



Synthesis and evaluation of paramagnetic caffeic acid phenethyl ester (CAPE) analogs

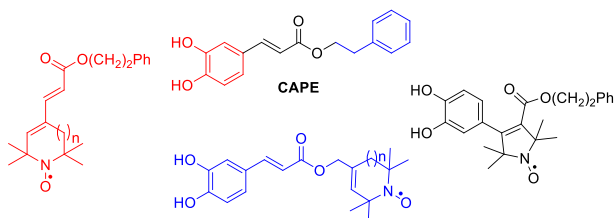
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Abstract

The structure–activity relationships of newly prepared caffeates, containing nitroxide moieties as potential antioxidants have been investigated and compared to those of known natural (caffeic acid phenethyl ester, CAPE) and unnatural (*N*-acetylcysteine, paramagnetic alcohols) antioxidants. The *in vitro* antioxidant activity was tested by 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging, while assays of cell protection against reactive oxygen species were carried out in the presence of 2',7'-dichlorofluorescein diacetate and H₂O₂. Paramagnetic esters without phenol motifs exhibited the lowest antioxidant activity, followed by paramagnetic alcohols with moderate activity. Among the compounds investigated, paramagnetic phenolic compounds were the best antioxidants. As the new paramagnetic CAPE analogs were less cytotoxic than CAPE at 10 μM in NIH3T3 fibroblast cells but had similar antioxidant activity, they can be considered promising antioxidants.

Graphic abstract



Keywords Heterocycles · Radicals · Structure–activity relationships · Nitroxides · Antioxidant

Introduction

Caffeic acid phenethyl ester [2-phenylethyl (*2E*)-3-(3,4-dihydroxyphenyl)acrylate; CAPE, **1**] is a phenolic compound isolated from the propolis of honeybee hives [1]. Although CAPE is hydrolyzed to caffeic acid *in vivo*, it has a better pharmacokinetic profile than that of caffeic acid [2]. CAPE

possesses many biological activities, such as antioxidant [3–5], anti-inflammatory [6, 7], immunomodulatory [8], antiviral, and neuroprotective activities (Fig. 1) [9, 10].

The phenylpropanoid scaffold of caffeic acid (3,4-dihydroxycinnamic acid) is commonly used as a template to develop caffeic acid derivatives and new chemical entities possessing antioxidant properties. Wang et al. investigated the relationship between catechol ring modifications (fluorination, altered numbers of hydroxyl groups) and the cytoprotective, cytotoxic, and antioxidant properties of CAPE [11, 12]. Their results showed that at least one hydroxyl group is important for cytoprotection and that the catechol ring is required for antioxidant activity. CAPE inhibits the HIV-1 integrase enzyme, and this activity was enhanced with the insertion of a third hydroxyl group into the catechol ring [13]. The modification of the phenethyl side also may be beneficial, as caffeic acid 4-aminophenethyl ester showed

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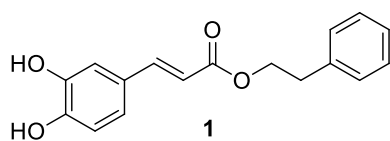


Fig. 1 Covalent structure of caffeic acid phenethyl ester (CAPE)

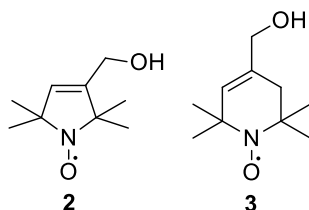


Fig. 2 Structure of paramagnetic alcohols to be investigated

better cytoprotection and 4-nitrophenethyl ester showed better cardioprotection, while the octyl ester showed better antileukemic activity than CAPE itself [14–16].

The unpaired electron of nitroxide stable free radicals allows them to take part in one-electron reactions such as oxidations and reductions, which makes nitroxides potent unnatural antioxidants [17]. Nitroxides may also function as intra- and extracellular superoxide dismutase (SOD) mimics or nonthiol radioprotectants [18–20]. Studies indicate that as antioxidants, nitroxides can exert cytoprotective action against oxidative stress induced by cytotoxic drugs or pathological processes such as ischemia–reperfusion and inflammation [21–23]. However, some piperidine-type nitroxides also exert pro-oxidative and cytotoxic effects, in particular on cancer cells [24, 25].

Several studies indicated that biomolecules (resveratrol, curcumin) modified with nitroxides had a beneficial influence on their activity as supplemented by ‘*in statu nascendi* acting’ antioxidants and radical scavengers [26–30]. Herein, we report the synthesis, antioxidant and cytoprotective activity of new CAPE analogs compared with those of the parent compound (CAPE) and regular antioxidant standards, such as *N*-acetylcysteine and Trolox. The antioxidant and cell protection activity of paramagnetic building block

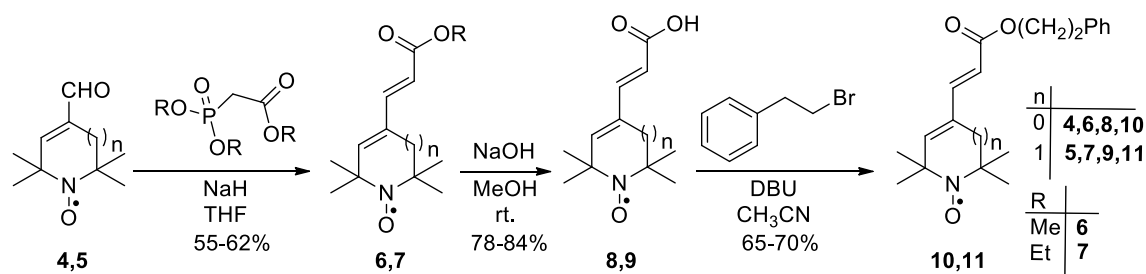
alcohols 3-(hydroxymethyl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-oxyl (**2**), 4-(hydroxymethyl)-2,2,6,6-tetramethyl-5,6-dihydropyridin-1(2*H*)-oxyl (**3**) (Fig. 2) were also evaluated [31, 32]. To obtain new paramagnetic analogs of CAPE, three strategies were followed: (1) replacement of the catechol ring with nitroxides, (2) replacement of the phenethyl group with nitroxide rings, and (3) incorporation the pyrroline radical in the caffeic acid scaffold.

