Bioorganic & Medicinal Chemistry 16 (2008) 8952–8958

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Fluorescent polycyclic ligands for nitric oxide synthase (NOS) inhibition

Jacques Joubert, Sandra van Dyk, Sarel F. Malan *

Pharmaceutical Chemistry, School of Pharmacy, North-West University, Private Bag 6001, Potchefstroom 2520, South Africa

ARTICLE INFO

Article history:
Received 20 May 2008
Revised 19 August 2008
Accepted 22 August 2008
Available online 27 August 2008

Keywords:
Fluorescent inhibitors
Nitric oxide synthase
Polycyclic cage
Neuroprotection

ABSTRACT

In recent years polycyclic compounds have been shown to exhibit pharmacological profiles of importance in the symptomatic and proposed curative treatment of neurodegenerative diseases (e.g., Parkinson's and Alzheimer's disease). These structures also show modification and improvement of the pharmacokinetic and pharmacodynamic properties of drugs in current use. Nitric oxide (NO) is a molecular messenger involved in a number of physiological processes in mammals. It is synthesised by nitric oxide synthase (NOS) from L-arginine and its overproduction could lead to a number of neurological disorders. The aim of this study was to synthesise a series of novel indazole, indole and other fluorescent derivatives conjugated to polycyclic structures for evaluation in NOS assays. NOS is a target system where fluorescent techniques and fluorescently labelled NOS inhibitors can be used for detecting the biophysical properties of enzyme–ligand interactions and thus facilitate development of novel inhibitors of neurodegeneration. This could lead to a greater insight into the neuroprotective mechanism and a possible cure/treatment for neurodegenerative diseases. A series of compounds incorporating polycyclic structures such as 3-hydroxy-4-aza-8-oxoheptacyclo[9.4.1.0.^{2,10}0.^{3,14}0.^{4,9}0.^{9,13}0^{12,15}]tetradecane and amantadine as well as suitable fluorescent moieties were selected for synthesis. In the biological evaluation the oxyhaemoglobin (oxyHb) assay was employed to determine the activity of the novel compounds at an enzymatic level of NOS. IC_{50} values of the novel fluorescent compounds were compared to that of aminoguanidine (AG) and 7-nitroindazole (7-NI), two known NOS inhibitors, and showed moderate to high affinity (IC_{50} values ranging from 7.73 μM to 0.291 μM) for the NOS enzyme.

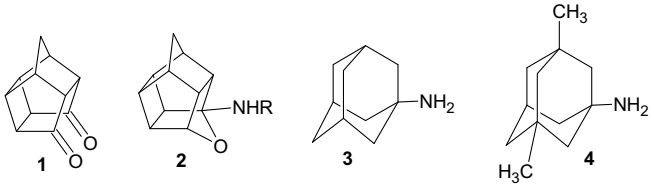
1. Introduction

Investigations into the synthesis and chemistry of novel saturated polycyclic hydrocarbon 'cage' compounds have been the aim of several research groups. The medicinal potential of these compounds was realised with the discovery that amantadine (**3**) exhibits antiviral activity. Subsequent to this discovery, it was found that amantadine could be benificial to patients with Parkinson's disease. It expresses its anti-Parkinsonian activity by increasing extracellular dopamine (DA) levels via DA re-uptake inhibition[1] or DA release and NMDA receptor antagonism.[2] Interest in the pharmacology of polycyclic cage amines was further stimulated when the dimethyl derivative of amantadine, memantine (**4**), was found to be a clinically well tolerated NMDA receptor antagonist.[3]

A structural similarity exists between the polycyclic cage structure of adamantane amines and that of the pentacycloundecyl amines.[4] Pentacycloundecylamines derivatives (**2**) are derived from Cookson's diketone (Pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione) (**1**), the so called 'bird cage' compound, obtained from the intramolecular photocyclisation of the Diels Alder adduct of *p*-benzoquinone and cyclopentadiene.[5] (Scheme 1)

Nitric oxide synthases are a family of enzymes in the body that contributes to neurotransmission, the immune system and vasodilitation. It does so by synthesis of nitric oxide and L-citrulline from the terminal nitrogen atom of L-arginine in the presence of NADPH and oxygen[7] (O_2) (Scheme 2) via the intermediate N^G-hydroxy-L-arginine.[8]

