# Antioxidant activity of α-pyridoin and its derivatives: possible mechanism†

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α-Pyridoin (**1**, 1,2-di(2-pyridyl)-1,2-ethenediol) is a unique enediol antioxidant. To explore the detailed antioxidant mechanism of α-pyridoin, we synthesized α-pyridoin and its 5,5′- or 6,6′-bis-substituted derivatives (**2–7**) and compared their capacities to scavenge galvinoxyl radical (GO•) and protect human red blood cells (RBCs) from oxidative haemolysis. It was found that the compounds (**5** and **6**) with a methyl or methoxy group at the 5-position exhibit significantly higher GO•-scavenging and anti-haemolysis activities than other derivatives and vitamin C. Kinetic analysis of the GO•-scavenging reaction and the effect of added base on the reaction rate revealed that in ethyl acetate, the reaction occurs primarily by the direct hydrogen atom transfer (HAT mechanism). However, in ethanol that supports ionization, the kinetics of the process is mostly governed by sequential proton loss electron transfer (SPLET mechanism).



## Introduction

A large body of clinical and experimental evidence shows that reactive oxygen species (ROS) and free radicals are involved in many pathological conditions such as cancer, aging and atherosclerosis, and that fruits, vegetables, and beverage-derived antioxidants (vitamin E, vitamin C, β-carotene, flavonoids and resveratrol) may have beneficial effects in protecting against these diseases.[1–6] Among these naturally occurring antioxidants, vitamin C (ascorbic acid, VC) (Fig. 1) shows very effective activity by scavenging ROS and regenerating vitamin E.[7,8]

The well-known defects of VC, such as its susceptibility to thermal and oxidative degradation and high hydrophilicity, has led to tremendous interest in derivatives with increased stability and lipophilicity.[9–15] The active site of VC is the 2,3-enediol moiety conjugated with the carbonyl group of a five-membered lactone. Based on the active site, Mashino and co-workers[16,17] have succeeded in synthesizing a series of unique enediol antioxidants, α-pyridoin and its derivatives (ArOHs) (**1–7**, Fig. 1), whose enediol form is stabilized by the intramolecular hydrogen bonding of the pyridine nitrogen and the hydroxyl group.[18,19] Mashino and co-workers have compared the antioxidant capacity of the compounds to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH•), to inhibit lipid peroxidation in rat liver microsome, and to protect human promyeloid leukemia cell lines (HL-60) from oxidative stress, and found that **5** and **6** are good candidates for a pharmacologically useful enediol antioxidant.[16,17]

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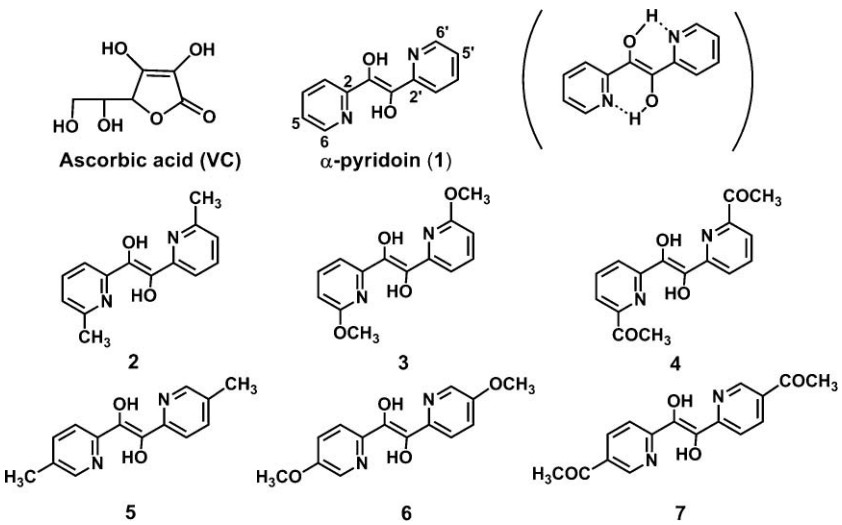



**Fig. 1** Molecular structures of α-pyridoin (**1**) and its derivatives.

However, their detailed antioxidant mechanisms still need to be further explored. The formal abstraction of enediolic hydrogen atoms can proceed *via* at least three different mechanisms:[20] direct hydrogen atom transfer (HAT mechanism, eqn (1)), sequential proton loss electron transfer (SPLET mechanism, eqn (2)) and electron transfer followed by proton transfer (ETPT mechanism, eqn (3)). Which mechanism is responsible for the antioxidant reaction of the compounds? How does solvent environment influence the antioxidant mechanisms? How many radicals can be scavenged by one molecule of the compounds in the antioxidant reaction? To resolve the key questions, and in connection with our interest in kinetics and mechanisms of natural antioxidants and their synthetic analogues,[21–24] we report herein a quantitative kinetic study of the scavenging reaction of α-pyridoin and its derivatives (ArOHs) (**1**–**7**, Fig. 1) towards galvinoxyl radical (GO•) in ethanol and ethyl acetate at 25 °C. The antioxidant effect of ArOHs against oxidative haemolysis of human red blood cells (RBCs) were also investigated.

