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# A KINETIC STUDY OF THE GENERATION AND DECOMPOSITION OF SOME PHENOTHIAZINE FREE RADICALS FORMED DURING ENZYMATIC OXIDATION OF PHENOTHIAZINES BY PEROXIDASE-HYDROGEN PEROXIDE

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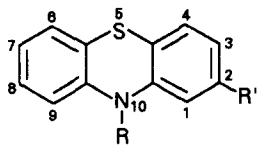
**Abstract**—A kinetic study of the oxidation of four different phenothiazines (Pts) by peroxidase-hydrogen peroxide was carried out. The free radical formed during the enzymatic oxidation suffers a non-enzymatic breakdown and the overall system was analysed and characterized. The non-enzymatic breakdown of the cation radical does not occur through a disproportionation mechanism but through a more complex mechanism. The kinetic parameters of the overall system were determined for the different Pts. These experimental data may serve in the understanding of the pharmacological action of Pts.

The widespread use of phenothiazines (Pts‡) in the treatment of mental illness has inspired much research into their chemical properties and reactions [1]. The following is a generalized structure of the Pts:




The compounds formed by substitution of halogens and other functional groups onto the Pt ring vary greatly according to the type of pharmacological activity and potency [2]. The influence of the 2-position substituent and the structure of the 10-position side chain on the antipsychotic activity of Pts has been studied, leading to the conclusion that changes in drug structure at these positions cause variations in this activity [2, 3].

The Pts can be divided into three groups with respect to the 10-position substituent (see Table 1) [4]: (a) aliphatic lateral chain, such as chlorpromazine (CPZ) and triflupromazine (TPMZ), with low antipsychotic potency. (b) Piperidine lateral chain, such as thioridazine (THZ). (c) Piperazine lateral chain, such as fluphenazine (FPZ) and trifluoperazine (TPZ), compounds of higher antipsychotic potency.

The oxidation of Pts occurs in distinct univalent steps. The unpaired electron intermediate of the free radical resulting from the first oxidation is called a "semiquinone", based on its structural similarities to the hydroquinone–quinone system. The oxidation of Pts by different agents has been used to detect the presence of these substances in urine and other systems [5–7]. Several investigators have suggested that the cation radicals formed by the oxidation of Pts are important intermediates in the metabolism of these drugs [8, 9].

Several studies of the mechanism by which the PT cation radicals ($Pts^{+\cdot}$) are generated and by which these radicals decay have been carried out [10–13]. Piette *et al.* [14] observed the formation of a free radical of CPZ ($CPZ^{+\cdot}$) during enzymic oxidation by peroxidase(POD)-hydrogen peroxide. An electronically excited species is generated during the POD oxidation of Pts, probably during the breakdown of their respective cation radicals [15].

POD usually catalyses a one electron oxidation of xenobiotic substrate to a free radical [16, 17], which can suffer a series of reactions as dismutate or undergo a further one electron oxidation to an electrophilic two electron oxidation product or undergo a reaction with nucleophiles. Thus, the free radical generated when the enzyme acts on CPZ suffers a series of chemical reactions as has been demonstrated [18].

This paper deals with the oxidation of four different Pts by $H_2O_2$ catalysed by POD, yielding a cation radical which suffers a non-enzymatic breakdown. The kinetic behaviour of the overall system is analysed and characterized. This information may be useful for understanding the pharmacological action of Pts. The oxidation of TPMZ, THZ and FPZ, representatives of the three different groups (a), (b) and (c) indicated earlier, has been studied and a comparative study of the 2-trifluorophenothiazine series (FPZ, TPZ, TPMZ) has been carried out.

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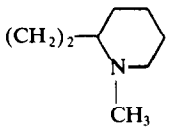
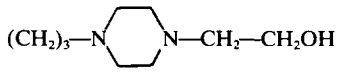
‡ Abbreviations: Pt, phenothiazine; $Pt^{+\cdot}$, PT cation radical; POD, peroxidase; E, ferriperoxidase:porphyrin-$Fe^{III}$ (resting form); CoI, compound I:$^{+\cdot}$porphyrin-$Fe^{IV}$=O; CoII, compound II:porphyrin-$Fe^{IV}$=O; CPZ, chlorpromazine; FPZ, fluphenazine; THZ, thioridazine; TPZ, trifluoperazine; TPMZ, triflupromazine.