Results and discussion

The Horner–Wadsworth–Emmons reaction of paramagnetic aldehydes 1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-carbaldehyde (**4**) [31] with triethyl phosphonoacetate and 1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridine-4-carbaldehyde (**5**) [32] with trimethyl phosphonoacetate led to the formation of paramagnetic α,β -unsaturated esters ethyl (*E*)-3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)acrylate (**6**) [33] and methyl (*E*)-3-(1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)acrylate (**7**), respectively. Their hydrolysis was conducted at room temperature for 2 h with a 10% aq. NaOH/methanol solution (2.0 equiv. NaOH) to yield, after acidification with aq. H₂SO₄, carboxylic acids (*E*)-3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)acrylic acid (**8**) [31] and (*E*)-3-(1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)acrylic acid (**9**), respectively. Then, the paramagnetic carboxylic acids were treated with 2-phenethyl bromide in dry acetonitrile in the presence of 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) to obtain compounds phenethyl (*E*)-3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)acrylate (**10**) and phenethyl (*E*)-3-(1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)acrylate (**11**), in which the catechol ring of CAPE is replaced with dihydropyrrole- or tetrahydropyridine-type nitroxides (Scheme 1).

For the replacement of the phenethyl moiety of CAPE, the first step was to alkylate (*E*)-3-(3,4-diacetoxyphenyl)acrylic acid (**12**) [34] with paramagnetic allylic bromides 3-(bromomethyl)-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxy (**13**) [35] and 4-(bromomethyl)-2,2,6,6-tetramethyl-5,6-dihydropyridin-1-yloxy (**14**) [36].

Scheme 1



The alkylation was conducted in CH_3CN in the presence of DBU base at ambient temperature to give compounds (1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-yl)-methyl (*E*)-3-(3,4-diacetoxyphenyl)acrylate (**15**) and (1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)-methyl (*E*)-3-(3,4-diacetoxyphenyl)acrylate (**16**) in 60–71% yield. To obtain paramagnetic caffeic acid esters (1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-yl)methyl (*E*)-3-(3,4-dihydroxyphenyl)acrylate (**17**) and (1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)methyl (*E*)-3-(3,4-dihydroxyphenyl)acrylate (**18**), the acetyl protecting groups were removed by the addition of three equivalents K_2CO_3 in $\text{CH}_2\text{Cl}_2/\text{MeOH}$ mixture (Scheme 2).

To keep both catechol and phenethyl groups, the carbon–carbon double bond of CAPE seemed to be an accessible point for the synthesis of a new paramagnetic derivative. 4-Bromo-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-carboxylic acid (**19**) [37] was alkylated with 2-phenethyl bromide to yield phenethyl 4-bromo-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-carboxylate (**20**). The Suzuki reaction of **20** and 2-[3,4-bis(methoxymethoxy)phenyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**21**) [38] in the presence of $\text{Pd}(\text{PPh}_3)_4$ and Na_2CO_3 under a N_2 atmosphere afforded phenethyl 4-[3,4-bis(methoxymethoxy)phenyl]-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-carboxylate (**22**) in moderate yield. The methoxymethyl protecting groups were removed by refluxing in MeOH containing a catalytic amount of aq. HCl, resulting in nitroxide phenethyl 4-(3,4-dihydroxyphenyl)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-carboxylate (**23**) (Scheme 3).

The new nitroxides were tested in a Trolox-equivalent antioxidant capacity assay (TEAC), which monitors electron and proton donor activity [39, 40]. The method is based

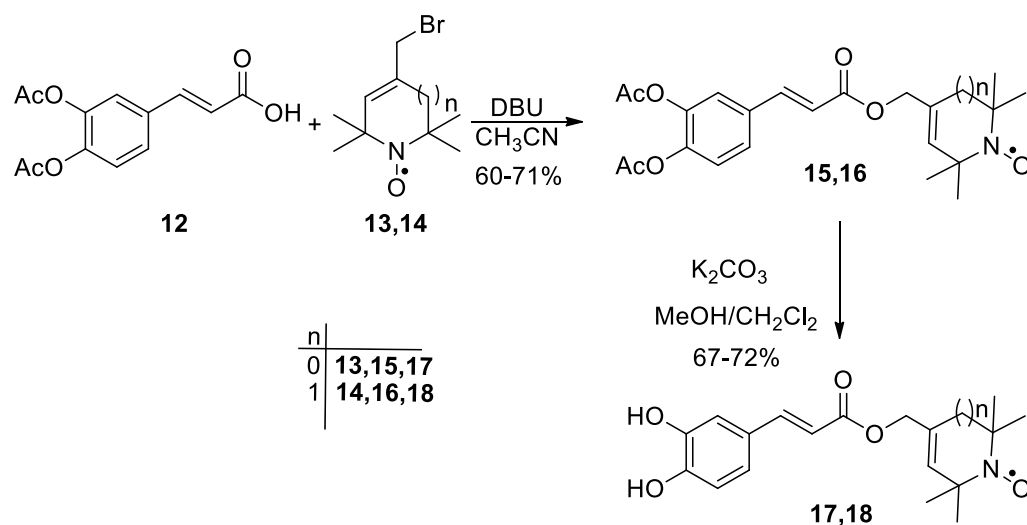
on the reduction of green-colored 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS^+), which is detected at 734 nm.

Our results suggest that the catechol functionality is necessary for good antioxidant activity, as the TEAC values of paramagnetic alcohols **2** and **3** nonphenolic esters **7**, **10**, and **11**; and acid **9** were below 0.2, while the caffeic acid derivatives CAPE (**1**), **17**, **18**, and **23** showed good antioxidant activity. Compounds **17** and **18** have even greater proton- and electron-donating properties than Trolox and CAPE. Nevertheless, in the case of compound **23**, the vicinity of nitroxide and catechol had adverse effect on antioxidant activity (Fig. 3), which can be the consequence of an intramolecular redox reaction between the nitroxide and catechol rings [41].

Cytotoxicity of CAPEs was analyzed by the WST assay. As shown in Fig. 4, 10 μM CAPE (**1**) decreased cellular viability (0.63 ± 0.09 , relative to DMSO), and the reference compound *N*-acetylcysteine (NAC) increased it (1.36 ± 0.24). We did not observe any statistical significance in the difference in cellular viability between the CAPE analogs and DMSO; thus, these new compounds have less cytotoxic activity than CAPE. Compounds **1**, **17**, **18**, and **23** exerted some toxicity toward fibroblasts, while the paramagnetic alcohols and nonphenolic esters were not toxic. This result suggests the responsibility of the catechol ring for cytotoxicity (Fig. 4), but this is still tolerable.