$$\mathrm{ArOH + X^{\bullet} \rightarrow ArO^{\bullet} + XH} \quad (1)$$

$$\mathrm{ArOH \xrightarrow{-H^+} ArO^- \xrightarrow{X^{\bullet}} ArO^{\bullet} + X^- \xrightarrow{H^+} ArO^{\bullet} + XH} \quad (2)$$

$$\mathrm{ArOH + X^{\bullet} \rightarrow ArOH^{\bullet +} + X^- \rightarrow ArO^{\bullet} + XH} \quad (3)$$

## Results and discussion

### GO•-scavenging activity of ArOHs

GO• is a relatively stable phenoxyl radical, and has been widely used to assess radical-scavenging activity of phenolic compounds and their abilities to transfer labile H atoms to radicals.[25] Therefore, the reactions of ArOHs shown in Fig. 1 and GO• were studied with UV-vis spectroscopy. When α-pyridoin (**1**) was reacted with GO• in ethyl acetate at 25 °C, the absorption band at 428 nm due to GO• decayed immediately. If a large excess of α-pyridoin (**1**) was employed, the decay of GO• occurred with pseudo-first-order kinetics (eqn (4)) as shown in Fig. 2. Plotting this pseudo-first-order rate constant ($k_{obs}$) *versus* the concentration of α-pyridoin (**1**) gave a straight line (the inset of Fig. 2), from which the second-order rate constant ($k$) for the GO•-scavenging reaction by α-pyridoin (**1**) could be obtained. Other ArOHs gave the same

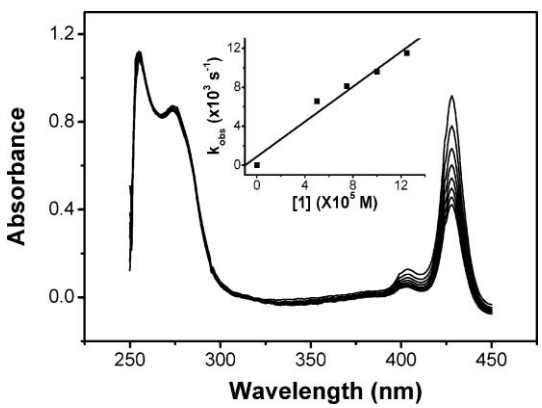



**Fig. 2** Spectral changes observed upon addition of **1** (50 μM) to an ethyl acetate solution of GO• (5 μM) at 298 K (Interval: 2 min). Inset: plot of the pseudo-first-order rate constant ($k_{obs}$) *vs.* the concentration of **1**.

**Table 1** GO•-scavenging and *anti*-haemolysis activities of α-pyridoin (**1**) and its derivatives (ArOHs)

| | $k$/M$^{-1}$ s$^{-1}$ (GO•)[a] | | | | | |
|---|---|---|---|---|---|---|
| ArOHs | Ethyl acetate | Ethanol | $n_{GO^{\bullet}}$[b] | $IC_{50}$/ μM[a] | $t_{eff}$/ min[c] | $n_{LOO^{\bullet}}$[d] |
| **1** | 88.3 ± 3.3 | 4364.0 ± 3.3[e] | 1.5 | 6.9 ± 0.5 | 34.0 | 1.0 |
| **2** | 103.4 ± 8.7 | 8969.8 ± 6.0[e] | 1.4 | 7.3 ± 0.5 | 48.9 | 1.5 |
| **3** | 4.25 ± 0.3 | 10.3 ± 0.8 | 1.3 | 7.5 ± 0.6 | 62.0 | 1.9 |
| **4** | 17.3 ± 1.3 | 76.0 ± 3.3 | 1.2 | 8.2 ± 0.2 | 46.0 | 1.4 |
| **5** | 956.4 ± 1.8 | 15633.9 ± 8.1[e] | 1.7 | 5.9 ± 0.2 | 71.4 | 2.2 |
| **6** | 133.7 ± 2.1 | 9909.2 ± 18.4[e] | 1.2 | 8.5 ± 0.4 | 66.7 | 2.0 |
| **7** | 22.2 ± 1.6 | 95.4 ± 5.3 | 0.7 | 15.6 ± 0.9 | 56.8 | 1.7 |
| VC | 33.2 ± 2.5 | 4429.1 ± 15.3[e] | 0.8 | 13.3 ± 0.8 | 40.2 | 1.2 |
| Trolox | | | | | 59.3 | 2.0 |