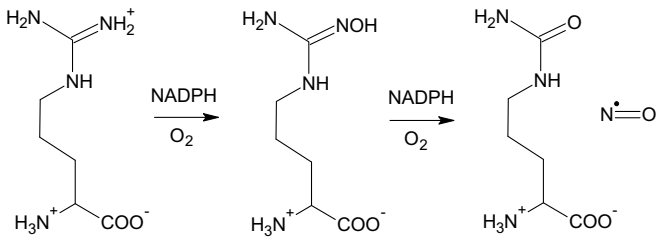
Three distinct NOS enzymes have been identified and characterised as products of different genes, with different subcellular localisation, regulation, catalytic properties, and inhibitor sensitivity; neuronal NOS (nNOS) and endothelial NOS (eNOS), which are constitutively expressed, and inducible NOS (iNOS).[7,8] nNOS and eNOS are physiologically activated by steroid hormones or neurotransmitters such as NO, dopamine, glutamate and glycine that increase



Scheme 1. Structural similarities between the polycyclic compounds.[4]

* Corresponding author. Tel.: +27 18 2992266; fax: +27 18 2994243.
E-mail address: sarel.malan@nwu.ac.za (S.F. Malan).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.08.049



Scheme 2. Reaction catalysed by NOS.[6]

the intracellular calcium concentrations. iNOS, in contrast, is Ca^{2+} independent and is expressed in a broad range of cell types. This form of NOS is induced after stimulation with cytokines and exposure to microbial products. After permanent activation, it continuously produces high concentrations of NO.[9]

NOS is the only known enzyme that has several cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4) and calmodulin.[6] Although NO mediates several physiological functions, a number of disease states are associated with either the overproduction or underproduction of NO, making the NOS pathway an attractive target for the development of therapeutics.[7] Overproduction by NOS has been implicated in a number of clinical disorders, including acute (stroke) and chronic (Alzheimer's, Parkinson's and Huntington's diseases) neurodegenerative diseases, convulsions and pain.[10]

The majority of known NOS inhibitors are nonselective or iNOS selective and only a few compounds are able to selectively inhibit nNOS. These include 7-NI,[11] 1-(2-trifluoromethyl-phenyl)-imidazole (TRIM),[12] some aromatic amidines[13] and amino acid derivatives (for example, some aminoguanidines).[14,15] TRIM has been reported to be relatively selective for nNOS, but with low potency while the nitroindazole family were found to be potent nNOS inhibitors.[11,12] Molecular tools capable of providing mechanistic insights into the production of NO and/or the inhibition of the NOS enzymes thus remain of interest. The development of neuroprotective agents is therefore orientated towards the synthesis of novel structures that interfere with a specific step of the complex chemical signalling system involving NOS and the inhibition of the enzyme itself.

In recent years the use of fluorescent detection methods, that is, confocal laser scanning microscopy, flow cytometry and image analysis, in nonradioactive assays have found widespread applicability in receptor and enzyme pharmacology. Fluorescent ligands are used to determine receptor and enzyme properties like receptor internalisation and sub cellular localisation, the thermodynamics and kinetics of ligand binding and to assess the nature of the microenvironment of the ligand binding site.[16–18]

The main objective of this study was to synthesise fluorescent NOS inhibitors and to explore their neuroprotective ability/potential. The fluorescent compounds for this study were selected on the basis of their spectroscopic properties, ease of synthesis and structural similarities to 7-NI to exhibit NOS inhibition. It is hypothesised that the novel fluorescent compounds may have the ability to inhibit NOS, as the compounds selected as fluorescent ligands have structural similarities to 7-NI, which is reported to be a selective nNOS inhibitor.[11,19] The fluorescent compounds synthesised include *N*-methylanthranilic acid, indazole-3-carboxylic acid, 1-fluoro-2,4-dinitrobenzene, 1-cyanoisoindole, 1-thiocyanoisoindole and 1-nitroisoindole conjugated to 3-hydroxy-4-aza-8-oxoheptacyclo[9.4.1.$0^{2,10}$.$0^{3,14}$.$0^{4,9}$.$0^{9,13}$.$0^{12,15}$]tetradecane (Table 1) and amantadine (Table 2).

The anthranilic and indazole complexes were obtained through the intermediate complexes with CDI and DCC and yielded the fluorescent esters (**5**, **6**) and amides (**7**, **8**) on reaction with the primary alcohol and amine, respectively. The dinitrobenzene complex was obtained through amination (**9**) with the primary amine of amantadine and the fluorescent isoindoles were obtained with the reaction of *o*-phthaldialdehyde with amantadine in the presence of sodium cyanide, sodium thiocyanate or sodium nitrate to form the fluorescent isoindoles (**10**, **11**, **12**).

