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Novel Inhibitors of Neuronal Nitric Oxide Synthase with Potent Antioxidant Properties

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Abstract—A series of hybrid compounds possessing an nNOS pharmacophore linked to an antioxidant fragment has been synthesized. Among them, compound **8d**, a propofol derivative, displayed the greatest dual potencies against nNOS ($IC_{50} = 0.12\,\mu M$) and lipid peroxidation ($IC_{50} = 0.4\,\mu M$) accompanied with e/nNOS selectivity (67.5). This shows that nNOS was able to accommodate very bulky groups such as di-*tert*-butyl or di-*iso*-propyl phenol in its active site.

A selective or a general loss of neurones is responsible for many acute or chronic neurological disorders. These pathophysiological situations, such as cerebral ischemia, involve an enhanced formation of free radicals in brain tissue. Both reactive oxygen species (e.g., $O_2^{\bullet-}$) and reactive nitrogen species (e.g., $NO^{\bullet}$) participate in the inflammatory process and contribute to neuronal death. $NO^{\bullet}$ reacts rapidly with $O_2^{\bullet-}$ in aqueous media to form the highly reactive peroxynitrite ($ONOO^-$) with harmful effects on neuronal cells.

NO is synthesised from L-arginine by NO synthase (NOS). Three isoforms have been identified from three distinct genes: neuronal NOS (nNOS); inducible NOS (iNOS) and endothelial NOS (eNOS).

Scavenging reactive oxygen species or inhibiting the NO synthesis has been shown to result in an enhanced neuronal survival after cerebral ischemia. In pioneering experiments,[1] we have shown that the combination of a NOS inhibitor and an antioxidant (e.g., superoxide scavenger) in a model of acute neurodegeneration represented by focal transient ischemia was not additive but synergistic to reduce neuronal damage. This provides us with a basis for further studies towards the development of a novel strategy for the treatment of stroke that would consist in the administration of a drug possessing both activities. Consequently, a series of compounds was synthesized where the NOS pharmacophore was covalently linked to the antioxidant. As a prerequisite to the design of these drugs, they should be devoid of vascular effects. This could be achieved by selectively targeting nNOS without reducing circulating $NO^{\bullet}$ from eNOS.

Our first attempt to produce hybrid compounds led to the discovery of BN 80933.[2] This bifunctional agent provided promising neuroprotection as demonstrated in a rat model of transient cerebral ischemia.[3]

During our investigation towards potent and selective nNOS inhibitors, phenyl-2-thiophenecarboximidamide[4] **4a** was chosen among NOS pharmacophores which could be easily incorporated into our structures. It is now well accepted[5] that a selective and/or potent nNOS inhibition is dependent on the presence of a basic center in the inhibitor structure. A short structure–activity study was conducted around **4a** in order to determine the most suitable amino-linker to be used.

These amino-amidine derivatives **4b–e** were accessible through a short, straightforward strategy. As outlined

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in Scheme 1, they were prepared from commercially available derivatives **1** upon catalytic hydrogenation of the nitro group that provided the requisite substituted aniline intermediates **2**. The weak nucleophilicity of the aniline group oriented our chemical strategy towards reactive amidine precursors such as thioimidate reagents and in particular *S*-methyl-2-thiophenethiocarboximide. HI,[6] which allowed us to obtain the desired amidines **3** in fair to good yields. In the last step, Boc protection was removed under classical acidic conditions to provide the hydrochloride salt of the requisite amino-amidine **4b–e**.

The compounds were tested on the two constitutive isoforms of NO Synthase (nNOS and eNOS) and their selectivity was calculated. Inhibition was determined by measuring their effect on the conversion by NOS of [^{3}H]L-arginine into [^{3}H]L-citrulline.[3]

As shown in Table 1, different inhibitory potencies on nNOS were observed as the distance between the amino group and the arylamidine increased. The different substitution pattern in **4d** and **4e** led, however, to similar activities but with a higher selectivity in favor of **4d**. Hence, **4d** was selected as candidate to be linked to the antioxidant part of the molecule.

