New Cholinesterase Inhibitor, Lipoic Acid-Nitrone Derivatives

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Lipoic acid (LA) is a multifunctional antioxidant against a variety of ROS. Nitrone acts as free radical spin trap and exhibits neuroprotective activity. Thus, LA-nitrone derivatives (**6**, **7**, **8**, and **9**) were synthesized and screened as an antioxidant and inhibitors for cholinesterases. Even though the antioxidant effect of LA-nitrone derivatives was not improved, they turned out to be effective inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in μM range.

Key Words : Lipoic acid, Nitrone, Antioxidant, Cholinesterase inhibitor

Introduction

The reactive oxygen species (ROS) cause a lot of human diseases such as intestinal diseases, atherosclerosis, cardiac diseases, Alzheimer's disease, chronic neurodegenerative disorders, reperfusion injuries, respiratory disorders, inflammation, diabetes, cancer, and aging.[1-4]

Lipoic acid (LA) is a 1,2-dithiolane analogue and it is rapidly reduced to dihydrolipoic acid (DHLA) by cytosolic and mitochondrial dehydrogenases inside a cell.[5,6] Both LA and its reduced form, DHLA, act as a multifunctional antioxidant against a variety of ROS.[5,6] LA is a cofactor of enzymatic mitochondrial decarboxylation reactions and is an indispensable molecule for producing an adequate amount of ATP from glucose via the citric acid cycle.[7] The other functions of LA are the assistant for regeneration and *de novo* syntheses of endogenous antioxidants such as glutathione and α-tocoperol, thioredoxin, and vitamin C,[8] the chelator for metal ions such as iron,[9,10] and the repairing agent for oxidatively damaged macromolecules.[11-13] LA exhibits anti-amyloidogenicity by inhibiting the formation and extension of fibrillar β amyloid (fAβ1-40) and fAβ (1-42) as well as destabilizing fAβs at pH 7.5 *in vitro*.[14]

Since LA has many benign effects, it is used as a dietary supplement and applied to medical treatments such as diabetes,[15-17] ischemia-reperfusion injury,[18] cataract formation,[19] neurodegeneration,[20-22] and hypertension.[23] LA also decreases exhaled nitric oxide concentrations in anesthetized endotoxemic rats.[24]

Due to its lipophilicity, LA easily crosses over the blood-brain barrier and results in accumulation in all neuronal cell types.[25] Many LA derivatives such as LA-*L*-dopa and LA-dopamine,[26] LA-Trolox (a water-soluble analogue of vitamin E),[27] LA-amphiphilic hybrid of α-phenyl-*N*-*tert*-butyl nitrone (PBN) (PBNLP),[28] LA-nitric oxide synthase inhibitors,[29] and LA-*N*-alkyl-substituted morpholine[30] have been synthesized to improve their biological activities and to give a synergic effect. LA dimers have been synthesized as an oral auxiliary drug for type 2 diabetic patients.[31] The activity of LA-chroman analogues were evaluated against reperfusion arrhythmias.[32]

Nitrone-based free radical spin traps such as α-phenyl-*N*-*tert*-butyl nitrone (PBN) exhibit neuroprotective activity.[33,34] Recently, Durand *et al*. reported the synthesis of a novel series of amphiphilic glycosylated nitrones derived from PBN for antioxidant activity.[28]

In the present paper, we report the synthesis of the covalent LA-nitrone derivatives between LA and the simple nitrones (**1**, **2**, **3**, and **4**) and their biological activities.

Results and Discussions

Nitrone-based free radical spin traps such as α-phenyl-*N*-*tert*-butyl nitrone (PBN) have been developed as an antioxidant.[33,34] Therefore, the novel compounds coupled between LA and nitrone functional group would expect to show synergic effects as an antioxidant. The nitrone compounds in Figure 1 have been synthesized by COS Biotech, Inc. Four nitrone compounds were directly coupled to lipoic acid without any further purification. Nitrone **1**, **3**, and **4** were coupled to LA with CDMT/NMM condition at room temperature to result in **5** (71% isolated yield), **7** (42% isolated yield), and **8** (44% isolated yield), respectively. The coupling reaction between nitrone **2** and LA with EDCI/DMAP condition resulted in **6** with 50% isolated yield (Figure 2).