Table 1
Fluorescent derivatives of 3-hydroxy-4-aza-8-oxoheptacyclo[9.4.1.0.^{2,10}.$0^{3,14}$.$0^{4,9}$.$0^{9,13}$.$0^{12,15}$]tetradecane

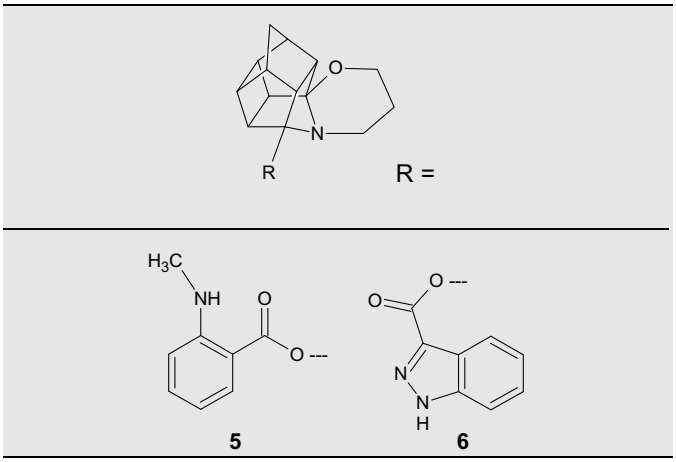
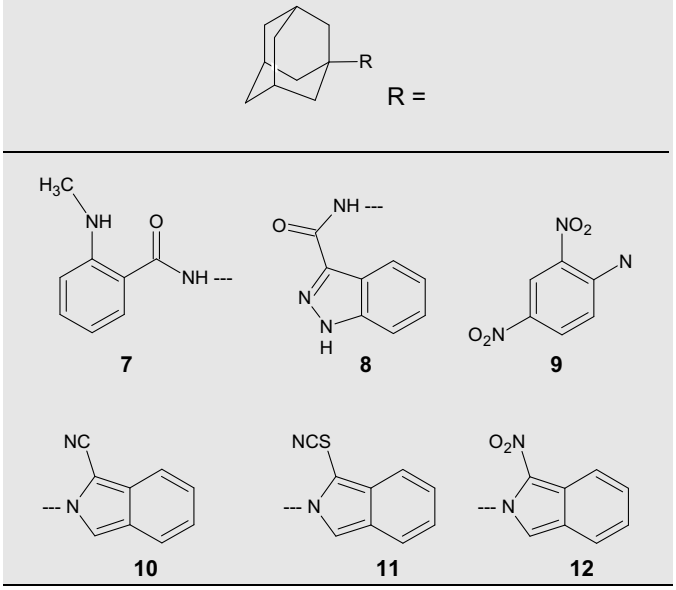


Table 2
Fluorescent derivatives of amantadine



2. Results and discussion

The synthesised compounds were all obtained as oils or amorphous solids from chromatography or were crystallised from organic solvents and the structures were confirmed using ^{1}H and ^{13}C NMR, MS and IR. The oxyhaemoglobin (oxyHb) assay[20] was employed to determine the activity of the novel compounds at an enzymatic level of NOS. This assay is principally based on the reaction of NO with oxyHb and the formation of methaemoglobin (metHb). In order to determine the amount of NO formed, the change in absorbance difference between 401 and 421 nm is measured during the initial linear phase of the reaction (Fig. 1). If

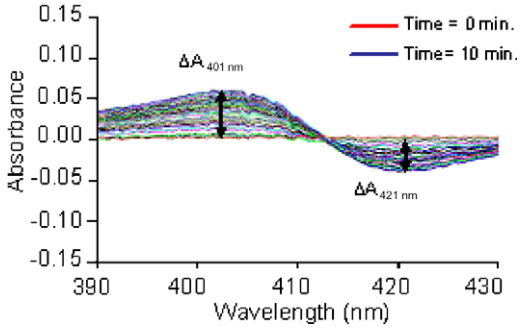


Figure 1. A typical spectrophotometric recording of compound 10 at a specific concentration. Continuous scans between 390 nm and 430 nm were performed as the oxyHb was converted to metHb.