The intracellular oxidative stress within NIH3T3 cells was evaluated by 2',7'-dichlorofluorescein diacetate (DCFDA) [42, 43], which is taken up by the cells, wherein nonspecific esterases cleave the acyl groups, forming 2',7'-dichlorodihydrofluorescein (H_2DCF), which can be oxidized to fluorescent DCF by reactive oxygen species (ROS). Therefore, the limited formation of DCF (and fluorescence intensity)

Scheme 2



Scheme 3

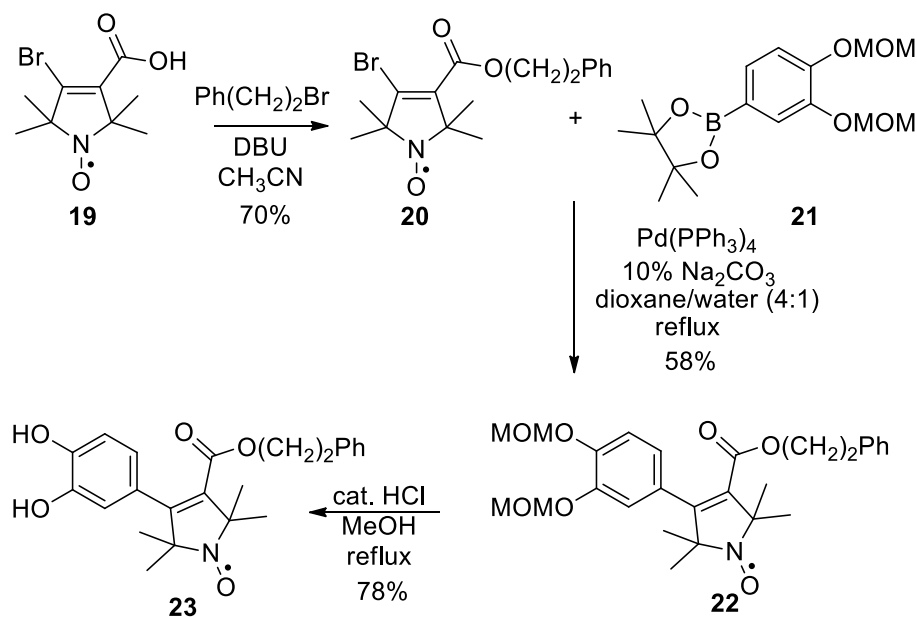
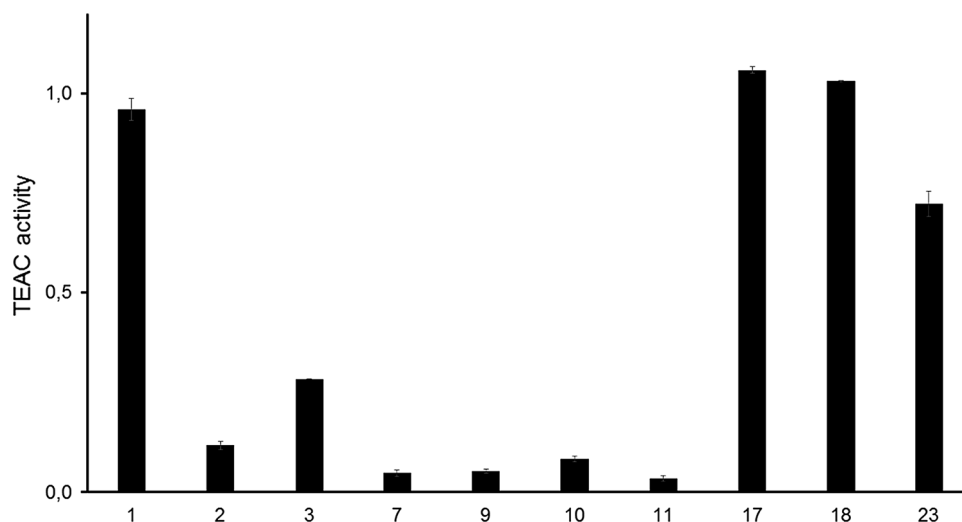


Fig. 3 Trolox equivalent antioxidant capacity (TEAC) presents the antioxidant activity of nitroxides measured by ABTS radical scavenging assay. Data represent the mean \pm SD (n=6)



indicates the enhanced ROS scavenging activity of the investigated compound. All of the studied compounds decreased the ROS formation in cells: CAPE (**1**), **17**, **18**, and NAC significantly decreased intracellular oxidative stress (Fig. 5). This activity cannot be attributed to the presence of nitroxide functionality, as compounds **2**, **3**, and **23** were not active, but retaining the original caffeic acid ester scaffold was advantageous, as in the case of compounds **1**, **17**, and **18**.

Reactive oxygen species are involved in the pathogenesis of many diseases. The hydroxyl radical is the most dangerous ROS and can be generated from H_2O_2 in the presence of transition metal ions including Fe^{2+} and Cu^+ in certain processes. To

confirm the antioxidant properties of new caffeic acid phenethyl ester analogs, the cytoprotective effect of the paramagnetic CAPEs against hydrogen peroxide (H_2O_2) was tested. H_2O_2 treatment decreased cellular viability (0.41 ± 0.08), but CAPE (**1**), **17**, and **23**, containing caffeic acid ester scaffold, prevented H_2O_2 -induced cytotoxicity. It is interesting to note that compounds **7**, **9**, **10**, and **11** did not exhibit antioxidant protection in this assay, while alcohols **2**, **3** and compound **18** produced a moderate protective effect (Fig. 6).