The radical scavenging effect and cholinesterase (ChE)

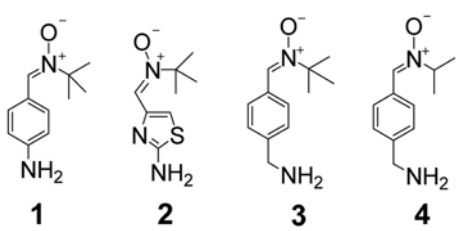

Figure 1. Nitrone compounds for coupling with LA.

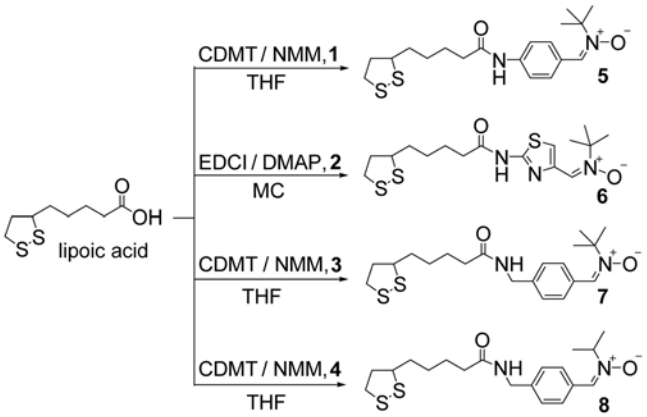

Figure 2. Synthesis of LA-Nitrone Compounds.

Table 1. Biological activity of LA-nitrone compounds

sample	ΔA (%)	AChE inhibition (%)		BuChE inhibition (%)	
concentration (mg/mL)	0.1	0.1	0.01	0.1	0.01
lipoic acid	64.9	10.5	0.0	14.7	5.0
1	13.2	38.8	0.0	17.6	15.9
2	22.4	45.8	5.8	38.2	9.3
3	25.4	74.2	0.0	69.4	10.8
4	25.5	55.6	0.0	24.3	11.3
5	19.1	58.0	24.9	56.7	20.7
6	17.5	93.7	66.9	83.4	37.6
7	25.4	93.0	64.9	61.6	13.5
8	26.9	67.1	29.9	70.0	19.4

ΔA is the absorbance difference at 518 nm

inhibitory effect of LA-nitrone compounds were tested and the results are shown in Table 1. The antioxidative effect of LA-nitrone compounds was checked by DPPH (1,1-diphenyl-2-picrylhydarzyl) assay, in which radical scavenging effect of DPPH was expressed as percentage decrease [ΔA(%)] with respect to control values. There is no improvement in ΔA values of LA-nitrones compared to the corresponding nitrones. Also ΔA values of LA-nitrones were much less than that of lipoic acid. The inhibition effects of AChE and BuChE were checked by the Ellman's coupled enzyme assay.[35]

Since the inhibition effect of compound **1-8** on ChEs was decreased at the low concentration, there is no inhibitory saturation effect. Compound **6** and **7** are the most potent inhibitors of AChE among the nine compounds. Compound **3**, **6** and **8** are the most potent inhibitors of BuChE. Neither lipoic acid nor nitrones inhibited cholinesterases as effective as LA-nitone compound **6**. IC_{50} values for **3**, **6**, **7** and **8** were measured for AChE and BuChE (Table 2). Compound **6** shows the thiazole in the nitrone moiety is better than phenyl ring for binding to the enzyme active site.

Table 2. IC_{50} value of the LA-nitrone compounds **3**, **6**, **7** and **8**

Compound	IC_{50} for AChE (μM)	IC_{50} for BuChE (μM)
3	278.8	247.2
6	19.1	46.4
7	19.1	114.5
8	128.7	128.5

Conclusions

Four nitrone compounds (**1**, **2**, **3**, and **4**) are covalently connected to LA to give LA-nitone derivatives (**5**, **6**, **7**, and **8**). IC_{50} values of **6** are 19.1 and 46.4 μM for AChE and BuChE, respectively. Growing evidence suggests that the neurobiological basis of senile dementia in Alzheimer's disease (AD) and related dementias is a loss of cholinergic neurons. It results in decline in acetylcholine (ACh) in brain regions which regulate behavioral and emotional responses. This cholinergic deficit can be partly corrected by inhibiting ChEs. In healthy brain, AChE predominates (80%) and BuChE is considered to play a minor role in regulating brain ACh levels. In the AD brain, BuChE activity rises while AChE activity remains unchanged or declines. Therefore, both enzymes are likely to have involvement in regulating ACh levels and represent legitimate therapeutic targets to ameliorate the cholinergic deficit. Recent researches have thought that dual inhibitors of AChE and BuChE may provide more sustained efficacy over the course of AD and may help to slow disease progression.[36] Further investigations will be carried out to check the activity against AD with LA-nitrone compounds.