[a] Data are expressed as the mean ± S.D. for three determinations. [b] Calculated from the slope of the straight line in Fig. 5. [c] Data are the average of three determinations, which were reproducible with deviation less than ±10%. [d] Calculated from the equation $t_{eff} = n_{LOO^{\bullet}}[ArOH]/R_i$. [e] The rates were measured by the second-order kinetics with the ratio of [ArOH] : [GO•] being 1 : 1.

second-order kinetics and their second-order rate constants are listed in Table 1. It can be seen from Table 1 that GO•-scavenging activity of ArOHs in ethyl acetate follows the sequence of **5** > **6** > **2** > **1** > VC > **7** > **4** > **3**, in line with the previous observation in the DPPH•-scavenging by ArOHs with **5** and **6** being the most reactive ones.[16] By comparing the $k$ values for ArOHs, it is clear that the introduction of electron-donating groups (EDG), such as methyl and methoxy, in the 5-position, remarkably increases the GO•-scavenging activity. It is also noticeable that GO•-scavenging activity of **5**, **6**, **2** and **1** is significantly higher than that of VC.

$$-\mathrm{d[GO^{\bullet}]}/\mathrm{d}t = k\,[\mathrm{ArOH}][\mathrm{GO^{\bullet}}] = k_{obs}[\mathrm{GO^{\bullet}}] \quad (4)$$

Although ethanol and ethyl acetate have the same hydrogen-bond-accepting activity ($\beta_2^H = 0.45$), ethanol has much higher dielectric constants ($\varepsilon = 24.30$) than ethyl acetate ($\varepsilon = 6.02$) and hence has a greater ability to support ionization of the substrate.[26] To investigate the influence of solvent environment on the GO•-scavenging activity by ArOHs, the solvent of ethanol was selected and reaction kinetics were performed at the same temperature. The results are summarized in Table 1. The structure–activity relationship obtained in ethanol is similar to that obtained in ethyl acetate. However, the rate constants for the reaction of GO• with ArOHs in ethanol are 2–100 times larger than that in ethyl acetate.

The kinetic difference between ethanol and ethyl acetate should arise from their different mechanisms.[20,26] Recently, Litwinienko and Ingold clearly demonstrated the occurrence of SPLET in the reaction of DPPH• with some phenolic compounds in methanol and ethanol.[20,26] In contrast to ethanol, ethyl acetate has a low ability to support ionization of the substrate and the reactions occur primarily by the HAT mechanism.[20,26] In solvents that support ionization, such as alcohols and water, enediol (ArOH) may be in equilibrium with the corresponding enediolate anion ($ArO^-$) for the acidity of enediolic O–H.[16] As a matter fact, $ArO^-$ is a much stronger electron donor than the parent molecule. If $ArO^-$ acts as the electron donor and the SPLET mechanism (eqn (2)) is operative in solvents that support ionization, the addition of base could increase the second-order rate constant ($k$) to a limiting

value by enhancing phenol deprotonation. When the electron donor is the parent molecule and an ETPT mechanism occurs (eqn (3)), the addition of base could also increase the second-order rate constant ($k$) to a limiting value by the coordination of base to a radical cation ($ArOH^{\bullet+}$).[27] However, the two electron transfer mechanisms should be distinguished using the difference of basicity. In SPLET mechanism, the limiting $k$ value should be the same regardless of the basicity (see below), which could be due to the same limiting-value of [$ArO^-$]. Nevertheless, in the ETPT process, the limiting $k$ value should be different and dependent on the difference of basicity.[27] In addition, in the HAT process (eqn (1)), the measured rate constant should be expected to remain the same with the addition of a small amount base. However, the basicity of solvent can affect the HAT process. The stronger the basicity of solvent is, the stronger hydrogen bond acceptor is, and the HAT process should be slower according to the kinetic solvent effect.[28] As a whole, the basicity should be important for the SPLET, ETPT or HAT mechanisms.

To rationalize the reaction mechanism, the effect of base on the radical-scavenging rates of α-pyridoin (**1**) in ethanol and ethyl acetate was examined. As shown in Fig. 3, the rate constant increased with increasing pyridine concentration to reach a constant value. When pyridine was replaced by 2,6-lutidine, a stronger base than pyridine, the limiting rate constant was similar to that in the case of pyridine (Fig. 3), further supporting that the actual electron donor is $ArO^-$ and a SPLET mechanism (eqn (2)) does operate in ethanol. However, in ethyl acetate with the lower dielectric constant, the addition of base showed no effect on the radical-scavenging rate for α-pyridoin (**1**) (the inset of Fig. 3), a result indicating the only occurrence of HAT.

