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# Nitrone derivatives of trolox as neuroprotective agents

György T. Balogh,[a,*] Krisztina Vukics,[a,*] Árpád Könczöl,[b] Ágnes Kis-Varga,[a] Anikó Gere[a] and János Fischer[a]

[a]*Gedeon Richter Ltd, Budapest H-1475, 10. PO Box 27, Hungary*
[b]*Budapest University of Technology and Economics, Budapest, Műegyetem rkp. 3. H-1111, Hungary*

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**Abstract**—Synthesis of nitrone derivatives of trolox is described. Their biological evaluation was performed in vitro for scavenging different free radicals, inhibiting $Fe^{2+}$-induced lipid peroxidation, and in vivo in a permanent middle cerebral artery occlusion model in mice. New compounds exert pharmacological activities comparable to or better than those of trolox or nitrone-type reference compounds.


## 1. Introduction

Much evidence suggests that biological oxidation in the human body generates highly pathogenic free radicals and other reactive oxygen species such as hydroxyl radical (•OH), superoxide anion ($•O_2^-$), peroxynitrite ($ONOO^-$) and lipid peroxide radicals (ROO•), causing cellular injury. These pathological events have important roles in many degenerative disorders, for example, atherosclerosis, rheumatoid arthritis, and several neurodegenerative diseases such as Alzheimer's disease, Parkinson disease, ischemic conditions and stroke.[1] Therefore, protection against these toxic molecules would have a benefit in the treatment of the diseases mentioned herein.

Nitrone compounds are among the widely used free radical trapping agents[2] (Fig. 1). They trap especially C-centred radicals on their α-C atom, producing a more stable nitroxyl radical. The beneficial effect of α-phenyl-*N*-*tert*-butylnitrone (PBN) was discovered in the 1990s. Its administration decreased the mortality rate in several ischemia models in rodents.[3] However, because of its effective dose and therapeutic window PBN is not acceptable as a medicine.

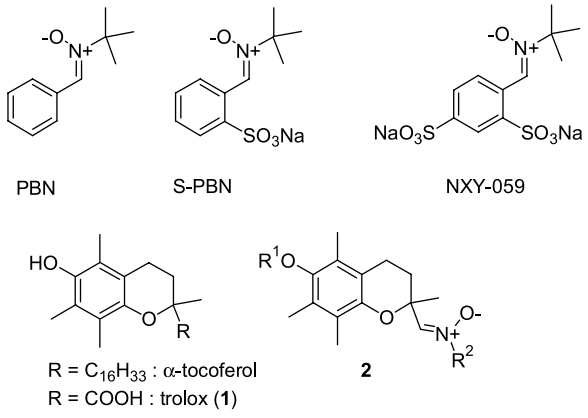


**Figure 1.**

Research for more effective and therapeutically acceptable free radical trapping agents for medicinal purposes has resulted in new compounds in recent years such as S-PBN and NXY-059,[4] of which the latter currently is in Phase III clinical trials for the indication of stroke.

During our work on nitrone compounds,[5] we aimed at the synthesis of new molecules keeping the nitrone component, but combining it with other types of antioxidant fragment the possessing ability to trap O- or N-centred radicals as well. Trolox (**1**), an α-tocoferol (Vitamin E) derivative, is one of the most powerful antioxidants, with relatively high selectivity for scavenging peroxynitrite and hydroxyl radical. We hoped that the combina-

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* Corresponding authors. Fax: +36 1 2606650; e-mail addresses: gy.balogh@richter.hu, k.vukics@richter.hu

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tion of these two different types of free radical trapping abilities would be favourable. For this purpose, we proposed to synthesize and perform biological evaluation of compounds **2**, derivatives of trolox holding a nitrone moiety in the position 2. We intended to separate the free radical trapping ability of the nitrone component from that of trolox itself; therefore we synthesized 'soft' (*O*-acetyl) and 'hard' (*O*-methyl) *O*-protected derivatives of **2**.