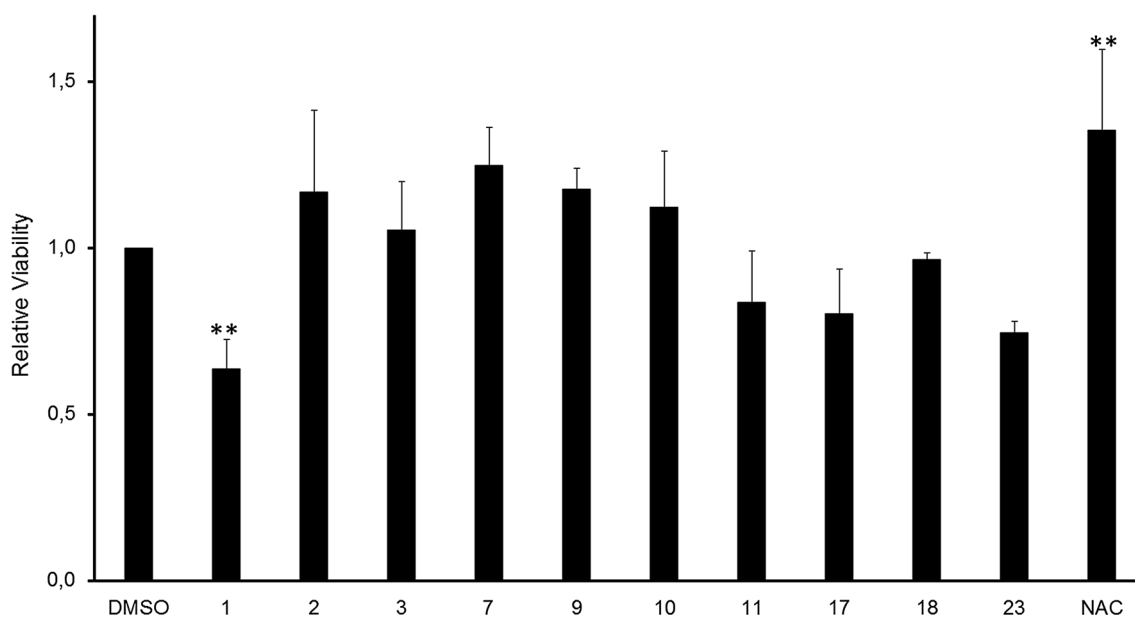


Fig. 4 Cytotoxicity of CAPEs were analyzed by WST assay. Cellular viability of NIH3T3 cells treated with nitroxides, CAPE or NAC (10 μ M) for 24 h. Data represent the mean \pm SD ($n=6$). *Statistically significant ($p < 0.05$) when compared to control

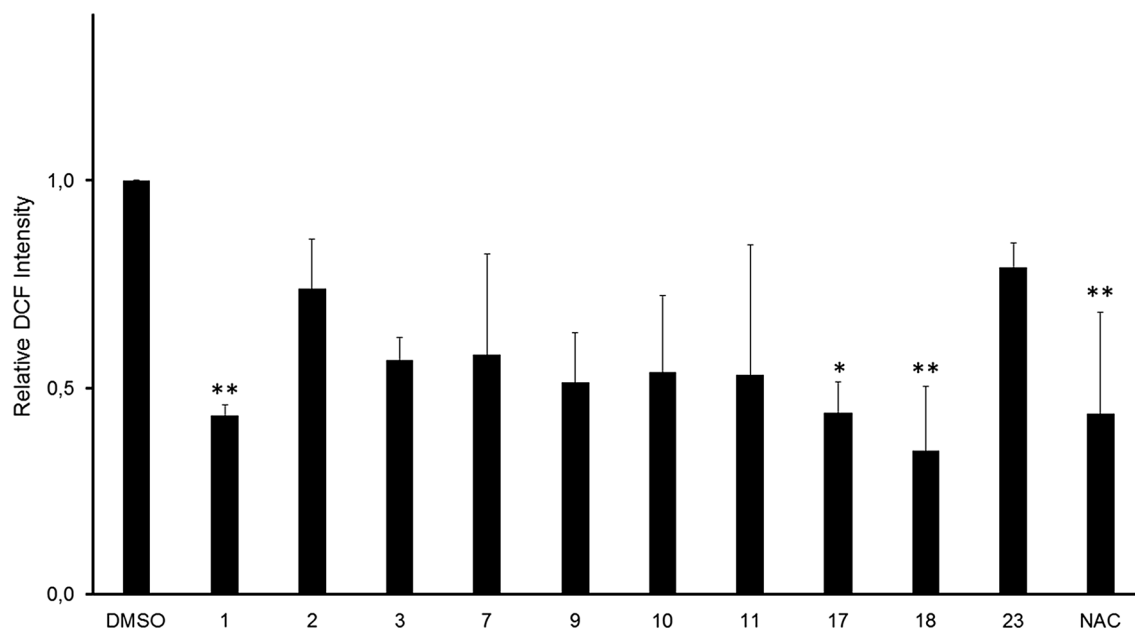


Fig. 5 Intracellular oxidative stress was evaluated by the fluorescence intensity of 2',7'-dichlorofluorescein. Cells were treated with 10 μ M each compound for 24 h. After treatment, cells were stained with

20 μ M DCFDA for 30 min at 37 $^{\circ}$ C. Data represent the mean \pm SD ($n=6$). *Statistically significant ($p < 0.05$) when compared to control

Conclusion

Three structural fragments of caffeic acid phenethyl ester were modified with nitroxide free radicals to study their antioxidant structure–activity relationships. The main

modifications were the replacement of the catechol ring or phenethyl group with 5- and 6-membered nitroxides and the incorporation of pyrrolidine type of nitroxide into the CC double bond of the acrylate. The toxicity and antioxidant properties of the new compounds were compared to those of CAPE (**1**) and regular antioxidant standards.