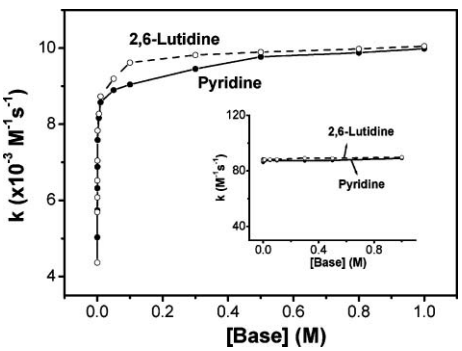



**Fig. 3** Plot of $k$ *vs.* [base] for the reaction of **1** with GO$^{\bullet}$ in the presence of pyridine (●) and 2,6-lutidine (○) in ethanol and ethyl acetate (inset) at 298 K.

To investigate whether the two enediolic hydrogen atoms contribute to the GO$^{\bullet}$-scavenging reaction, the antioxidant stoichiometric factor, $n_{GO^{\bullet}}$, of ArOHs in methanol was determined by UV-vis spectroscopy. The $n_{GO^{\bullet}}$ value means the number of GO$^{\bullet}$ reduced by one molecule of antioxidant. For all of the compounds tested, the reaction is biphasic, with a fast decay in absorbance in the first minutes, followed by a slower step in which degradation products are involved, until equilibrium is reached. As shown in Fig. 4, the absorption of GO$^{\bullet}$ at 428 nm decayed with the addition of **3** to a methanol solution of GO$^{\bullet}$, and steady state was achieved after 30 min. Therefore, the reaction time of 30 min was selected. Excellent linear correlations for concentration *vs.*

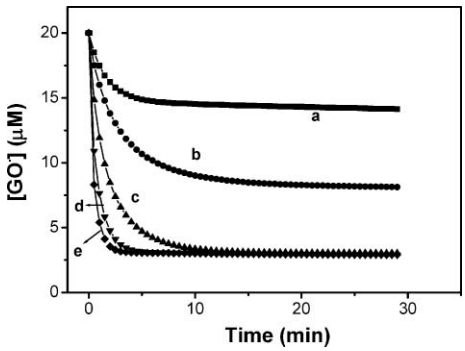



**Fig. 4** Reaction curves between 20 μM GO$^{\bullet}$ and different concentrations of **3**. (a) 2 μM; (b) 5 μM; (c) 10 μM; (d) 15 μM; (e) 20 μM.

absorbance were obtained for all the compounds tested (Fig. 5) and the concentrations giving 50% reduction in the absorbance of 20 μM GO$^{\bullet}$ solution ($IC_{50}$) were determined from the linear curves (Table 1). The $n_{GO^{\bullet}}$ value can also be obtained from the slope of the straight line (Table 1). It is seen from the results listed in Table 1 that **5** has the lowest $IC_{50}$ value and the highest $n_{GO^{\bullet}}$ value among the compounds tested, and hence possesses the highest GO$^{\bullet}$-scavenging activity. The fact that the stoichiometric factor, $n_{GO^{\bullet}}$, of the compounds (**1–6**) is larger than 1 (Table 1) suggests that the second GO$^{\bullet}$ must be involved in the antioxidation reaction.

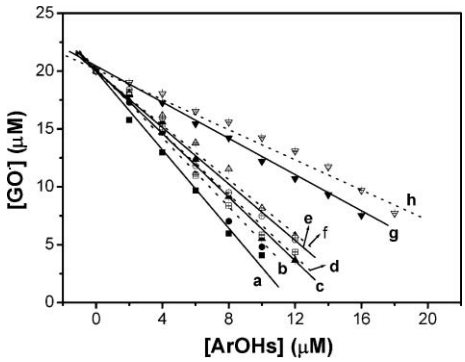



**Fig. 5** GO$^{\bullet}$ (20 μM) scavenging activity of α-pyridoin and its derivatives, and ascorbic acid (ArOHs) in methanol at room temperature. The reaction time was 30 min. (a) **5**; (b) **1**; (c) **2**; (d) **3**; (e) **4**; (f) **6**; (g) **VC**; (h) **7**.

### Inhibition of RBC haemolysis by ArOHs

In contrast to homogenous solution (ethyl acetate and ethanol), human RBCs are heterogeneous media and useful in the evaluation of antioxidant properties of the compounds with different lipophilicity. Therefore, the antioxidative effect of ArOHs was investigated in a RBC model to further evaluate the influence of microenvironment on the antioxidant activity.

Fig. 6 shows the 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced RBC haemolysis under an aerobic atmosphere. It can be seen from line a in Fig. 6 that haemolysis did not take place at once when RBCs were incubated with 50 mM AAPH. The endogenous antioxidant such as vitamin E and/or ubiquinol-10 present in the RBC membrane, protected RBCs against AAPH-induced haemolysis[29] until they were exhausted completely, resulting in the inhibition time ($t_{inh}$) (82.8 min).