Table 1. The different types of Pt and their substituents

| Pt | Substituents: 2-position | Substituents: 10-position | Type |
|---|---|---|---|
| THZ | $SCH_3$ |  | (b) |
| TPMZ | $CF_3$ | $(CH_2)_3—N—(CH_3)_2$ | (a) |
| FPZ | $CF_3$ |  | (c) |
| TPZ | $CF_3$ |  | (c) |

2-trifluorophenothiazine series

Table 2. Molar absorptivities of $Pts^{+\cdot}$

| $Pt^{+\cdot}$ | $\lambda_{max}$ | $\varepsilon$ ($M^{-1}$ $cm^{-1}$) |
|---|---|---|
| $TPMZ^{+\cdot}$ | 500 | 7450 ± 232 |
| $TPZ^{+\cdot}$ | 500 | 6538 ± 141 |
| $FPZ^{+\cdot}$ | 500 | 6796 ± 141 |
| $THZ^{+\cdot}$ | 633 | 7682 ± 275 |

## MATERIALS AND METHODS

Horseradish POD (EC 1.11.1.7), type VI (320 U/mg; 1 U will catalyse the formation of 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 at 25°) was purchased from Sigma (Germany) and its concentration calculated using $\varepsilon_{403} = 102.3\ mM^{-1}\ cm^{-1}$ [19]. Solutions of $H_2O_2$ were prepared from Perhydrol 30% (E. Merck, Germany) and concentrations were determined using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ [20]. Pts were purchased from Sigma and other chemicals were of analytical grade and supplied by E. Merck.

Spectrophotometric measurements were carried out using a Beckman DU-7 spectrophotometer equipped with an IBM-PC for data capture. Temperature was controlled by a Hetofrig circulating bath with a precision of ±0.1°. The perchlorate salts of $Pts^{+\cdot}$ in solid form were prepared according to the method of Levy *et al.* [21]. The molar absorptivities were calculated in 6 M $H_2SO_4$ and were as shown in Table 2. The $Pts^{+\cdot}$ were monitored spectrophotometrically at $\lambda_{max}$ as indicated earlier [3, 6, 21, 22].

Data analysis of progress curves was performed by means of non-linear regression [23, 24].

## RESULTS

### *Kinetic analysis*

The oxidation of different Pts by POD-hydrogen peroxide to a cation radical is coupled to a non-enzymatic second order reaction causing the breakdown of the free radical. The overall system can be described as follows:

$$2Pt + H_2O_2 \xrightarrow[POD]{V_0} 2Pt^{+\cdot} + 2H_2O \quad (1)$$

$$2Pt^{+\cdot} \xrightarrow{k_{app}} \text{Products} \quad (2)$$

where $V_0$ is the enzymatic reaction rate and $k_{app}$ the apparent second order constant of radical breakdown.

In the kinetic analysis of this coupled reaction system it has been assumed that the concentrations of Pt and $H_2O_2$ remain constant during the assay time and the intermediate $Pt^{+\cdot}$ and products do not inhibit the peroxidase [18].

The accumulation of $[Pt^{+\cdot}]$ is described by:

$$\frac{d[Pt^{+\cdot}]}{dt} = V_0 - k'_{app}[Pt^{+\cdot}]^2 \quad (3)$$

where $k'_{app} = 2k_{app}$. The integrated form is:

$$[Pt^{+\cdot}] = \sqrt{V_0/k'_{app}}\,\frac{1 - e^{-2\sqrt{V_0 k'_{app}}\,t}}{1 + e^{-2\sqrt{V_0 k'_{app}}\,t}} \quad (4)$$

and in the steady-state it can be defined as:

$$[Pt^{+\cdot}]_{ss} = \sqrt{V_0/k'_{app}}. \quad (5)$$

### *Data analysis of experimental recordings*

Figure 1 shows an experimental recording of the intermediate $FPZ^{+\cdot}$ vs time. These recordings allow us to estimate $V_0$ of the tangent of the progress curve $t \to 0$ and, according to Eqn (5), the altitude of the curve gives $[FPZ^{+\cdot}]_{ss}$. $k'_{app}$ can then be calculated by using the same Eqn (5). The values of $V_0$ and $k'_{app}$ can be introduced in Eqn (4) but this does not reproduce the experimental behaviour exactly. However, these values may be used as initial estimations to fit the experimental data to Eqn (4) by non-linear regression. The final estimation of both kinetic parameters leads to a successful overlay between experimental and calculated data.