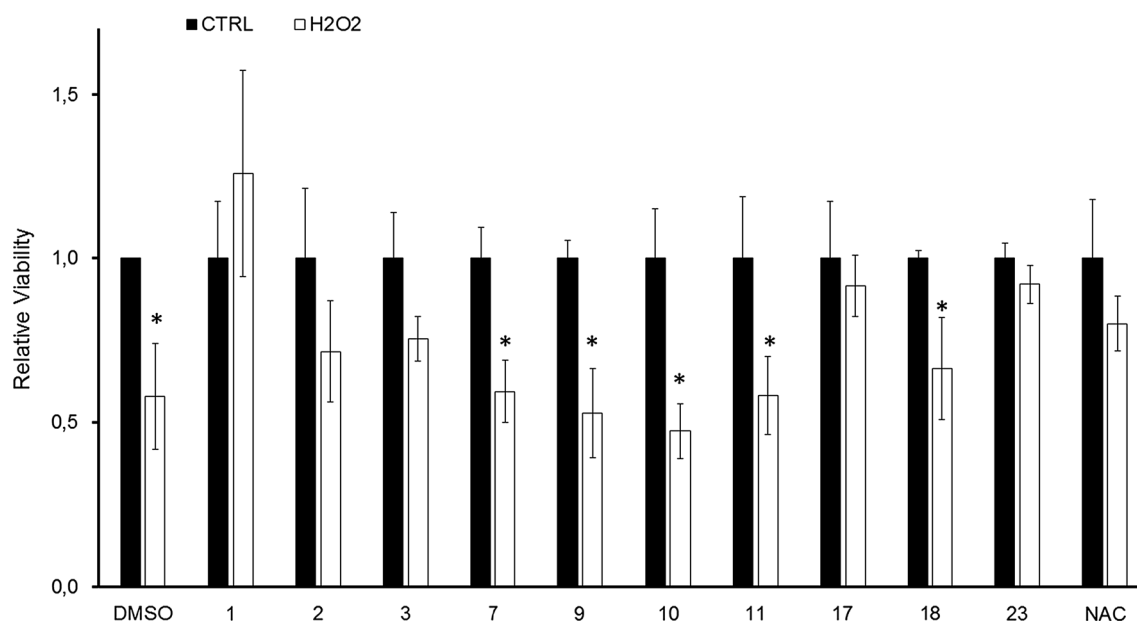


Fig. 6 To determine the cytoprotective activity of CAPEs toward H₂O₂ were analyzed using WST assay. Cells were treated with H₂O₂ (500 μM) with CAPEs (10 μM), respectively, for 24 h. Data represent the mean ± SD (*n* = 6). *Statistically significant (*p* < 0.05) when compared to control

Regarding the ABTS assay, paramagnetic nonphenolic esters **7**, **10**, and **11** and alcohols **2** and **3** have poor ABTS⁺ scavenging activity, which is not surprising because esters can be electron donors but not proton donors. As expected, compounds **1**, **17**, **18**, and **23** were found to be good proton and electron donor antioxidants.

The results of the cytotoxicity assays suggest that the catechol ring motif increases cytotoxicity, but not significantly. The most toxic compounds were CAPE (**1**) and compound **23**, but when nitroxides were the esterifying groups, as in the case of compounds **17** and **18**, the toxicity of the resulting compound was lower than that of **1** and **23**. In DCF assays, compounds **1**, **17**, and **18** were the most active in intracellular ROS scavenging and had the same or slightly greater antioxidant effects than NAC. In cell viability assays performed under H₂O₂-induced stress, compounds **1**, **17**, and **23** were the most protective molecules, as all showed better activity than the standard (NAC). However, the results of the assays of nitroxide derivatives were contradictory, as compounds **17** and **18** with nitroxide and catechol structural units were the best proton and electron donor antioxidants and the most protective against H₂O₂-induced cytotoxicity.

These findings support our hybrid drug design strategy and are in good accordance with our previous findings. The caffeic acid moiety should remain intact, and proper esterification, in our case with nitroxides, decreases its cytotoxicity while simultaneously improving its protective activity against H₂O₂-induced oxidative stress. Our results can be a good starting point in the search for new caffeic acid-based lead compounds, which is in progress in our laboratories.

Experimental

Elemental analyses were performed on a Fisons EA 1110 CHNS elemental analyzer, and the results were found to be in good agreement (±0.3%) with the calculated values. Melting points were determined with a Boetius micro-melting point apparatus. Mass spectra were recorded on a Thermoquest Automass Multi in EI mode (70 eV). ¹H NMR spectra were recorded with Bruker Avance 3 Ascend 500 at 500 MHz and ¹³C NMR spectra at 125 MHz in CDCl₃ or DMSO-*d*₆ at 298 K in the presence of five equivalents of hydrazobenzene (DPPH/radical). The IR spectra were taken with a Bruker Alpha FT-IR instrument with ATR support on a ZnSe plate. Spectroscopic measurements were performed on Specord 40 UV/VIS Spectrophotometer at 732 nm. Flash column chromatography was performed on Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially available plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel GF₂₅₄. Compounds **1** [34], **2** [31], **3** [32], **4** [31], **5** [32], **12** [33], **13** [34], **14** [35], **19** [36], and **21** [37] were synthesized according to published procedures. All other reagents were purchased from Aldrich or Molar Chemicals.

(E)-Methyl 3-(1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)acrylate (7, C₁₃H₂₀NO₃) To a stirred suspension of 0.3 g NaH (12.5 mmol) in 10 cm³ THF 910 mg trimethyl phosphonoacetate (5.0 mmol) was added at 0 °C under a N₂ atmosphere. After stirring for 10 min, 910 mg aldehyde **5** (5.0 mmol) was added, and the mixture was

stirred and refluxed for another 30 min. After cooling to room temperature, 20 cm³ brine and 20 cm³ ether were added, the organic phase was separated, the aqueous phase was washed with 2 × 20 cm³ ether, and then the combined organic phase was dried (MgSO₄), filtered, evaporated, and the residue was purified by flash column chromatography to give 655 mg (55%) **7** as a pale orange solid. M.p.: 66–68 °C; *R*_f=0.55 (hexane–EtOAc, 2:1); ¹H NMR (500 MHz, CDCl₃ + (PhNH)₂): δ = 7.38 (d, *J* = 15.8 Hz, 1H), 5.93 (s, 1H), 5.86 (d, *J* = 15.7 Hz, 1H), 3.82 (s, 3H), 2.26 (s, 2H), 1.35 (s, 6H), 1.26 (s, 6H) ppm; ¹³C NMR (125 MHz, CDCl₃ + (PhNH)₂): δ = 167.7, 146.8, 144.3, 128.5, 116.1, 60.1, 57.2, 51.6, 38.9, 25.9, 25.1 ppm; IR (neat): $\bar{\nu}$ = 1712, 1642, 1624 cm⁻¹; MS (70 eV): *m/z* (%) = 238 (M⁺, 93), 208 (28), 193 (30), 149 (100).