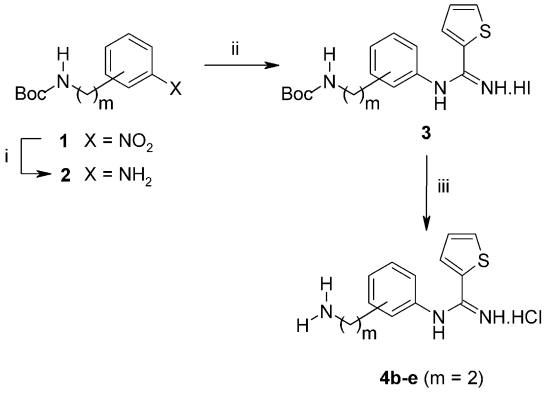
Of the free radical scavengers described in the literature, substituted phenols are the best known for their antioxidant properties.[7] Among the most potent ones, BHT (2,6-di-*tert*-butyl-4-methylphenol) was chosen as the antioxidant pharmacophore. For the connection between the NOS pharmacophore and the antioxidant part of the molecule we used a flexible linker. Optimization of the size of the molecule was related to the impact of the bulkiness of the substituted phenols on the nNOS and lipid peroxidation inhibitory activities.

The synthesis of the dual inhibitors (Scheme 2) was accomplished in two steps starting from the amino-amidine derivative **4d** and the commercial **5a–c** or synthetic phenols **6** (Scheme 3). The peptidic coupling was achieved using classical carbodiimide/HOBT procedure to afford **7a–d** which were reduced to the required amines **8a–d** using BH_3–THF.

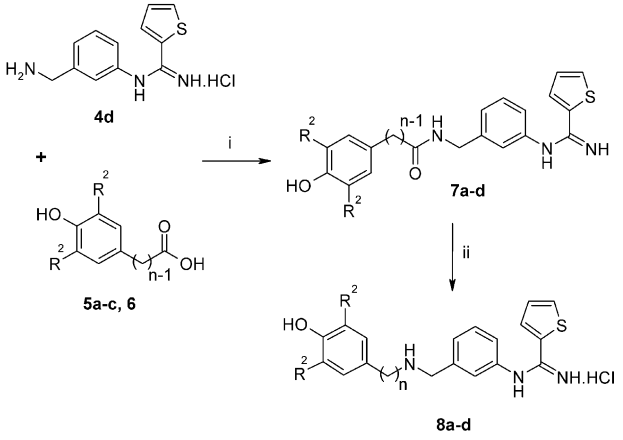
The synthesis of the 2,6-di-*iso*-propylphenol (propofol)[8] derivative **6** is illustrated in Scheme 3. After protection of the hydroxyl group of **9**,[9] the acetyl derivative **10** was condensed with $(Ph)_3P{=}CH{-}CO_2Et$ in refluxing THF. The cinnamyl intermediate **11** was then successively hydrogenated and saponified leading to the required carboxylic acid derivative **6**.

Results and Discussion

Table 2 shows the IC_{50} values for nNOS and eNOS inhibition. The antioxidant potency of the new compounds was assessed for their ability to inhibit Fe^{2+}

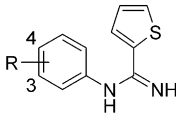


Scheme 1. (i) 10% Pd/C, H_2, 1.5 atm, EtOH, rt; (ii) *S*-methyl-2-thiophenethiocarboximide. HI,[6] iPrOH, 50–60 °C, 15 h; (iii) HCl in 1,4-dioxane or EtOAc, rt.



Scheme 2. (i) EDC, HOBT, Et_3N, CH_2Cl_2, rt; (ii) BH_3–THF 3 equiv, THF, reflux, 1.5 h then MeOH and HCl 3 N, reflux, 1 h.

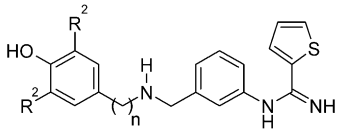
Table 1. nNOS and eNOS inhibition,[a] results for compounds **4a–e**



Compd	R	Isomer	Salt	nNOS Inhibition IC_{50}, μM	eNOS Inhibition IC_{50}, μM	Selectivity e/nNOS
4a	H		1HCl	2.2	13.9	6.3
4b	H_2N	4	1HCl	18	>100	>5.5
4c	H_2NCH_2	4	2HCl	0.2	37.1	195.3
4d	H_2NCH_2	3	2HCl	0.05	24.2	484
4e	$H_2NCH_2CH_2$	4	2HCl	0.08	25	312.5

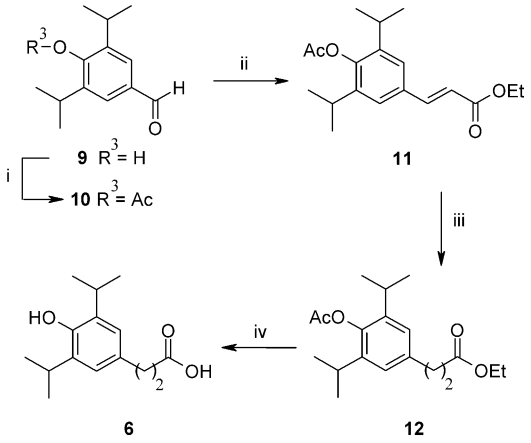
[a]NOS inhibition was determined at least two times according to ref 3.