## 2. Chemistry

Trolox nitrones **2a**–**f** were synthesized as depicted in Scheme 1. Trolox **1** was esterified to ethyl ester **3** in refluxing ethanol with catalytic *p*-toluenesulfonic acid. Trolox aldehyde **4** was prepared from **3** in two steps, first reduced to the corresponding alcohol with diisobutyl aluminium hydride at −70 °C, followed by Swern oxidation. Compound **4** was converted into its *O*-acetyl derivative **5** by acetylation with acetyl chloride and triethyl amine in methylene chloride at 0 °C. Methoxy trolox aldehyde **7** was obtained in a manner similar to **4** from methyl ester **6** prepared via methylation of trolox with methyl iodide in the presence of potassium carbonate in dimethyl formamide. Trolox nitrones **2a**–**f** were prepared by the condensation of the appropriate aldehyde and *N*-alkylhydroxyl amine.[5,6] According to the homonuclear $^{1}H$–$^{1}H$ NOE experiments of nitrones **2a**–**f**, each nitrone proved to be *Z*-isomer.

## 3. Biology

Free radical scavenging properties of compounds **2a**–**f** and reference molecules (PBN, NXY-059 and trolox) were tested against pathophysiologically relevant reactive oxygen and nitrogen species (·OH, $ONOO^-$, NO and ROO·).

The ·OH scavenging capacity of the compounds was determined by the inhibition of ·OH induced damage of 2-deoxyribose. Arising thiobarbituric acid reactive products were measured at a wavelength of 535 nm.[7,8]

The $ONOO^-$ scavenging capacity of the compounds was measured by pyrogallol red bleaching assay (542 nm). $ONOO^-$ was prepared essentially by the method of Hughes and Nicklin.[8,9]

The NO scavenging capacity of the compounds was tested by direct capturing of NO from cupferron. Free NO levels were measured indirectly by its stable, spontaneously arising oxidative metabolites, nitrite and nitrate with Griess–Ilosvay reagent at a wavelength of 540 nm.[10]

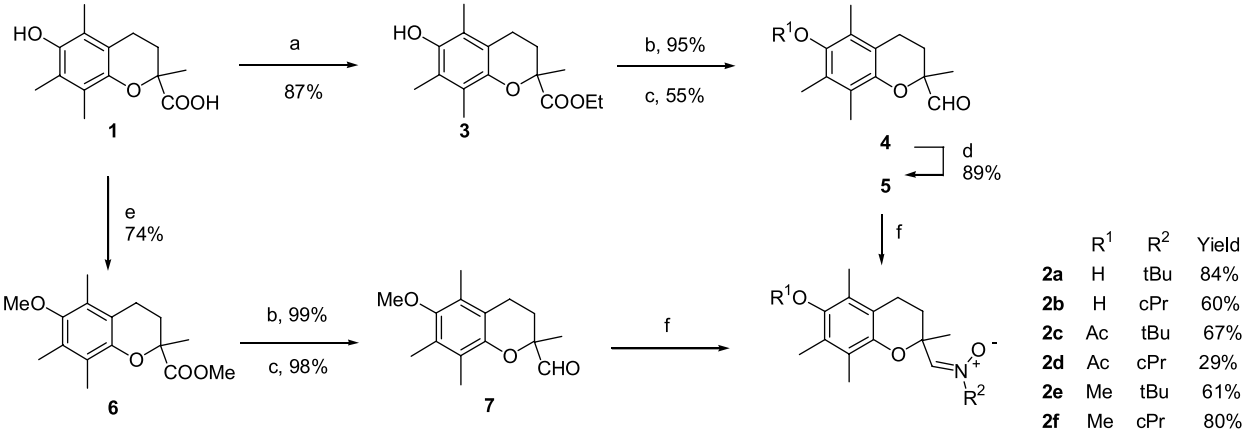
Total peroxyl radical-trapping antioxidant parameter (TRAP) of the compounds was determined by the spectrophotometric measurement (417 nm) of a relatively stable cation radical of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), which is generated in a one-electron oxidation. Peroxyl radical was formed by thermal decomposition of the water-soluble azo compound, 2,2′-azobis(2-methylpropion-amide) dihydrochloride (AAPH).[8,11]

The ability of **2a**–**f** to inhibit $Fe^{2+}$-induced lipid peroxidation (LPO) in mitochondria prepared from rat brain was determined by spectrophotometric measurement of thiobarbituric acid reactive substances (TBARs).[12,13]

A permanent middle cerebral artery (MCA) occlusion test,[14,15] a model of focal ischemia, was used to investigate the efficacy of some selected compounds (**2a**, **2b** and **2d**) in vivo.