Experimental Section

^{1}H-NMR, and ^{13}C-NMR spectra were recorded on a Varian Mercury 400 (400 MHz) and Bruker ARX-300 (300 MHz). Melting points were determined on SMP3. High-resolution mass spectra (HRMS) were recorded on a JMS-700 Mstation mass spectrometer under fast atom bombardment (FAB) conditions with nitrobenzyl alcohol (NBA) as the matrix in the Korea Basic Science Institute (Seoul), Korea. Flash column chromatography was performed using E. Merck silica gel (60, particle size 0.040-0.063 mm). Analytical thin layer chromatography (TLC) was performed using pre-coated TLC plates with silica Gel 60 F254 (E. Merck). All of the synthetic reactions were carried out under argon atmosphere with dry solvent, unless otherwise noted. Tetrahydrofuran (THF) was distilled from sodium/benzophenone immediately prior to use and methylene chloride (CH_2Cl_2) was dried from calcium hydride. All chemicals were reagent grade unless otherwise specified. The lipoic acid, CDMT, NMM, and EDCI were obtained from Sigma-Aldrich Chemical Co. and used without purification. The nitrones, **1**, **2**, **3**, and **4**, were obtained from Cos Biotech Inc. (Daejeon).

Free radical scavenging activity. The DPPH (1,1-Diphenyl-2-picrylhydarzyl) radical scavenging effect was carried out according to the method first employed by M.S. Blois. The 100 μL of sample solution was added to 900 μL of DPPH solution in ethanol (1.01×10^{-4} M). After incuba-

tion at room temperature for 30 min, the absorbance of this solution was determined at 518 nm using spectrophotometer and the remaining DPPH was calculated. All experiments were carried out in triplicate. Results were expressed as percentage decrease with respect to control values. The each fraction was evaluated at the final concentration at 100 μg/mL in the assay mixture.

Cholinesterase assay. ChE-catalyzed hydrolysis of the thiocholine esters was monitored by following production of the anion of thiocholine at 412 nm by the Ellmans coupled assay. Assays were conducted on HP8452A or HP8453A diode array UV-visible spectrophotometers and the cell compartments were thermostated by circulating water or Peltier temperature controller, respectively. Acetylthiocholine (ATCh) and butyrylthiocholine (BuTCh) were used as substrates for AChE and BuChE, respectively.

5-[1,2]Dithiolan-3-yl-pentanoic acid phenylamido-*t*-butyl nitrone (5). Lipoic acid (100 mg, 0.48 mmol) was dissolved in THF. CDMT (102 mg, 0.58 mmol) and NMM (147 mg, 1.46 mmol) were added to the lipoic acid solution. The mixture was stirred for 1 h and then nitrone **1** (93 mg, 0.48 mmol) was added to the solution at room temperature. The reaction mixture was stirred for 24 h at room temperature. The reaction mixture was poured into water. The resultant solution was extracted with methylene chloride three times. The combined organic extracts were treated with brine and dried over anhydrous $MgSO_4$. After the organic solvent was removed under vacuum, the crude product was then purified by flash chromatography using hexane/ethylacetate (1 : 3) as the eluant to give **5** as a white solid (130 mg, 71% yield).

mp 148-150 ^oC; ^1H NMR ($CDCl_3$, 400 MHz): 8.27 (d, J = 9.2 Hz, 2H), 7.59 (d, J = 8.8 Hz 2H), 7.50 (s, 2H), 3.57 (m, 1H), 3.19 (m, 2H), 2.45 (m, 3H), 1.87 (m, 1H), 1.66 (m, 6H), 1.58 (s, 9H), 1.61 (s, 9H), 1.50 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) 172.43, 140.12, 129.97, 129.56, 119.05, 70.66, 56.54, 40.44, 38.69, 37.70, 34.85, 29.06, 28.52, 25.33; HR-FAB-MS. Exact mass calcd. for $C_{19}H_{29}N_2O_2S_2$ (M + H): 381.1665, found: 381.1667.