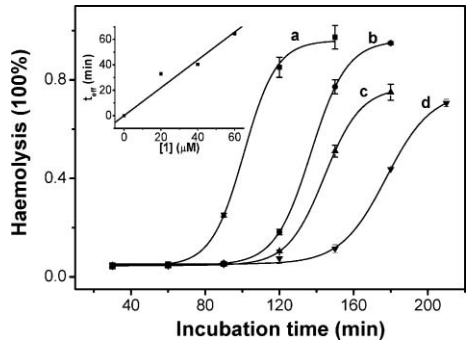



**Fig. 6** Inhibitory effect of α-pyridoin (**1**) against 50 mM AAPH-induced haemolysis of 5% human RBCs in 0.15 M PBS (pH 7.4) under an aerobic atmosphere at 37 °C. The initial concentrations of **1** were: (a) 0; (b) 20 μM; (c) 40 μM; (d) 60 μM. The inset shows the relationship between the additional or effective inhibition time, $t_{eff}$, and the initial concentration of **1**. Data are expressed as the mean of three RBC samples.

Addition of ArOHs into the 5% RBC suspension significantly increased the intrinsic inhibition time of the RBCs (Fig. 6 and 7). The inhibition time produced by ArOHs depended on the concentration of the ArOH as illustrated in the inset of Fig. 6 and on the specific ArOH used as shown in Fig. 7. For example, when initiated with 50 mM of AAPH, the inhibition time produced by 20 μM of α-pyridoin (**1**) was 116.8 min (Fig. 6). It corresponds to the additional or effective inhibition time, $t_{eff}$, produced by the antioxidant being 34.0 min.

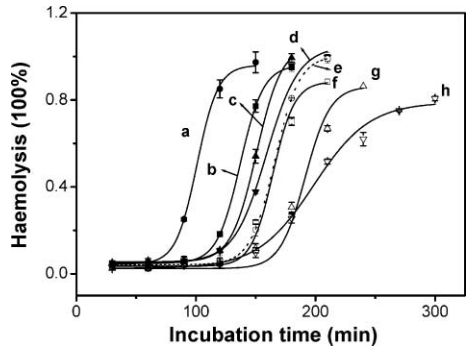


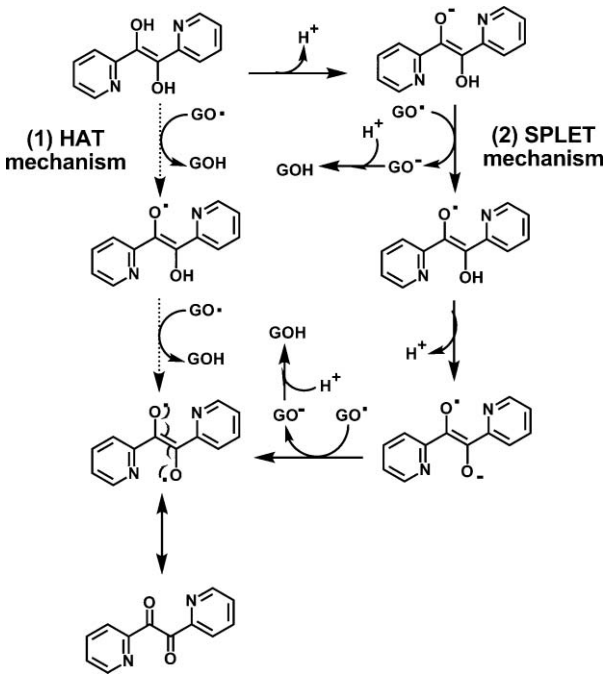

**Fig. 7** Inhibition of AAPH-induced haemolysis of human RBCs by α-pyridoin and its derivatives (ArOHs). The experimental conditions were the same as described in the legend of Fig. 6 with $[ArOH]_0 = 20$ μM. (a) Native RBCs; (b) inhibited with **1**; (c) inhibited with **4**; (d) inhibited with **3**; (e) inhibited with **2**; (f) inhibited with **7**; (g) inhibited with **5**; (h) inhibited with **6**. Lines for VC are not shown for clarity. Data are expressed as the mean of three RBC samples.

The $t_{eff}$ can be expressed as eqn (5).[23] Here, $R_i$ is the apparent rate of chain initiation, and $n_{LOO^\bullet}$ is the stoichiometric factor that denotes the number of $LOO^\bullet$ trapped by one molecule of antioxidant. It can be seen from the equation that $n_{LOO^\bullet}$ can be obtained if $R_i$ is known. As a matter of fact, it is difficult to measure $R_i$ directly. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, is always selected as the reference antioxidant whose $n$ is taken as 2.[30] Therefore, we determined the $t_{eff}$ produced by Trolox with different concentrations (10, 20, 30 and 40 μM) (data not shown). Plotting this $t_{eff}$ *versus* the concentration of Trolox gave a straight line, from whose slope $n_{LOO^\bullet}/R_i$ could be obtained (eqn (5)). The $R_i$ value was calculated to be $1.21 \times 10^{-8}$ M $s^{-1}$ in 50 mM AAPH-induced RBC haemolysis. On the basis of the $R_i$ value and eqn (5), the $n_{LOO^\bullet}$ values of compounds **1–7** and VC can be deduced, and are listed in Table 1. It can be concluded from the $t_{eff}$ and $n$ values that the anti-haemolysis activity of the compounds (**5**, **6** and **3**) is significantly higher than the other α-pyridoin derivatives and VC.

