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Novel Lipoic Acid Analogues that Inhibit Nitric Oxide Synthase

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Abstract—The synthesis and biological activity of novel lipoic acid analogues are reported. Lipoic acid and structural homologues coupled to arylthiophene amidine via carboxamide linkers are metabolic antioxidants capable of protecting neuronal cells against glutamate cytotoxicity, preventing loss of intracellular glutathione, and inhibit nitric oxide synthase. © 2002 Published by Elsevier Science Ltd.

Alpha-lipoic acid (LA) has been termed the 'ideal' antioxidant,[1] a readily absorbed and bioavailable compound capable of scavenging a number of free radicals. LA is readily absorbed from the diet, transported, taken up by cells and reduced to dihydrolipoic acid (DHLA) in various tissues including brain and is considered a metabolic antioxidant (Fig. 1).[1,2]

The antioxidant activity of LA is attributed to its capacity to regenerate endogenous antioxidants for example vitamin E, C and notably glutathione (GSH), and thus maintains an intracellular antioxidant balance.[3,4] GSH is the most important thiol antioxidant but it cannot be directly administrated whereas LA can.[1,3] Since LA raises intracellular GSH levels, it has been used for treating diseases in which oxidative stress plays a major role.

The enzyme nitric oxide synthase (NOS) plays various roles both in normal and pathological processes.[5] Overproduction of nitric oxide (NO) is implicated in different inflammatory and neurologic disorders and represents a potential therapeutic target.[6,7]

We have previously reported that co-administration of a NOS inhibitor and an antioxidant, notably N^G-nitro-L-arginine and di-*tert*-butyl-hydroxybenzoic acid produced a synergistic protective effect in the reduction of neuronal damage during ischemia-reperfusion.[8] More recently, we demonstrated that the co-administration of LA **2** and NOS inhibitors, notably compound **3** provided a greater protective effect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced depletion of brain dopamine in mice[9] than the expected sum of the compounds administered individually. MPTP is a neurotoxin which induces selective destruction of dopaminergic neurons in the substantia nigra pars compacta and leads to a dramatic decrease of dopamine.[10]

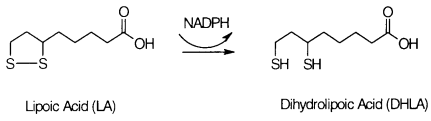

Figure 1. Lipoic acid is reduced to dihydrolipoic acid in vivo.

Based on these in vivo results, the neurotoxicity of NO,[11–13] and the observed decrease in intracellular GSH in certain neurodegenerative models[14] we have synthesised new lipoic acid derivatives that inhibit NOS and are metabolic antioxidants.

In this report, the design, synthesis and a preliminary examination of the in vitro activity of these novel lipoic acid analogues will be discussed.

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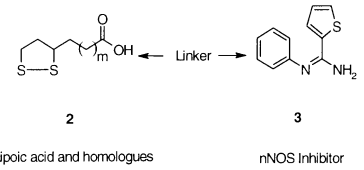


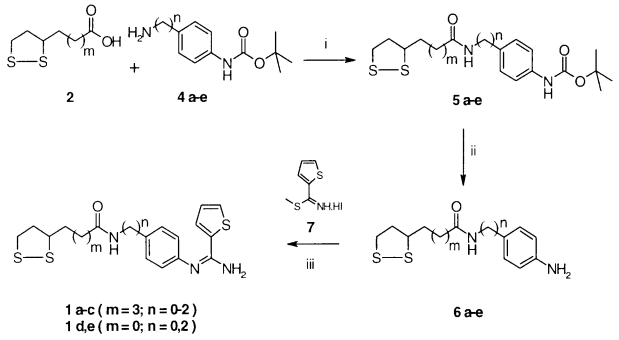
Figure 2. Design of lipoic acid analogues possessing nNOS activity.