### *Effect of* $E_0$

The progress curves of the accumulation of $Pt^{+\cdot}$ are strongly dependent on enzyme concentration.

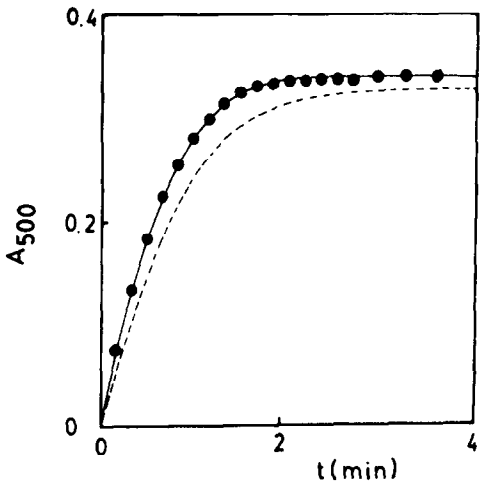



Fig. 1. Data analysis of a progress curve. The reaction medium at 25° contained 10 mM sodium acetate buffer, pH 4.0, 0.33 mM FPZ, 1 mM $H_2O_2$ and 20.5 U/L POD. (●) Experimental results. (– – –) Eqn (4) simulation by means of the initial estimation of the parameters, $V_0 = 0.794 \pm 8.10^{-3}$ ($\mu$M/sec) and $k'_{app} = 340 \pm 9$ ($M^{-1}\,sec^{-1}$). (——) Eqn (4) simulation by means of the parameters obtained by non-linear regression from Eqn (4), $V_0 = 0.968 \pm 7.10^{-3}$ ($\mu$M/sec) and $k'_{app} = 384 \pm 6$ ($M^{-1}\,sec^{-1}$).

The intermediate level $[Pt^{+\cdot}]_{ss}$ increased with enzyme concentration as Fig. 2A shows. The $V_0$ values obtained from Eqn (4) are directly proportional to enzyme concentration (Fig. 2B). The oxidation of CPZ by POD plus $H_2O_2$ has been investigated thoroughly by Piette *et al.* [14] and can be described for Pt derivates by Eqns (6–8):

$$E + H_2O_2 \xrightarrow{k_1} CoI + H_2O \quad (6)$$

$$CoI + Pt \xrightarrow{k_2} CoII + Pt^{+\cdot} \quad (7)$$

$$CoII + Pt \xrightarrow{k_3} E + H_2O + Pt^{+\cdot}. \quad (8)$$

The rate constant of the formation of CoI of POD, $k_1$, in Eqn (6) has been reported as being in the order of $2 \times 10^7\,M^{-1}\,sec^{-1}$ [25, 26]. The formation rate of CoI ($V_1$) is:

$$V_1 = k_1[E][H_2O_2] \quad (9)$$

where [E] = [POD] when $t \to 0$. The calculated rate for [POD] = 4.2 U/L and $[H_2O_2]$ = 1 mM is 6.6 $\mu$M/sec, more than 30 times faster than the $V_0$ of the fastest oxidized Pt derivative, which is 0.21 $\mu$M/sec for TPZ at the same POD concentration. This indicates that formation of CoI is not rate-limiting. The reaction of substrates with CoI (Eqn 7) is generally not rate-limiting because $k_2$ is, for most substrates, about 40 times larger than $k_3$ (Eqn 8) [25, 26]. Therefore, the rate-limiting step under the conditions used in this work must be the reaction of Pt with CoII (Eqn 8) [14]. From the data of Fig. 2B and using Eqn (10), the $k_3$ values for the various Pts used can be calculated:

$$V_0 = 2k_3[POD][Pt]. \quad (10)$$

The calculated $k_3$ values are presented in Table 3.

On the other hand $k'_{app}$ is independent of the enzyme concentration as Eqn (2) predicts.