## 4. Results and discussion

Our in vitro and in vivo biological results suggest that the combination of trolox with a nitrone moiety was beneficial (Table 1). Our new compounds **2a**–**f** exerted better efficacy in in vitro tests than reference compounds having nitrone component alone, although NXY-059 is considered a modest free radical trapping agent according to the literature,[4] and other mechanisms are supposed to be involved explaining its good efficacy in vivo. Nevertheless, even O-protected compounds, espe-




| | $R^1$ | $R^2$ | Yield |
|---|---|---|---|
| **2a** | H | tBu | 84% |
| **2b** | H | cPr | 60% |
| **2c** | Ac | tBu | 67% |
| **2d** | Ac | cPr | 29% |
| **2e** | Me | tBu | 61% |
| **2f** | Me | cPr | 80% |

**Scheme 1.** Reagents and conditions: (a) EtOH, pTsOH, reflux, 5 h; (b) DIBAL-H, toluene, −70 °C; (c) (1) oxalyl chloride, DMSO, $CH_2Cl_2$, −70 °C, (2) $Et_3N$; (d) acetyl chloride, $Et_3N$, $CH_2Cl_2$, 0 °C, 1.5 h; (e) MeI, $K_2CO_3$, DMF, 40 °C; (f) $R_2NHOH$, EtOH, reflux, 3 h.

**Table 1.** Free radical scavenging properties and inhibition of lipidperoxidation for compounds **2a–f**

| Compds | ·OH $IC_{50}$ (mM)[a] | $ONOO^-$ $IC_{50}$ (mM)[a] | NO $IC_{50}$ (mM)[a] | TRAP $IC_{50}$ (μM)[a] | LPO $IC_{50}$ (μM)[a] |
|---|---|---|---|---|---|
| **2a** | 0.17 (±0.03) | 3.2 (±1.4) | 6.8 (±2.2) | 5.5 (±2.0) | 2.0 (±0.6) |
| **2b** | 0.15 (±0.01) | 1.1 (±0.3) | 12 (±1.8) | 6.0 (±1.2) | 0.68 (±0.04) |
| **2c** | na | na | 19 (±1.2) | 895 (±110) | 10 (±1.3) |
| **2d** | na | 0.59 (±0.22) | 7.4 (±0.8) | 117 (±9) | 3.7 (±0.6) |
| **2e** | na[b] | 3.7 (±0.7) | 22 (±4) | 2940 (±350) | 23 (±2.4) |
| **2f** | na[b] | 1.2 (±0.3) | 11 (±1.4) | 296 (±48) | 20 (±1.8) |
| Trolox | 0.06 (±0.01) | 0.55 (±0.09) | 19 (±1.2) | 10 (±0.6) | 25 (±0.6) |
| PBN | 43 (±11) | na | 77 (±4.2) | 3065 (±480) | 16 (±1.3) |
| NXY-059 | 1.5 (±0.12) | na | na | 437 (±123) | 5491 (±58) |

[a] Values are means of three experiments, standard deviation is given in parentheses (na = not active).
[b] Solubility problems.

cially cyclopropyl-nitrones (**2d** and **2f**) where only the nitrone function is responsible for free radical trapping, exceeded the efficacy of PBN and NXY-059.

In comparison with trolox, **2a** and **2b** were comparable to trolox in vitro, slightly weaker for ·OH, but slightly stronger for peroxyl radical trapping (TRAP). The inefficacy of O-protected derivatives **2c–f** in ·OH scavenging capacity measurements indicates the favoured role of the free hydroxyl group of trolox in the ·OH capturing process. The comparable activities of **2d–f** to trolox in $ONOO^-$ test are yet to be explained. Moreover, in the LPO test, there was a marked difference in efficacy, producing 13 and 37 times lower $IC_{50}$ values for **2a** and **2b** than trolox, respectively.

According to our pMCAo measurements trolox had no effect. NXY-059 decreased infarct area by 18%, comparable to O-methylated trolox-nitrone **2d** causing a 17% of reduction in infarct. The combined trolox–nitrone molecules **2a** and **2b** had slightly superior efficacy when compared to NXY-059, reducing the infarct area by 27% and 26%, respectively (Fig. 2).

