from 0.90 to 3.01 in the presence of acetic acid. Meanwhile, except for IsbNH, the TEAC values of PtzNH and PozNH for trapping $ABTS^{+\cdot}$ increased as well. DpaNH can react with $ABTS^{+\cdot}$ in the presence of pyridine. CazNH does not scavenge DPPH and $ABTS^{+\cdot}$ perhaps because the aromatic tricyclic ring in CazNH makes the hydrogen atom in the N–H group difficult to abstract.

Scheme 1 The HAT and SPLET mechanisms of Ar_2NHs to scavenge DPPH and $ABTS^{+\cdot}$



Conclusion

In conclusion, either DPPH or $ABTS^{+\cdot}$ can be scavenged by PozNH, PtzNH and IsbNH. In addition, $ABTS^{+\cdot}$ can be scavenged by DpaNH. The TEAC analysis revealed that the antioxidant capacities of some Ar_2NHs were greater than for TroH. Furthermore, the reaction rates of Ar_2NHs and DPPH were accelerated by the addition of acetic acid, suggesting that SPLET mechanism is the main basis for this increase in activity. However, the addition of acetic acid and pyridine decreased the reaction rates of Ar_2NHs and $ABTS^{+\cdot}$, suggesting the HAT mechanism may be the best explanation for this reaction.

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