$$t_{eff} = n_{LOO^\bullet}[ArOH]/R_i \quad (5)$$

## Mechanism and structure–activity relationship

The acidity of enediolic O–H in α-pyridoin (**1**)[16] makes it possible to dissociate in ethanol and water, which supports ionization, and the SPLET reaction is therefore feasible. The $GO^\bullet$-scavenging reaction rate of α-pyridoin (**1**) in ethanol was raised for more than 2-fold to achieve the same limiting value by the addition of pyridine and 2,6-lutidine. This result is fully consistent with the partial dissociation of these ArOHs and the occurrences of SPLET in ethanol. Thus, in ethanol, α-pyridoin (**1**) should be the first to lose a proton to form the enediolate anion, and the latter then undergoes electron transfer to form the corresponding radical (Scheme 1). The fact that the $n_{GO^\bullet}$ value of α-pyridoin (**1**) is 1.50 suggests that the resulting radical will further undergo the second electron transfer with $GO^\bullet$ to form the corresponding α-pyridil (Scheme 1). The product has previously been elucidated by oxidizing α-pyridoin derivatives with iodine in dichloromethane.[17] It should be pointed out that α-pyridoin and its derivatives have some acidic (OH groups) and basic (nitrogens) sites. Therefore, it is possible that hydroxyl groups in α-pyridoin protonate nitrogens within the same molecule and that α-pyridoin exists in zwitterionic form depending on the pH value of the aqueous solution. In contrast to the pyridine ring conjugated to the double bond




**Scheme 1** Proposed mechanisms of $GO^\bullet$-scavenging by α-pyridoin in ethyl acetate (HAT mechanism, dotted line) and ethanol (SPLET mechanism, solid line).

(electron withdrawing group) in α-pyridoin, the nitrogen atom of pyridine or 2,6-lutidine has a relatively high electron density. Therefore, the basicity of pyridine or 2,6-lutidine is stronger than that of α-pyridoin, which is in favor of the migration of a proton from the internal nitrogen heteroatom to the external base (pyridine or 2,6-lutidine).

On the other hand, the relatively low rate constants for the reaction of ArOHs with GO• in ethyl acetate compared with the rate constants in ethanol (Table 1), and the addition of base having no effect on the reaction rate for α-pyridoin (**1**), indicate clearly that in ethyl acetate with a low ability to ionize ArOHs, the reaction occurs primarily by the HAT mechanism (Scheme 1).

It can be seen from the results listed in Table 1 that **5** and **6** exhibit significantly higher GO•-scavenging and anti-haemolysis activities than the other α-pyridoin derivatives and VC. That is, the introduction of an EDG (methyl and methoxy) at the 5-position remarkably increases the antioxidant activity. As a matter of fact, it is hard to predict precisely the substituent effect on the relative antioxidant activity sequence of ArOHs for the different mechanism and microenvironment. In ethyl acetate, the GO•-scavenging reaction occurs primarily by the HAT mechanism. Consequently, the enhancement in the GO•-scavenging activity of **5** and **6** can be explained by the fact that the introduction of an EDG (methyl and methoxy) at the 5-position reduces the bond dissociation enthalpy of enediolic O–H. We have recently also found that the introduction of an EDG helps to improve the antioxidant activity of resveratrol analogues.[21] However, in ethanol and water that supports ionization, the SPLET reaction is predominant. The introduction of an EDG can decrease the acidity of the α-pyridoin derivatives and hence decrease $ArO^-$ concentration, but the EDG's destabilizing effect on the enediolate anion will increase the electron transfer rate. Thus, the EDG's overall effect on SPLET reaction result in the fact that the compound **5** bearing the weak EDG (5-methyl) is the most active one instead of compound **6** bearing the strong EDG (5-methoxy). In addition, it is also noticeable that compound **3** has a low activity in the GO•-scavenging reaction, but exhibits enhanced anti-haemolysis activity. This is in accordance with the previous observation in the inhibition of lipid peroxidation of rat liver microsomes by the compound.[16] The compound was proven previously to be the most lipophilic among the α-pyridoin derivatives examined.[16] This indicates clearly that the lipophilicity of the compound is also the important factor to influence the efficiency of antioxidant in heterogeneous media (RBCs).