Chemistry

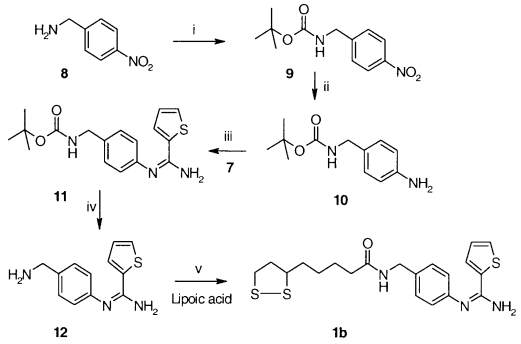
The concept is to covalently link lipoic acid and structural homologues of lipoic acid **2** to a selective neuronal NOS (nNOS) inhibitor[15] **3** via a chemical linker as shown in Figure 2.

Lipoic acid (**2**, $m=3$) and trisnorlipoic acid[16] (**2**, $m=0$) were coupled to the corresponding *N*-Boc-protected phenylamines **4a**–**e** ($n=0$–2) using peptide coupling conditions as shown in Scheme 1, followed by removal of the Boc protecting group of **5a**–**e** under acidic conditions to afford intermediates **6a**–**e**. The anilines **6a**–**e** were then coupled with *S*-methyl-2-thiophene-imidate[17] **7** to give the desired lipoic acid analogues[18] **1a**–**e**.

Removal of the *tert*-butyl carbamate protecting groups of **5** under acidic conditions led to moderate yields due to low solubility of **5** in organic solvents as well as, in some cases, extensive polymerisation due to the oxidation of the 1,2-thiolane moiety producing disulphide polymers.[19] This was overcome by coupling **2** in the final step, for example in the synthesis of compound **1b** as shown in Scheme 2. The amine function of *p*-nitrobenzylamine **8** was protected as a *tert*-butyl carbamate, followed by catalytic hydrogenation of the nitro moiety of **9** using 10% Pd/C to the required aniline. The aniline **10** was then reacted with *S*-methyl-2-thiophene-imidate **7**, followed by acidic deprotection of **11** to afford the benzylamine **12**, which was coupled to lipoic acid to provide the desired lipoic acid analogue **1b**. The analogues described in Schemes 1 and 2 were prepared in racemic form.



Scheme 1. (i) HOBT, Et_3N, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDCl), DCM; (ii) HCl, ethanol/diethyl-ether/acetone/dichloromethane; (iii) *i*PrOH, *S*-methyl-2-thiophene-imidate **7**, 60 °C.



Scheme 2. (i) BOC_2O, Et_3N, DCM, 70%; (ii) 10% Pd/C, H_2, DCM/THF, 68%; (iii) *i*PrOH, **7**, 60 °C, 99%; (iv) HCl, EtOAc, 92%; (v) HOBT, Et_3N, EDCl, DCM, 40%.

Pharmacology

Results and discussion

The compounds were evaluated in vitro for their capacity to inhibit nNOS. Inhibition was determined by measuring their effect on the conversion by NOS of [^{3}H]L-arginine into [^{3}H]L-citrulline.[20] As can be seen from Table 1 the lipoic acid derivatives **1a**–**e** have retained nNOS activity and have comparable IC_{50} values to that of the parent compound **3** while LA **2** itself is inactive.

The neuroprotective effect was measured by their ability to protect mouse hippocampal neuronal HT22 cell lines[21] from oxidative stress caused by glutamate. The cells do not express NOS and lack ionotropic glutamate receptors; thus, when exposed to high concentrations of glutamate the cells die via a form of programmed cell death called oxidative glutamate toxicity.[22] Toxicity is due to the inhibition of cystine uptake by the glutamate which leads to GSH depletion and cell death. The protective effects of the compounds against glutamate toxicity is expressed as EC_{50}, calculated relative to the viability of cells which have not been subjected to the action of glutamate, considered as 100% viability.[23]