(E)-3-(1-Oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)acrylic acid (9, C₁₂H₁₈NO₃) To a solution of 1.19 g **7** (5.0 mmol) in 30 cm³ MeOH, 4 cm³ aq. 10% NaOH (10.0 mmol) was added, and the mixture stirred for 2 h at ambient temperature. The mixture was diluted with 10 cm³ water, the methanol was evaporated off, and the residue pH was adjusted to 2 at 0 °C by adding aq. 5% H₂SO₄ solution. The acid formed was extracted with 2 × 2 cm³ CHCl₃, and the combined organic phase was dried (MgSO₄), filtered, and evaporated. The residue was purified by flash column chromatography (hexane–EtOAc, 2:1) to give 870 mg (78%) compound **9** as a yellow solid. M.p.: 156–158 °C; *R*_f=0.25 (CHCl₃–Et₂O, 2:1); ¹H NMR (500 MHz, CDCl₃ + (PhNH)₂): δ = 7.25 (d, *J* = 16 Hz, 1H), 6.01 (d, *J* = 15.7 Hz, 1H), 5.84 (s, 1H), 2.59 (s, 2H), 1.49 (s, 6H), 1.42 (s, 6H) ppm; ¹³C NMR (125 MHz, CDCl₃ + (PhNH)₂): δ = 173.6, 142.1, 136.5, 125.5, 123.6, 64.4, 62.3, 36.5, 23.9 (brs, 4C) ppm; IR (neat): $\bar{\nu}$ = 3250–2500, 1671, 1613 cm⁻¹; MS (70 eV): *m/z* (%) = 224 (M⁺, 76), 194 (32), 179 (72), 149 (100).

General procedure for the synthesis of phenethyl esters **10**, **11**, **20**

To the solution of 212 mg **8** or 224 mg **9** or 263 mg **19** (1.0 mmol) in 5 cm³ CH₃CN, 167 mg DBU (1.1 mmol), and 277 mg (2-bromoethyl)benzene (1.1 mmol) were added, and the mixture was allowed to react for 24 h at room temperature. Then, the solvent was evaporated, and the residue was purified by flash column chromatography (hexane–EtOAc, 4:1) to give the title compounds in 65–70% yield.

Phenethyl (E)-3-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)acrylate (10, C₁₉H₂₄NO₃) Dark yellow solid; yield 233 mg (70%); m.p.: 61–63 °C; *R*_f=0.59 (hexane–EtOAc, 2:1); ¹H NMR (500 MHz, CDCl₃ + (PhNH)₂): δ = 7.37 (t, *J* = 6.9 Hz, 2H), 7.26–7.22 (m, 3H), 6.15 (d, *J* = 16.3 Hz, 1H), 5.99 (s, 1H), 4.46 (t, *J* = 7.1 Hz, 2H), 3.05

(t, *J* = 7.1 Hz, 2H), 1.41 (s, 6H), 1.32 (s, 6H) ppm; ¹³C NMR (125 MHz, CDCl₃ + (PhNH)₂): δ = 166.9, 141.2, 140.1, 138.6, 137.9, 129.4, 128.6, 126.7, 119.9, 69.7, 67.7, 65.1, 35.3, 25.5, 24.9 ppm; IR (neat): $\bar{\nu}$ = 1698, 1603, 1455 cm⁻¹; MS (70 eV): *m/z* (%) = 314 (M⁺, 6), 299 (5), 284 (18), 105 (100), 91 (44).

Phenethyl (E)-3-(1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)acrylate (11, C₂₀H₂₆NO₃) Orange solid; yield 215 mg (65%); m.p.: 60–61 °C; *R*_f=0.60 (hexane–EtOAc, 2:1); ¹H NMR (500 MHz, CDCl₃ + (PhNH)₂): δ = 7.37 (d, *J* = 6.6 Hz, 2H), 7.27 (t, *J* = 7.3 Hz, 3H), 5.91 (s, 1H), 5.83 (d, *J* = 15.8 Hz, 1H), 4.44 (t, *J* = 7.1 Hz, 2H), 3.04 (t, *J* = 7.1 Hz, 2H), 2.24 (s, 2H), 1.34 (s, 6H), 1.25 (s, 6H) ppm; ¹³C NMR (125 MHz, CDCl₃ + (PhNH)₂): δ = 167.2, 146.8, 144.3, 137.9, 129.4, 128.55, 128.53, 126.6, 116.3, 64.9, 60.1, 57.2, 38.9, 35.3, 26.1, 25.0 ppm; IR (neat): $\bar{\nu}$ = 1705, 1636, 1617 cm⁻¹; MS (70 eV): *m/z* (%) = 328 (M⁺, 2), 179 (5), 149 (31), 105 (100).

Phenethyl 4-bromo-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-carboxylate (20, C₁₇H₂₁BrNO₃) Yellow solid; yield 256 mg (70%); m.p.: 77–79 °C; *R*_f=0.71 (hexane–EtOAc, 2:1); ¹H NMR (500 MHz, CDCl₃ + (PhNH)₂): δ = 7.32 (t, *J* = 7.4 Hz, 2H), 4.46 (t, *J* = 7.1 Hz, 2H), 3.04 (t, *J* = 7.1 Hz, 2H), 1.34 (s, 6H), 1.32 (s, 6H) ppm; ¹³C NMR (125 MHz, CDCl₃ + (PhNH)₂): δ = 163.1, 137.6, 136.9, 134.3, 129.4, 128.9, 128.6, 126.7, 72.0, 70.6, 65.3, 35.0, 24.5, 24.4 ppm; IR (neat): $\bar{\nu}$ = 1704, 1624, 1592 cm⁻¹; MS (70 eV): *m/z* (%) = 368 (M⁺, 15), 262 (5), 232 (6), 105 (100), 91 (83).

General procedure for the synthesis of protected, paramagnetic caffeic acids **15**, **16**

Caffeic acid diacetate **12** (2.64 g, 10.0 mmol), 1.67 g DBU (11.0 mmol), and 2.33 g **13** or 2.47 g **14** (10.0 mmol) were dissolved in 15 cm³ CH₃CN and the mixture was allowed to react for 24 h at ambient temperature. The solvent was then evaporated, and the residue was purified by flash column chromatography (hexane–EtOAc, 4:1) to give the title compounds in 60–71% yield.