## Experimental

### Materials

α-Pyridoin (**1**) and its derivatives (**2–7**) were prepared according to the available procedures,[16] and their structures and purity were confirmed by MS, $^1$H and $^{13}$C NMR, and HPLC (see the ESI†). Generally, the aromatic aldehyde was dissolved in a mixed solvent of methanol and water (v/v, 8 : 3), and sodium cyanide was added to the above-mentioned mixture, which was then refluxed at 80 °C for 10–20 min. The resulting yellow solid was collected by filtration, washed with methanol and $H_2O$ successively, and dried under reduced pressure to afford α-pyridoin or its derivatives (**1–7**), then recrystallized from DMSO to give pure compounds.

α-Pyridoin (2,2′-pyridoin) (**1**): 92% yield from 2-pyridinecarboxaldehyde. m.p. 141–143 °C; $^1$H NMR (400 MHz, $CD_3COCD_3$): $\delta$ = 7.36 (ddd, $^3J$ (H,H) = 8.4 Hz, $^3J$ (H,H) = 4.4 Hz, $^4J$ (H,H) = 1.2 Hz, 2H; H-5, H-5′), 7.89 (d, $^3J$ (H,H) = 8.4 Hz, 2H; H-3, H-3′), 8.00 (dt, $^3J$ (H,H) = 8.4 Hz, $^4J$ (H,H) = 1.6 Hz, 2H; H-4, H-4′), 8.59 (d, $^3J$ (H,H) = 4.4 Hz, 2H; H-6, H-6′), 13.00 ppm (s, 2H; –OH); $^{13}$C NMR (100 MHz, $CD_3COCD_3$): $\delta$ = 120.1, 122.5, 136.4, 138.8, 146. 9, 157.4 ppm; MS (EI) *m*/*z*: 214 [M$^+$].

6,6′-Dimethyl-2,2′-pyridoin (**2**): 93% yield from 6-methyl-2-pyridinecarboxaldehyde. m.p. 186–189 °C; $^1$H NMR (400 MHz, $CD_3COCD_3$): $\delta$ = 2.58 (s, 6H; –$CH_3$), 7.22 (d, $^3J$ (H,H) = 8.0 Hz, 2H; H-5, H-5′), 7.69 (d, $^3J$ (H,H) = 8.0 Hz, 2H; H-3, H-3′), 7.88 (t, $^3J$ (H,H) = 8.0 Hz, 2H; H-4, H-4′), 13.19 ppm (s, 2H; –OH); $^{13}$C NMR (100 MHz, $CD_3COCD_3$): $\delta$ = 24.1, 119.8, 128.8, 136.4, 138.6, 152.4, 159.5 ppm; MS (EI) *m*/*z*: 241.9 [MP$^+$].

6,6′-Dimethoxy-2,2′-pyridoin (**3**): 30% yield from 6-methoxy-2-pyridinecarboxaldehyde. m.p. 195–197 °C; $^1$H NMR (400 MHz, $CD_3COCD_3$): $\delta$ = 3.99 (s, 6H; –$OCH_3$), 6.78 (d, $^3J$ (H,H) = 8.0 Hz, 2H; H-5, H-5′), 7.46 (d, $^3J$ (H,H) = 8.0 Hz, 2H; H-3, H-3′), 7.89 (t, $^3J$ (H,H) = 8.0 Hz, 2H; H-4, H-4′), 12.25 ppm (s, 2H; –OH); $^{13}$C NMR (100 MHz, $CD_3COCD_3$): $\delta$ = 53.6, 108.9, 112.0, 134.5, 140.9, 152.4, 161.3 ppm; MS (EI) *m*/*z*: 273.9 [M$^+$].

6,6′-Diacetyl-2,2′-pyridoin (**4**): 50% yield from 6-acetyl-2-pyridinecarboxaldehyde. m.p. 202–204 °C (decomp.); $^1$H NMR (400 MHz, $CD_3COCD_3$): $\delta$ = 2.74 (s, 6H; –$COCH_3$), 8.00 (dd, $^3J$ (H,H) = 8.0 Hz, $^4J$ (H,H) = 1.2 Hz, 2H; H-3, H-3′), 8.17 (dd, $^3J$ (H,H) = 8.0 Hz, $^4J$(H,H) = 1.2 Hz, 2H; H-5, H-5′), 8.24 (t, $^3J$ (H,H) = 8.0 Hz, 2H; H-4, H-4′), 12.77 ppm (s, 2H; –OH); $^{13}$C NMR (150 MHz, DMSO-$d_6$): $\delta$ = 26.1, 120.5, 122.8, 135.1, 139.9, 149.4, 154.6, 196.6 ppm; MS (EI) *m*/*z*: 297.9 [M$^+$].