5-[1,2]Dithiolan-3-yl-pentanoic acid thiazol-2-ylamido-*t*-butyl nitrone (6). Lipoic acid (300 mg, 1.44 mmol) was dissolved in methylene chloride. EDCI (335 mg, 1.75 mmol) and DMAP (18 mg, 0.15 mmol) were added to the lipoic acid solution. The mixture was stirred for 15 min and then nitrone **2** (289 mg, 1.45 mmol) mg was added to the solution at room temperature. The reaction mixture was stirred for 24 h at room temperature. The reaction mixture was poured into water. The resultant solution was extracted with methylene chloride three times. The combined organic extracts were treated with brine and dried over anhydrous $MgSO_4$. After the organic solvent was removed under vacuum, the crude product was then purified by flash chromatography using ethylacetate as the eluant to give **6** as a white solid (280 mg, 50% yield).

mp 155-157 ^oC; ^1H NMR ($CDCl_3$, 400 MHz) 8.79 (s, 2H), 7.81 (s, 1H), 3.57 (m, 1H), 3.14 (m, 2H), 2.46 (m, 3H), 1.92 (m, 1H), 1.82 (m, 4H), 1.72 (s, 9H), 1.68 (m, 2H) (Figure 19); ^{13}C NMR ($CDCl_3$, 100 MHz) 170.28, 156.53, 141.38, 125.58, 118.09, 70.60, 56.48, 40.53, 38.79, 36.42, 34.86, 29.02, 28.47, 24.94; HR-FAB-MS. Exact mass calcd. for $C_{16}H_{26}N_3O_2S_3$ (M + H): 388.1182, found: 388.1190.

5-[1,2]Dithiolan-3-yl-pentanoic acid benzylamido-*t*-butyl nitrone (7). Following the procedure for the preparation of **5**, **7** was obtained from lipoic acid (200 mg, 0.97 mmol) and **3** (200 mg, 0.97 mmol) as a white solid (160 mg, 42% yield).

mp 106-108 ^oC; ^1H NMR ($CDCl_3$, 400 MHz) 8.25 (d, J = 8.4 Hz, 2H), 7.53 (s, 1H), 7.31 (d, J = 8.4 Hz, 2H), 5.79 (m, 1H), 4.47 (d, J = 5.6 Hz, 2H), 4.04 (s, 1H), 3.58 (m, 1H), 3.12 (m, 2H), 2.42 (m, 1H), 2.22 (t, 2H), 1.95 (m, 1H), 1.72 (m, 6H), 1.61 (s, 9H), 1.51 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) 172.63, 140.53, 130.44, 129.62, 129.28, 127.90, 71.09, 56.64, 43.64, 40.51, 38.75, 36.74, 34.89, 29.15, 28.62, 25.67; HR-FAB-MS. Exact mass calcd. for $C_{20}H_{31}N_2O_2S_2$ (M + H): 395.1821, found: 395.1824.

5-[1,2]Dithiolan-3-yl-pentanoic acid benzylamido-isopropyl nitrone (8). Following the procedure for the preparation of **5**, **8** was obtained from lipoic acid (300 mg, 1.45 mmol) and **4** (279 mg, 1.45 mmol) as a white solid (241 mg, 44% yield).

mp 111-113 ^oC; ^1H NMR ($CDCl_3$, 400 MHz) 8.21 (d, J = 8.0 Hz, 2H), 7.43 (s, 1H), 7.31 (d, J = 8.2 Hz, 2H), 5.80 (m, 1H), 4.47 (d, J = 6.0 Hz, 2H), 4.21 (m, 1H), 3.56 (m, 1H), 3.16 (m, 2H), 2.44 (m, 1H), 2.24 (t, 2H), 1.89 (m, 1H), 1.65 (m, 6H), 1.47 (d, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) 172.65, 140.61, 131.69, 130.11, 129.05, 127.93, 68.08, 56.64, 43.63, 40.51, 38.75, 36.72, 34.89, 29.16, 28.62, 25.67, 21.20; HR-FAB-MS. Exact mass calcd. for $C_{19}H_{29}N_2O_2S_2$ (M + H): 381.1665, found: 381.1667.

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