(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-yl)methyl (E)-3-(3,4-diacetoxyphenyl)acrylate (15, C₂₂H₂₆NO₇) Pale yellow solid; yield 2.95 g (71%); m.p.: 132–135 °C; *R*_f = 0.59 (CHCl₃–Et₂O, 2:1); ¹H NMR (500 MHz, CDCl₃ + (PhNH)₂): δ = 7.73 (d, *J* = 16 Hz, 1H), 7.48–7.45 (m, 2H), 6.48 (d, *J* = 16 Hz, 1H), 5.69 (s, 1H), 4.81 (s, 2H), 2.37 (2 s, 6H), 1.37 (s, 6H), 1.33 (s, 6H) ppm; ¹³C NMR (125 MHz, CDCl₃ + (PhNH)₂): δ = 168.0, 167.9, 166.2, 143.7, 143.3, 142.6, 139.3, 133.2, 132.4, 126.5, 123.9, 122.8, 119.9, 69.9, 67.8, 60.8, 25.6, 24.6, 20.6 ppm;

IR (neat): $\bar{\nu}$ = 1766, 1713, 1634 cm^{-1} ; MS (70 eV): m/z (%) = 416 (M^+ , 10), 317 (2), 205 (16), 105 (75), 43 (100).

(1-Oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)methyl (E)-3-(3,4-diacetoxyphenyl)acrylate (16, $\text{C}_{23}\text{H}_{28}\text{NO}_7$) Orange solid; yield 2.58 g (60%); m.p.: 57–59 °C; R_f = 0.64 (CHCl_3 – Et_2O , 2:1); ^1H NMR (500 MHz, CDCl_3 + $(\text{PhNH})_2$): δ = 7.72 (d, J = 15.9 Hz, 1H), 7.46–7.43 (m, 2H), 6.48 (d, J = 15.9 Hz, 1H), 5.61 (s, 1H), 4.65 (s, 2H), 2.34 (2 s, 6H), 2.18 (s, 2H), 1.34 (s, 6H), 1.28 (s, 6H) ppm; ^{13}C NMR (125 MHz, CDCl_3 + $(\text{PhNH})_2$): δ = 168.1, 167.9, 166.4, 143.6, 143.1, 142.5, 133.5, 133.3, 126.6, 126.5, 123.9, 122.8, 119.2, 67.7, 59.5, 57.4, 40.8, 26.17, 25.12 ppm; IR (neat): $\bar{\nu}$ = 1765, 1709, 1503 cm^{-1} ; MS (70 eV): m/z (%) = 430 (M^+ , 2), 247 (9), 205 (36), 163 (38), 121 (79), 43 (100).

General procedure for the synthesis of paramagnetic caffeic acids 17, 18

15 (2.08 g) or **16** (2.15 g) (5.0 mmol) was dissolved in 20 cm^3 MeOH – CH_2Cl_2 (1:1), 2.07 g K_2CO_3 (15.0 mmol) was added, and the mixture was stirred at 20 °C for 1 h. The solvent was evaporated, and the residue was partitioned between 10 cm^3 5% H_2SO_4 and 20 cm^3 EtOAc . After separation, the water phase was extracted with additional 2 \times 20 cm^3 EtOAc . The combined organic phases were dried over MgSO_4 , filtered, and evaporated, and the residue was purified by column chromatography (hexane– EtOAc) to obtain paramagnetic caffeic acids in 67–72% yield.

(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-yl)-methyl (E)-3-(3,4-dihydroxyphenyl)acrylate (17, $\text{C}_{18}\text{H}_{22}\text{NO}_5$) Yellow solid; yield 1.19 g (72%); m.p.: 123–125 °C; R_f = 0.36 (CHCl_3 – Et_2O , 2:1); ^1H NMR (500 MHz, $\text{DMSO}-d_6$ + $(\text{PhNH})_2$): δ = 7.03 (d, J = 5.1 Hz, 1H), 6.81 (d, J = 6.2 Hz, 1H), 6.6 (s, 1H), 6.34 (d, J = 15.3 Hz, 1H), 5.60 (s, 1H), 5.83 (d, J = 15.8 Hz, 1H), 4.69 (s, J = 7 Hz, 2H), 1.16, 1.12 (2 s, 12H) ppm; ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$ + $(\text{PhNH})_2$): δ = 166.6, 150.4, 146.1, 145.9, 140.3, 125.9, 121.9, 118.2, 116.3, 115.4, 114.2, 68.9, 66.9, 60.4, 26.1, 25.0 ppm; IR (neat): $\bar{\nu}$ = 3480–2700, 1717, 1603 cm^{-1} ; MS (70 eV): m/z (%) = 332 (M^+ , 3), 317 (1), 182 (72), 107 (100).

(1-Oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)methyl (E)-3-(3,4-dihydroxyphenyl)acrylate (18, $\text{C}_{19}\text{H}_{24}\text{NO}_5$) Orange-brownish solid; yield 1.16 g (67%); m.p.: 74–76 °C; R_f = 0.37 (CHCl_3 – Et_2O , 2:1); ^1H NMR (500 MHz, CDCl_3 + $(\text{PhNH})_2$): δ = 7.12 (s, 1H), 7.1 (d, J = 7.5 Hz, 1H), 6.28 (d, J = 15.8 Hz, 1H), 5.63 (s, 1H), 4.61 (s, 2H), 1.73 (s, 2H), 1.40 (s, 6H), 1.32 (s, 6H) ppm; ^{13}C NMR (125 MHz, CDCl_3 + $(\text{PhNH})_2$): δ = 167.4, 147.3, 145.8, 144.7, 131.5, 127.3, 127.1, 122.5, 115.6, 114.7,

114.4, 67.1, 61.5, 59.5, 39.7, 25.6, 24.5 ppm; IR (neat): $\bar{\nu}$ = 3500–2800, 1694, 1597 cm^{-1} ; MS (70 eV): m/z (%) = 346 (M^+ , 4), 307 (5), 234 (26), 163 (100).