*Effect of* $S_0$

The variation in substrate concentration shows a hyperbolic dependence of $V_0$ vs Pt concentration

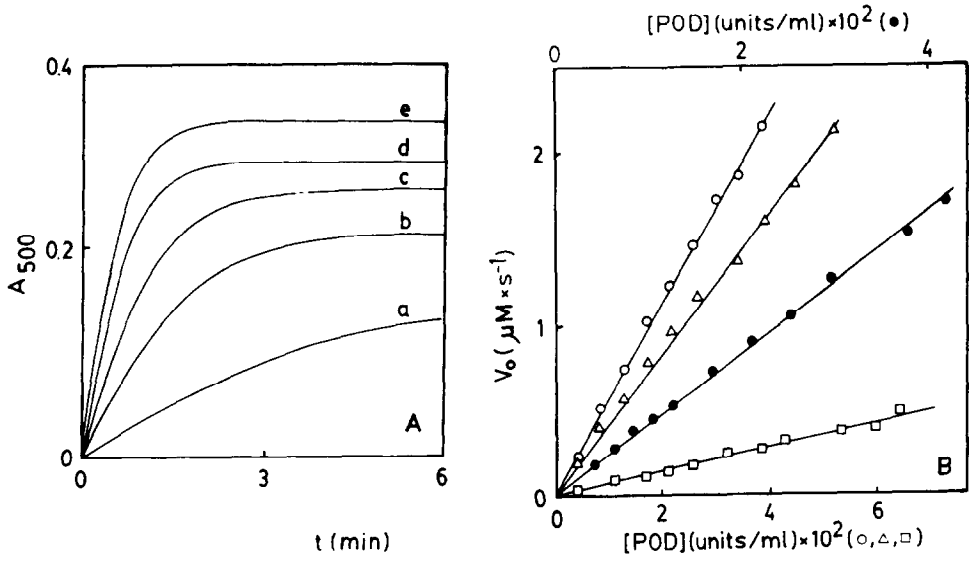



Fig. 2. (A) Progress curves for $FPZ^{+\cdot}$ accumulation. This is followed by the absorbance increase at 500 nm for different enzyme concentrations (U/L): (a) 3.84, (b) 7.68, (c) 12.8, (d) 16.6 and (e) 20.5. The reaction medium at 25° contained 10 mM sodium acetate buffer, pH 4.0, 1 mM $H_2O_2$ and 0.33 mM FPZ. (B) Plot of $Pt^{+\cdot}$ accumulation rate against enzyme concentration. The reaction medium contained 10 mM sodium acetate buffer, pH 4.0, 1 mM $H_2O_2$ and 0.33 mM Pt for (□) TPMZ, (○) FPZ and (△) TPZ, and 0.06 mM Pt for (●) THZ.

Table 3. Kinetic constants characterizing the oxidation of several Pts by POD-hydrogen peroxide

| Pt | $K_m$ (mM) | $k_3 \times 10^4$ ($M^{-1}$ $sec^{-1}$) | $k'_{app}$ ($M^{-1}$ $sec^{-1}$) |
|---|---|---|---|
| TPMZ | 1.31 ± 0.05 | 14 ± 1 | 35 ± 1 |
| TPZ | 0.52 ± 0.02 | 107 ± 4 | 84 ± 2 |
| FPZ | 0.60 ± 0.03 | 88 ± 4 | 398 ± 7 |
| THZ | 0.39 ± 0.02 | 412 ± 17 | 19 ± 1 |

The values of $k_3$ were obtained under the conditions indicated in the legend to Fig. 2 and the values of $K_m$ under the conditions indicated in the legend to Fig. 3. The values of $k'_{app}$ were obtained for a concentration of 0.33 mM of Pt, 1 mM $H_2O_2$ and 10 mM sodium acetate buffer, pH 4.0; POD concentration was 21.2 U/L for TPMZ, TPZ and FPZ and 4.2 U/L for THZ.

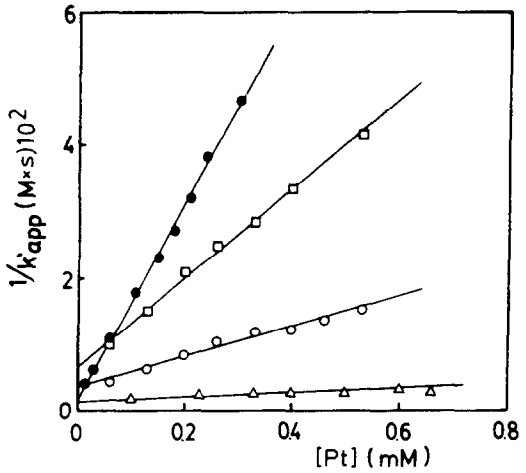



Fig. 3. Plot of the reciprocal observed rate constant against Pt concentration. The reaction medium at 25° contained 10 mM sodium acetate buffer, pH 4.0, 1 mM $H_2O_2$ and 14.8 U/L POD for (□) TPMZ, (○) TPZ and (△) FPZ, and 4.2 U/L POD for (●) THZ.