Compounds **1a**–**e** (Table 1) are comparable and in some cases as active as LA **2** in protecting HT22 cells from oxidative stress, the parent compound **3** being inactive. Glutamate challenge causes a marked decrease in the intracellular concentration of GSH. Thus using the enzymatic glutathione assay, based on the formation of 5-thio-2-nitrobenzoic acid as a result of the oxidation of

Table 1. nNOS inhibition and glutamate induced cytotoxicity results for compounds **1a**–**e**, **2**, **3**

Compd	nNOS inhibition IC_{50} (μM)[a]	Glutamate toxicity HT22 cells EC_{50} (μM)[a]	Prevention of GSH depletion IC_{50} (μM)[a]
3	4.00	na	na
2	na	19.6	121.6
(**1a**) $m=3$, $n=0$	3.50	11.1	46.9
(**1b**) $m=3$, $n=1$	2.30	6.3	64.8
(**1c**) $m=3$, $n=2$	1.04	11.7	37.1
(**1d**) $m=0$, $n=0$	4.40	9.6	11.9
(**1e**) $m=0$, $n=2$	1.15	1.8	12.3

[a]na, not active, up to 100 μM.

GSH by 5,5′-dithiobis(2-nitrobenzoic acid), total GSH was assayed.[24] Examination of Table 1 reveals that there is a trend towards the protection of HT22 cells with restoration of intracellular GSH levels ($R^2 = 0.6$). Especially interesting is the trisnorlipoic acid derivative, **1e**, which is 10 times more active in protecting neuronal cells against oxidative stress and 10 times more active in maintaining GSH levels than lipoic acid.

While the neuroprotective effect of the coadministration of **2** and **3** was shown in an in vivo model[9] it now remains to demonstrate the in vivo biological activity of these new dual activity compounds **1**.

In conclusion, in this preliminary study we have designed and synthesised molecules with dual activity, capable of inhibiting nNOS in vitro and acting as metabolic antioxidants. Some of these compounds are more effective than lipoic acid in protecting cells against glutamate cytotoxicity and in preventing glutamate induced loss of cellular glutathione.

Further studies are continuing to improve on nNOS inhibition and using other structural homologues of lipoic acid, for example, bisnor and tetranorlipoic acids.[25]

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18. Typical experimental procedure, Synthesis of **1a**: ***N*-{4-[(1,1-dimethylethoxy)carbonylamino]phenyl}-1,2-dithiolane-3-pentanamide** (**5a**). *N*-Boc-1,4-Phenylenediamine **4a** (1.84 g, 8.81 mmol), triethylamine (1.6 mL), hydroxybenzotriazole (1.70 g, 12.6 mmol), 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDCl), (4.82 g, 25.2 mmol) and additional triethylamine (1.6 mL) were added successively to a solution of lipoic acid (2.00 g, 9.694 mmol) in dichloromethane (40 mL). The reaction mixture was stirred overnight at 25 °C and diluted with water (100 mL). The product was extracted with dichloromethane (3×100 mL), dried over magnesium sulphate, filtered and concentrated under reduced pressure. The reddish brown powder obtained was suspended in diethyl ether (100 mL), filtered and rinsed with the same volume of ether to afford a salmon pink powder in 80.5% yield. Melting point: 190.0–195.0 °C. ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 1.39 (m, 2H, CH_2); 1.57 (s, 9H, BOC); 1.61 (m, 4H, CH_2); 1.88 (m, 1H, CH_2); 2.27 (m, 2H, CH_2); 2.50 (m, 1H, CH_2); 3.15 (m, 2H, CH_2); 3.63 (m, 1H, –S–CH–); 7.34 (d, 2H, arom, $J = 8.70$ Hz); 7.45 (d, 2H, arom, $J = 9.00$ Hz); 9.24 (s, 1H, CONH); 9.77 (s, 1H, CONH-BOC). ^{13}C NMR (DMSO-d_6, 100 MHz, δ): 35.00; 55.70; 101.52; 108.09; 123.78; 129.09; 130.42; 146.49; 148.15; 162.83; 163.91; 169.56.