Table 2. nNOS, eNOS and lipid peroxidation inhibitions for compounds **8a–d**



Compd	*n*	R^2	Salt	nNOS Inhibition IC_{50}, μM	eNOS Inhibition IC_{50}, μM	Selectivity e/nNOS	LPO Inhibition IC_{50}, μM
AR-R	—	—	2HCl	0.12	7.2	60	na
BHT	—	—	—	na	na	—	32.5
8a	1	tBu	2HCl	1.6	15.7	9.8	4.9
8b	2	tBu	—	1.4	15.5	11.1	2.4
8c	3	tBu	2HCl	0.47	4.9	10.4	0.4
8d	3	iPr	2HCl	0.12	8.1	67.5	0.4

na, not active.



Scheme 3. (i) CH_3COCl, Et_3N, CH_2Cl_2, 69%; (ii) $(Ph)_3P^+CH_2CO_2Et$, Br^-, nBuLi, THF, reflux, 15 h, 71%; (iii) 10% Pd/C, H_2, 2 atm, EtOH, rt, 97%; (iv) LiOH 3 equiv, H_2O, THF, 67%.

induced lipid peroxidation in rat brain microsomes.[10] AR-R (AR-R17477[5b]) and BHT (2,6-di-*tert*-butyl-4-methylphenol) were chosen as reference compounds for NOS and LPO tests, respectively.

At the beginning of our study, we found that compound **8a**, could inhibit both NO Synthase and lipid peroxidation. Although the inhibition of nNOS was modest compared to AR-R17477, **8a** demonstrated potent free radical scavenging activity. These encouraging results led us to prepare a series of analogues. The one carbon homologue **8b** showed potencies similar to **8a** except in the LPO test where the IC_{50} was somewhat better. Increasing the chain length led to the more potent nNOS inhibitor **8c**: $IC_{50} = 0.47\,\mu M$. Although selectivity between the two constitutive NOS isoforms (10 times) remained limited, we achieved a dramatic progress in the LPO test illustrated by the $IC_{50} = 0.40\,\mu M$ (80 times more potent than BHT). Clearly, the di-*tert*-butyl phenol group linked via a three-methylene chain to the NOS pharmacophore in **8c** greatly enhanced the antioxidant property and improved NOS inhibition by reaching a more accommodating (lipophilic) region of the active site of NOS.

Considering the modest solubility in aqueous media of **8c**, no further increase of the size of the molecule was undertaken but we rather focused on the phenol substitution pattern. In that way, we decided to exchange the di-*tert*-butyl group of the phenol for the less bulky di-*iso*-propyl, as in propofol.[8] Intermediate **6** was readily synthesized as described in Scheme 3 and coupled to the amino derivative **4d** (Scheme 2) to produce **8d**. We could not only enhance nNOS inhibition ($IC_{50} = 0.12\,\mu M$) to a satisfactory level but also achieve a good selectivity e/nNOS (>67), values which are similar to those of the reference AR-R17477. A comparison between the IC_{50} of **8c** and **8d** on nNOS and eNOS suggested that nNOS was more prone to steric bulkiness in this region of the active site than eNOS. The inhibitory potency of **8d** in the LPO test was as good ($IC_{50} = 0.40\,\mu M$) as for **8c**, confirming the excellent antioxidant capacity of this series of compounds. To complete this study, an iNOS inhibition test was performed on compounds **8a–d** showing that all of them exhibited an IC_{50} higher than 18 μM.

This study demonstrates the synthetic feasibility of dual nNOS/LPO inhibitors with inhibitory capacities in the low micromolar range. Unexpectedly nNOS was able to accommodate very bulky groups such as di-*tert*-butyl or di-*iso*-propyl fragments. Moreover, compound **8d** exhibits potent inhibitory properties versus nNOS and lipid peroxidation accompanied with acceptable e/nNOS and i/nNOS selectivity.

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