In conclusion, we synthesised new nitrone derivatives of trolox (**2a–f**) that exert good efficacy both in vitro and in vivo. They are comparable to trolox in scavenging free radicals and exceed nitrone-type references, for example, PBN or NXY-059. Compounds **2a–f** proved to be significantly more effective than reference compounds against lipid peroxidation and in permanent focal ischemia model. Therefore, **2a–f** are promising candidates for further development.

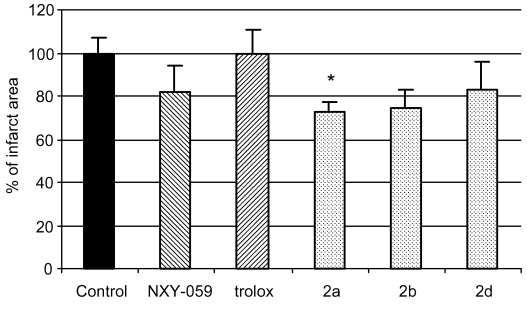



**Figure 2.** Neuroprotective activity of compounds **2a**, **2b**, **2d** and reference molecules in the pMCAo model in mice, single dose given 30 min after occlusion at 10 mg/kg intraperitoneally, $*p < 0.05$, $n = 4–10$.

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6. General procedure for the preparation of trolox nitrones **2a–f**: The appropriate trolox aldehyde (5 mmol) and *N*-alkyl hydroxylamine (12.5 mmol) in ethanol (20 mL) were stirred for 3 h under reflux. The solvent was evaporated in vacuum, and then methylene chloride and water were added to the residue. The layers were separated and the organic phase was washed with water, dried over anhydrous $Na_2SO_4$ and concentrated under vacuum.

The products were crystallized from an appropriate solvent, generally from diethyl ether/*n*-hexane, after purification by flash chromatography eluting with $CH_2Cl_2$/MeOH, if necessary. Physical properties of compounds **2a**–**f** are as follows: (**2a**): Mp: 170–172 °C (diethyl ether). $^1$H NMR (DMSO-$d_6$, 300 MHz, 30 °C): 7.47 (s, 1H), 6.62 (s, 1H), 2.90–2.76 (m, 1H), 2.56–2.40 (m, 1H), 2.36–2.17 (m, 1H), 2.06 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.85–1.68 (m, 1H), 1.55 (s, 3H), 1.32 (s, 9H). IR (KBr): 1592, 1452, 1234, 1165, 1108, 925, 785, 678 cm$^{-1}$. MS(EI): *m*/*z* 305 ($M^+$). HRMS: Calcd for $C_{18}H_{27}NO_3$: 305.1990. Found: 305.1996. **2b**: Mp: 152–154 °C (*n*-hexane/diethyl ether). $^1$H NMR (DMSO-$d_6$, 500 MHz, 30 °C): 7.46 (s, 1H), 7.02 (s, 1H), 3.90–3.81 (m, 1H), 2.89–2.77 (m, 1H), 2.55–2.45 (m, 1H), 2.32–2.20 (m, 1H), 2.07 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.77–1.65 (m, 1H), 1.55 (s, 3H), 1.14–1.00 (m, 2H), 0.67–0.54 (m, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): 4.0, 11.7, 12.6, 20.9, 21.4, 27.9, 43.4, 75.3, 96.0, 117.6, 120.5, 121.3, 122.7, 139.1, 144.0, 145.9. IR (KBr): 2927, 1603, 1455, 1326, 1258, 1165, 1089, 921, 816 cm$^{-1}$. MS(EI): *m*/*z* 289 ($M^+$). HRMS: Calcd for $C_{17}H_{23}NO_3$: 289.1679. Found: 289.1687. **2c**: Mp: 98.0–98.5 °C (petroleum benzine). $^1$H NMR ($CDCl_3$, 500 MHz, 30 °C): 6.69 (s, 1H), 3.07–2.95 (m, 1H), 2.63–2.52 (m, 1H), 2.43–2.32 (m, 1H), 2.32 (s, 3H), 2.13 (s, 3H), 2.03 (s, 3H), 1.95 (s, 3H), 1.96–1.85 (m, 1H), 1.69 (s, 3H), 1.44 (s, 9H). IR (KBr): 2984, 2934, 1759, 1570, 1465, 1369, 1212, 1077, 927 cm$^{-1}$. MS(FAB): *m*/*z* 348 ($MH^+$). HRMS: Calcd for $C_{20}H_{29}NO_4$: 347.2096. Found: 347.2112. **2d**: Mp: 188–191 °C (*n*-hexane/diethyl ether). $^1$H NMR ($CDCl_3$, 500 MHz, 50 °C): 6.75 (s, 1H), 3.06–2.96 (m, 1H), 2.63–2.53 (m, 1H), 2.48–2.36 (m, 1H), 2.31 (s, 3H), 2.13 (s, 3H), 2.04 (s, 3H), 1.96 (s, 3H), 1.92–1.81 (m, 1H), 1.69 (s, 3H), 1.40–1.28 (m, 2H), 0.76–0.62 (m, 2H). IR (KBr): 3076, 2936, 1755, 1592, 1369, 1216, 1193, 1083, 927 cm$^{-1}$. MS(FAB): *m*/*z* 332 ($MH^+$). HRMS: Calcd for $C_{19}H_{25}NO_4$: 331.1786. Found: 331.1789. **2e**: Mp: 110–112 °C (petroleum benzine). $^1$H NMR ($CDCl_3$, 300 MHz, 30 °C): 6.69 (s, 1H), 3.63 (s, 3H), 3.06–2.93 (m, 1H), 2.64–2.49 (m, 1H), 2.45–2.28 (m, 1H), 2.19(s, 3H), 2.12 (s, 3H), 2.11 (s, 3H), 1.99–1.83 (m, 1H), 1.68 (s, 3H), 1.44 (s, 9H). IR (KBr): 2980, 1576, 1453, 1357, 1256, 1093, 1002, 884, 734 cm$^{-1}$. MS(FAB): *m*/*z* 320 ($MH^+$). HRMS: Calcd for $C_{19}H_{29}NO_3$: 319.2145. Found: 319.2162. **2f**: Mp: 90–91 °C (*n*-hexane). $^1$H NMR (DMSO-$d_6$, 500 MHz, 30 °C): 7.08 (s, 1H), 3.92–3.84 (m, 1H), 3.53 (s, 3H), 2.88–2.79 (m, 1H), 2.57–2.47 (m, 1H), 2.33–2.22 (m, 1H), 2.10 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 1.79–1.69 (m, 1H), 1.57 (s, 3H), 1.14–1.02 (m, 2H), 0.67–0.56 (m, 2H). IR (KBr): 2935, 1582, 1459, 1403, 1256, 1200, 1102, 950, 740 cm$^{-1}$. MS(EI): *m*/*z* 303 ($M^+$). HRMS: Calcd for $C_{18}H_{25}NO_3$: 303.1834. Found: 303.1841.