Phenethyl 4-[3,4-bis(methoxymethoxy)phenyl]-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate (22, $\text{C}_{27}\text{H}_{34}\text{NO}_7$) To a deoxygenated solution of 545 mg **20** (1.5 mmol) in 20 cm^3 dioxane/water (4:1), 75 mg $\text{Pd}(\text{PPh}_3)_4$ (0.067 mmol), 490 mg pinacolate **21** (1.5 mmol), and 5 cm^3 10% Na_2CO_3 solution were added under N_2 gas, and then the reaction mixture was stirred and refluxed for 3 h. After consumption of the starting materials (followed by TLC), dioxane was removed by vacuum evaporation, 10 cm^3 H_2O was added, and the aqueous phase was extracted with 2 \times 20 cm^3 CHCl_3 . The combined organic phase was dried (MgSO_4), filtered, and evaporated. The residue was purified by flash column chromatography (hexane– EtOAc , 2:1), to offer 280 mg (58%) **22** as a brown oil. R_f = 0.38 (hexane– EtOAc 2:1); ^1H NMR (500 MHz, CDCl_3 + $(\text{PhNH})_2$): δ = 7.33 (t, J = 7.5 Hz, 2H), 7.21 (d, J = 8.3 Hz, 1H), 7.14 (d, J = 7.2 Hz, 2H), 7.07 (s, 1H), 6.82 (d, J = 6.4 Hz, 1H), 5.30 (2 s, 4H), 4.24 (t, J = 7.2 Hz, 2H), 3.58 (d, J = 3.3 Hz, 6H), 2.71 (t, J = 7.2 Hz, 2H), 1.54 (s, 6H), 1.35 (s, 6H) ppm; ^{13}C NMR (125 MHz, CDCl_3 + $(\text{PhNH})_2$): δ = 164.7, 154.4, 147.1, 146.6, 137.8, 133.3, 129.9, 128.8, 128.5, 126.5, 121.2, 117.4, 116.1, 95.8, 95.5, 71.4, 69.2, 64.6, 56.28, 34.7, 24.8, 24.3 ppm; IR (neat): $\bar{\nu}$ = 1707, 1602, 1506 cm^{-1} ; MS (70 eV): m/z (%) = 484 (M^+ , 6), 470 (1), 274 (30), 105 (100).

Phenethyl 4-(3,4-dihydroxyphenyl)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate (23, $\text{C}_{23}\text{H}_{26}\text{NO}_5$) 484 mg **22** (1.0 mmol) was dissolved in 10 cm^3 MeOH , then concentrated 0.1 cm^3 aq. HCl was added, and the mixture was refluxed for 10 min. After removal of the solvent, 10 cm^3 water was added, and the residue was neutralized with K_2CO_3 . EtOAc (20 cm^3) was added, the phases were separated, and the aqueous phase was extracted with 2 \times 20 cm^3 EtOAc . The combined organic phase was dried (MgSO_4), filtered, evaporated, and purified by flash chromatography (hexane– EtOAc 2:1) to give 310 mg (78%) title compound **23** as a brown oil. R_f = 0.57 (CHCl_3 – Et_2O , 2:1); ^1H NMR (500 MHz, CDCl_3 + $(\text{PhNH})_2$): δ = 7.09 (d, J = 7.3 Hz, 2H), 6.69 (s, 1H), 6.61 (s, 1H), 4.21 (t, J = 6.8 Hz, 2H), 2.69 (t, J = 6.7 Hz, 2H), 1.51 (s, 6H), 1.31 (2 s, 6H) ppm; ^{13}C NMR (125 MHz, CDCl_3 + $(\text{PhNH})_2$): δ = 164.3, 154.7, 144.2, 143.2, 137.6, 132.7, 128.8, 128.5, 127.3, 126.5, 119.5, 115.7, 114.9, 71.7, 69.7, 64.8, 34.7, 24.8, 23.8 ppm; IR (neat): $\bar{\nu}$ = 3600–2850, 1703, 1601, 1515 cm^{-1} ; MS (70 eV): m/z (%) = 396 (M^+ , 49), 381 (5), 366 (6), 105 (100).

ABTS scavenging assay

The measurements were taken on a Specord 40 instrument. ABTS was dissolved in PBS buffer (0.136 M NaCl, 0.0027 M KCl, 0.01 M Na₂HPO₄, 0.00176 M KH₂PO₄) to a 7-mM concentration. ABTS radical cation (ABTS^{•+}) was produced by reacting ABTS stock solution with potassium persulfate at a final concentration of 2.45 mM and allowing the mixture to stand in the dark at room temperature for 16 h before use. For the study of compounds, the ABTS^{•+} solution was diluted with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 37 °C. Stock solutions of new compounds and Trolox in DMSO were added to the diluted ABTS^{•+} solution, in final concentrations of 12.5, 10, 7.5, 2.5 μ M. After addition, the mixtures were incubated for 6 min at 37 °C before measuring their absorbance at 734 nm. All determinations were carried out three times. The percentage inhibition of absorbance at 734 nm is calculated with the usual formula: $(A_0 - A_{\text{antioxidant}})/A_0$, where A_0 is the absorbance of the diluted ABTS^{•+} solution. The concentration–response curves of new compounds were compared with the curve of Trolox.

Cell viability assays

Murine embryonic fibroblast NIH3T3 cells were maintained in DMEM (Cat no. 043-30085, Wako Pure Chemical, Osaka, Japan) supplemented with 10% newborn calf serum (NBCS) at 37 °C, 5% CO₂.

To determine the toxicity of the compounds, a WST-1 viability assay was employed as described previously. NIH3T3 cells (2000 per well) were seeded into all wells of 96-well plates. After 24-h incubation, the medium was replaced with CAPEs-containing medium (10 μ M) for 24 h. Then, the cells were treated with WST-1 solution [3.6 μ g/mm³ WST-1, 70 ng/mm³ 1-methoxyphenazine methosulfate in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH (pH 7.4); Dojindo, Kumamoto, Japan]. The cells were incubated for 1 h at 37 °C, and the absorbance of each well was recorded at 440 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific).

To determine the cytoprotective activity of CAPEs against H₂O₂, NIH3T3 cells were treated with H₂O₂ (500 μ M) and CAPEs (10 μ M). After 24 h of incubation, cellular viability was analyzed as described above [44].

Intracellular ROS detection

Intracellular oxidative stress was evaluated by 2',7'-dichlorofluorescein diacetate (DCFDA; Cat NO. 6883, Sigma-Aldrich) staining as previously reported. NIH3T3 cells were treated with each compound at 10 μ M for 24 h. After treatment, the cells were stained with 20 μ M DCFDA for

30 min at 37 °C. Then, the cells were trypsinized, and their fluorescence intensity was analyzed using an EC800 Analyzer (Sony Corporation, Tokyo, Japan).

Statistical analyses were done by Dunnett's test.

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