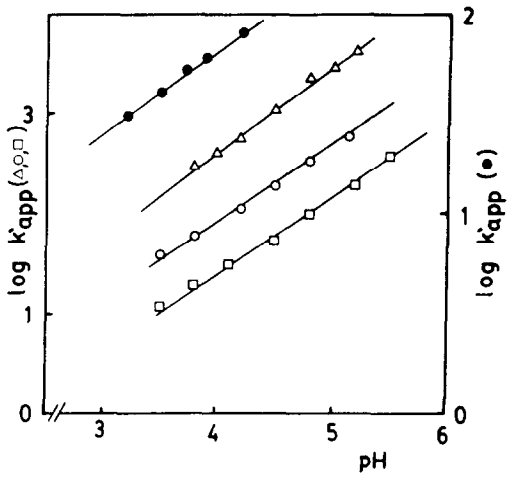



Fig. 4. Plot of log $k'_{app}$ against pH for different Pts. The reaction medium at 25° contained 10 mM sodium acetate buffer, 1 mM $H_2O_2$, 21.2 U/L POD and 0.33 mM Pt for (□) TPMZ, (○) TPZ and (△) FPZ, and 4.2 U/L POD and 0.12 mM Pt for (●) THZ.

allowing us to obtain the values of $K_m$ indicated in Table 3 for the different Pts. Inverse $k'_{app}$ vs Pt concentration is linear with non-zero $y$ intercept for all the Pts studied. (Fig. 3).

*Effect of pH*

The value of the optimum pH of the enzymatic step varied from one Pt to another, being 3.5 for TPMZ, 3.7 for THZ and 4.0 for TPZ and FPZ. The $Pts^{+\cdot}$ were observed to be more stable in reaction medium of low pH. The plot of log $k'_{app}$ against pH (Fig. 4) shows inverse kinetics of first order in hydronium ions.

*Effect of buffer*

At constant pH, when other conditions also remained constant, the breakdown reactions were first order in buffer concentration for TPMZ; however, for other Pts, $k'_{app}$ did not change with buffer concentration (Fig. 5).

## DISCUSSION

The reaction mechanism of $CPZ^{+\cdot}$, the most

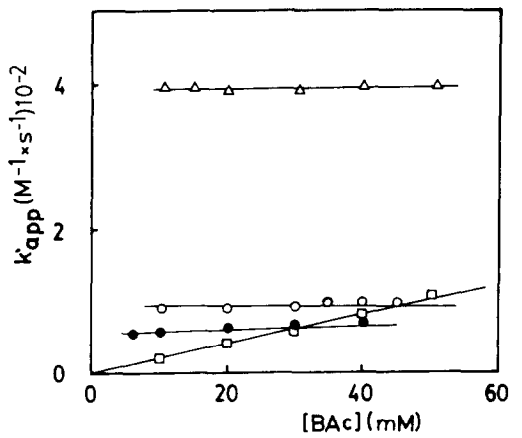



Fig. 5. Plot of the apparent rate constant $k'_{app}$ against buffer concentration. The reaction medium at 25° contained sodium acetate buffer, pH 4.0 (BAc), 1 mM $H_2O_2$, 21.2 U/L POD and 0.33 mM Pt for (□) TPMZ, (○) TPZ and (△) FPZ, and 1 mM $H_2O_2$, 4.2 U/L POD and 0.12 mM Pt for (●) THZ.