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10. NO was generated by thermal decomposition of cupferron at 40 °C. Increasing concentrations (0.3–100 mM) of samples in 0.5 mL ethanol were mixed with Griess–Ilosvay reagent consisting of 0.5 mL of 0.2% *N*-(1-naphthyl)ethylenediamine in 0.4 M HCl and 0.5 mL of 2% sulfanilamide in 4 M HCl and 0.5 mL of 0.2% cupferron in ethanol. After 30 min of incubation at 40 °C, while the NO formation reached its maximum, the absorption was measured at 540 nm.
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13. Mitochondria were prepared from whole brains of male LATI Wistar rats. Mitochondrial suspension (0.3 mg protein), 25 mM Tris–HCl buffer (pH 7.4), and 0.2 mM ascorbic acid were incubated in the presence or absence of reference substances and test compounds. Lipid peroxidation was induced by the addition of 0.2 mM $FeSO_4$ solution and the mixture was incubated for 60 min at 37 °C. The reaction was terminated with 20% trichloroacetic acid solution, and the samples were centrifuged. The supernatant was boiled with 0.5% thiobarbituric acid solution for 20 min. Optical density was determined at 532 nm. The inhibitory effect of the test compounds was expressed as the inhibition of TBARs formation.
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15. MCA occlusion was performed by electrical coagulation on the right MCA of male NMRI mice. Test compounds were administered i.p. at a dose of 10 mg/kg 30 min after occlusion. Animals were maintained for 48 h under normal conditions, and then their brains were perfused transcardially with an injection of 2,3,5-triphenyltetrazolium chloride (TTC). Animals were decapitated and the infarct area of the brain surface was determined planimetrically by means of an image analyzer system (Digicell 5.0 ASK).