5,5′-Dimethyl-2,2′-pyridoin (**5**): 15% yield from 5-methyl-2-pyridinecarboxaldehyde. m.p. 198–201 °C; $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ = 2.36 (s, 6H; –$CH_3$), 7.69 (d, $^3J$ (H,H) = 8.0 Hz, 2H; H-3, H-3′), 7.84 (dd, $^3J$ (H,H) = 8.0 Hz, $^4J$ (H,H) = 1.6 Hz, 2H; H-4, H-4′), 8.43 (s, 2H; H-6, H-6′), 12.92 (s, 2H; –OH) ppm; $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ = 17.8, 118.4, 131.3, 134.4, 138.7, 146.1, 153.0 ppm; MS (EI) *m*/*z*: 242 [M$^+$].

5,5′-Dimethoxy-2,2′-pyridoin (**6**): 48% yield from 5-methoxy-2-pyridinecarboxaldehyde. m.p. 162–164 °C; $^1$H NMR (400 MHz, $CD_3COCD_3$): $\delta$ = 3.96 (s, 6H; –$OCH_3$), 7.58 (dd, $^3J$ (H,H) = 9.2 Hz, $^4J$ (H,H) = 2.8 Hz, 2H; H-4, H-4′), 7.79 (d, $^3J$ (H,H) = 9.2 Hz, 2H; H-3, H-3′), 8.29 (d, $^4J$ (H,H) = 2.8 Hz, 2H; H-6, H-6′), 12.41 ppm (s, 2H; –OH); $^{13}$C NMR (100 MHz, $CD_3COCD_3$): $\delta$ = 56.3, 120.7, 123.8, 134.2, 134.6, 150.4, 155.0 ppm; MS (EI) *m*/*z*: 274 [M$^+$].

5,5′-Diacetyl-2,2′-pyridoin (**7**): 13% yield from 5-acetyl-2-pyridinecarboxaldehyde. m.p. 199–202 °C; $^1$H NMR (400 MHz, $CD_3COCD_3$): $\delta$ = 2.70 (s, 6H; –$COCH_3$), 8.03 (d, $^3J$ (H,H) = 8.4 Hz, 2H; H-3, H-3′), 8.53 (dd, $^3J$ (H,H) = 8.4 Hz, $^4J$ (H,H) = 2.0 Hz, 2H; H-4, H-4′), 9.20 (d, $^4J$ (H,H) = 2.0 Hz, 2H; H-6, H-6′), 13.16 ppm (s, 2H; –OH); $^{13}$C NMR (150 MHz, DMSO-$d_6$): $\delta$ = 26.9, 119.3, 130.1, 136.8, 137.7, 147.2, 157.9, 196.0 ppm; MS (EI) *m*/*z*: 298.0 [M$^+$].

GO• was purchased from Acros (98%, New Jersey, USA). AAPH and trolox were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) and used as received. Other chemicals used were of analytical grade.

### Assay for GO•-scavenging activity

Typically, an aliquot of ArOHs at more than 10-fold excess of the concentration of GO• was added to a quartz cell (10 mm i.d.), which contained GO• (5 μM) in ethyl acetate. UV-visible spectra changes associated with this reaction were measured at 25 °C with a Hitachi 557 spectrophotometer. The rates of hydrogen transfer were determined by monitoring the absorbance change at 428 nm due to GO•. In the case of ethanol, the rates of the compounds **1**, **2**, **5**, **6** and VC were by second-order kinetics with the ratio of [ArOH] : [GO•] being 1 : 1.

The $IC_{50}$ value of ArOHs in the scavenging of GO• was determined by monitoring the absorbance of GO• (20 μM) at 428 nm in methanol during a 30 min observation.

### Assay for haemolysis

Human RBCs were separated from heparinized blood that was drawn from a healthy donor. The RBCs were washed with phosphate-buffered saline (PBS) at pH 7.4, and then centrifuged at 2000 rpm for 10 min to obtain a constantly packed cell volume. The 5% suspension of RBCs in PBS (pH 7.4) was incubated under air at 37 °C for 5 min, into which a PBS solution of AAPH was added to initiate haemolysis. The reaction mixture was shaken gently while being incubated at 37 °C. The extent of haemolysis was determined spectrophotometrically as described previously.[29] Briefly, aliquots of the reaction mixture were taken out at appropriate time intervals, diluted with 0.15 M NaCl, and centrifuged at 2000 rpm for 10 min to separate the RBCs. The percentage haemolysis was determined by measuring the absorbance of the supernatant at 540 nm and compared with that of complete haemolysis by treating the same RBC suspension with distilled water.

## Conclusions

This work demonstrates that α-pyridoin (**1**) and its derivatives (**2–7**) are effective antioxidants in the scavenging of GO• and inhibition of RBC haemolysis. The observation that the compounds (**5** and **6**) are the most reactive among the α-pyridoin derivatives and VC examined, gives us useful information for antioxidant drug design. Furthermore, the study on the reaction kinetics gives us important information for understanding the reactive mechanism of the unique enediol antioxidants.

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