***N*-(4-Aminophenyl)-1,2-dithiolane-3-pentanamide** (**6a**). A stream of hydrogen chloride gas was slowly bubbled through a solution of intermediate **5a** (6.5 g; 16.4 mmol) in a mixture of ether/ethanol/acetone/dichloromethane (1/1/1/1, 200 mL) at 0 °C for 15 min. The temperature was allowed to rise to ambient temperature and left to stir overnight. A stream of argon was passed through the reaction mixture and the solvents evaporated under reduced pressure. The residue was dissolved in dichloromethane (200 mL), washed with a cold saturated solution of sodium bicarbonate (3×100 mL) followed by brine (100 mL). The organic phase was dried over magnesium sulphate, filtered and concentrated under reduced pressure. Purification was carried out on a silica-gel column (mobile phase: 50% ethyl acetate in heptane followed by 5% ethanol in dichloromethane) to afford a beige coloured solid in 29% yield. Melting point: 55.0–60.0 °C. NMR ^{1}H (DMSO-d_6, 400 MHz, δ): 1.38 (m, 2H, CH_2); 1.57 (m, 4H, CH_2); 1.87 (m, 1H, CH_2); 2.22 (m, 2H, CH_2); 2.40 (m, 1H, CH_2); 3.18 (m, 2H, CH_2); 3.62 (m, 1H, –S–CH–); 4.78 (s, 2H, NH_2); 6.48 (d, 2H, arom, $J = 8.64$ Hz); 7.20 (d, 2H, arom, $J = 8.64$ Hz); 9.39 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6, 100 MHz, δ): 27.34; 30.64; 36.43; 38.29; 40.37; 58.40; 116.03; 123.14; 130.83; 146.82; 172.27.

***N*-{4-[[(2-thienyl)(imino)methyl]amino]phenyl}-1,2-dithiolane-3-pentanamide hydrochloride** (**1a**). Intermediate **6a** (0.703 g; 2.37 mmol) was dissolved in 2-propanol (15 mL) and *S*-methyl-2-thiophene thiocarboximide hydroiodide **7** (1.014 g, 3.56 mmol) was added in one portion. After heating at 60 °C for 15 h, the reaction mixture was concentrated to dryness under reduced pressure. The residue was taken up in dichloromethane (100 mL), washed with a saturated solution of sodium bicarbonate (3×100 mL) followed by brine (3×100 mL). The organic phase was dried over magnesium sulphate, filtered and evaporated under reduced pressure. The free base was dissolved in dichloromethane (30 mL) and the solution cooled to 0 °C before the dropwise addition of a 1 N hydrochloric acid solution in anhydrous diethyl ether (6.3 mL). After stirring for 15 h at 25 °C, the solid formed was filtered and rinsed with diethyl ether and dried to afford a light beige solid in 77% yield. Melting point: 258.7–258.9 °C. ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 1.43 (m, 2H, CH_2); 1.62 (m, 4H, CH_2); 1.88 (m, 1H, CH_2); 2.38 (m, 3H, CH_2); 3.18 (m, 2H, CH_2); 3.63 (m, 1H,–S–CH–); 7.20–8.20 (m, 7H, arom.); 8.79

(broad s, 1H, NH^+); 9.78 (broad s, 1H, NH^+); 10.36 (s, 1H, CONH); 11.49 (broad s, 1H, NH^+). NMR ^{13}C (DMSO-d_6, 100 MHz, δ): 25.10; 28.54; 34.37; 36.44; 38.33; 56.34; 120.18; 126.58; 128.81; 129.08; 129.29; 134.63; 134.95; 139.68; 156.77; 171.71. IR:$\nu_{C=N}$ (amidine):1580 cm^1; $\nu_{C=O}$ (amide): 1659 cm^{-1}. Elemental analysis for $C_{19}H_{23}N_3OS_3 \cdot 0.25H_2O \cdot HCl$: Theoretical: C50.59%, N9.32%, S21.32%, H5.59%. Found: C50.91%, N9.15%, S21.12%, H5.56%.

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