intensively studied of the Pts, has been examined by Cheng *et al.* [10, 11]. Hammerich and Parker [12, 13] have proposed the following mechanism:

$$CPZ^{+\cdot} + RCOO^- \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} CPZ^{\cdot} - OCOR \quad (11)$$

$$CPZ^{\cdot} - OCOR + CPZ^{+\cdot} \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} CPZ^{+} - OCOR + CPZ \quad (12)$$

$$CPZ^{+} - OCOR + H_2O \underset{k_{-3}}{\overset{k_3}{\longleftrightarrow}} CPZ(OCOR)OH + H^+ \quad (13)$$

$$CPZ(OCOR)OH \xrightarrow{k_4} CPZ\text{-sulfoxide} + R\text{-}COOH. \quad (14)$$

The expression for $k'_{app}$ can therefore be obtained:

$$k'_{app} = \frac{C_1[\text{buffer}]}{[H^+]([CPZ] + C_2)} \quad (15)$$

where $C_1$ and $C_2$ represent combinations of rate constants involved in the mechanism (Eqns 11–14). Our experimental data for TPMZ indicate the following characteristics of the system: (a) second order kinetics in $Pt^{+\cdot}$ concentration, since experimental recordings are described by Eqns (4) and (5) (Fig. 1). (b) Linear plot of $1/k'_{app}$ vs Pt concentration according to Eqn (15) (Fig. 3). (c) Linear plot of $\log k'_{app}$ vs pH according to Eqn (15) (Fig. 4). (d) Linear plot of $k'_{app}$ vs buffer concentration according to Eqn (15) (Fig. 5). It can be concluded from the experimental results that TPMZ follows the mechanism described for CPZ giving sulfoxide as product [18]. Nevertheless, for THZ, TPZ and FPZ, although the first three points are fulfilled, the fourth is not, revealing a modification of the earlier mechanism. This would appear to indicate that a nucleophilic reagent other than $RCOO^-$ is involved in the cation radical attack, possibly the side chain amine in 10-position with $pK_a = 3.9$ for TPZ and FPZ, giving a hydroxylated product [27].

The kinetic parameters of the overall system corresponding to both the enzymatic oxidation and the non-enzymatic reaction were determined for the various Pts. As regards the enzymatic reaction, the values obtained for $K_m$ (Table 3) indicate that the affinity of the enzyme is highest for TPMZ, followed by FPZ > TPZ > THZ. The values of $k_3$ are also indicated in Table 3. Pts display a high donating power, which is modified according to the substituent on the 2-position of the ring. Thus, the high value of $k_3$ for THZ is in accordance with the weak electron-withdrawing substituent ($-SCH_3$) in this position (see Table 1). When the 2-substituent is a more powerful electron withdrawer, as in the case of trifluoromethyl (TPMZ, FPZ and TPZ), the electron-donating power of the Pt ring decreases and, as a consequence, so do the values of $k_3$ (see Table 3). These values for the 2-trifluorophenothiazine series are in the order TPMZ < FPZ < TPZ. In TPMZ, the amine of the side chain ($pK_a = 9.2$) is protonated at pH 4.0 (under the assay conditions of this work) and may cause electronic interaction between the right system and the amine, thus decreasing the electron-donating power of the Pt, which would explain the lower $k_3$ value (Table 3). The piperazine ring in TPZ and FPZ (with $pK_a$ values of the side chain of 3.9 and 8.1) would cause stearic hindrance for this interaction thus giving a higher value for $k_3$. In FPZ the $CH_2OH$ in the lateral chain is protonated at pH 4.0 and this group may interact with the Pt nucleus, and therefore the $k_3$ value for FPZ is lower than for TPZ.

The rate of decay of $Pts^{+\cdot}$ varies widely depending upon the nature of the substituents at positions 2 and 10 of the Pt nucleus. $THZ^{+\cdot}$ is the most stable, as indicated by the values of $k'_{app}$ for the various Pts summarized in Table 3. The substituent in the 2-position with electron-withdrawing groups caused the formation of a cation radical that was more electron deficient and thus more subject to nucleophilic attack, as reflected by the higher $k'_{app}$ value for the 2-trifluorophenothiazine series. According to the $k'_{app}$ values, $TPMZ^{+\cdot}$ is the most stable cation radical of the 2-trifluorophenothiazine series. Indeed, the high stability of the $TPMZ^{+\cdot}$ has been indicated in others works [2]. The presence of a piperazine ring may cause a more unstable radical. The results indicate that $FPZ^{+\cdot}$ is less stable than $TPZ^{+\cdot}$ but the reason for this difference is not clear, since the side chain differs by only one $CH_2OH$ group.

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