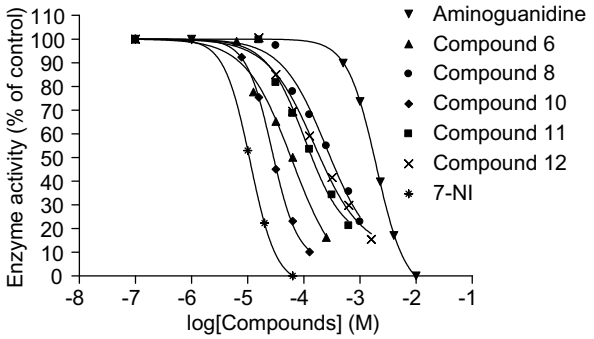


Figure 3. Inhibition curves of compounds with meaningful activities are superimposed to compare their IC_{50} values, 7-NI and aminoguanidine were used as reference compounds.

the change in absorbance at 401 nm is plotted against time and the change in absorbance over time at 421 nm is subtracted, the slope of this resulting curve is an indication of the increase in molar amount of metHb, and is identical to the molar amount of NO generated (Fig. 2). From this inhibition data the IC_{50} values were calculated and compared.

The inhibition curves of the selected compounds were superimposed on a single graph and the IC_{50} values were calculated. From the calculated IC_{50} values; compounds **6**, **8**, **10**, **11** and **12** revealed promising results as possible NOS inhibitors. 100% inhibition of NOS could not be obtained for the novel synthesised fluorescent structures as solubility becomes a limiting factor at higher concentrations.

When the compounds are compared to 7-NI (Fig. 3), one can clearly see that none of the structures showed as high activity as 7-NI (IC_{50} = 0.111 μM). All the compounds however showed more potent inhibitory activity than aminoguanidine (IC_{50} = 19.41 μM) (Fig. 3). Aminoguanidine is reported to be a selective iNOS inhibitor[21] and the lower activity observed could, to a certain degree, be attributed to this fact. Compounds **5**, **7** and **9** showed low or no inhibition (Table 3) of the NOS enzyme when compared to 7-NI and aminoguanidine.

The indazole structures, compounds **6** (IC_{50} = 0.35 μM) and **8** (IC_{50} = 2.53 μM) showed significant NOS activity. Both of these compounds exhibit better inhibition of the enzyme than the free indazole-3-carboxylic compound, IC_{50} = 9.65 μM; (Fig. 4). This confirms that both the polycyclic tetradecane and the adamantane moieties increase the activity of the indazole structures. The increased activity of the compounds conjugated to the polycyclic structures could be because of the higher lipophilicity and membrane permeability of these compounds.

Compound **10** proved to be the best inhibitor of the novel fluorescent compounds with a potent IC_{50} value of 0.291 μM. This 1-cyanoisoindole adamantane compound showed a 10-fold increase in activity when compared to the 1-tiocyanoisoindole adamantane and 1-nitroisoindole adamantane compounds (**11** and **12**) (Fig. 3 and Table 3).

All compounds showed an acceptable difference of excitation and emission wavelengths and Stoke shifts varied from 29 to 80 nm. Compound **6**, the second most potent compound showed the highest Stoke shift of 80 nm (Table 3).

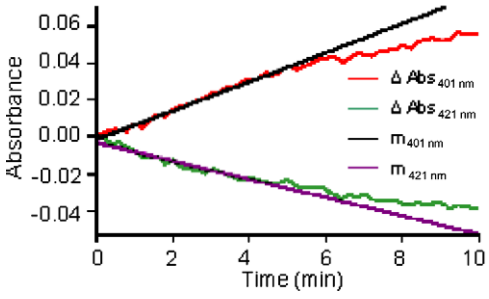


Figure 2. The change in absorbance at 401 nm and 421 nm versus time was calculated and the difference of the respective slope values [$(m\delta_{A(401\ nm)}) - (m\delta_{A(421\ nm)})$] gives an indication of enzyme activity.

3. Conclusion

We have identified a series of fluorescent structures with moderate to high affinity for the NOS enzyme, which may be utilised for further in vitro and in vivo studies using modern imaging techniques (e.g., confocal laser scanning microscopy, flow cytometry or multiphoton microscopy).

The potential of these novel fluorescent polycyclic structures as NOS inhibitors with structural similarity to 7-NI, a selective nNOS inhibitor,[11,17] the documented attenuation of excessive influx of Ca^{2+} into neuronal cells by NMDA receptor and L-type Ca^{2+} channel modulation[22] and effects on dopamine re-uptake and release,[23] indicate that these novel compounds may find application as multipotent drugs in neuroprotection.

These compounds thus have potential as useful pharmacological tools to investigate enzyme–ligand or receptor–ligand interactions in the quest for effective neuroprotective strategies and could lead to a greater insight into the neuroprotective mechanism.

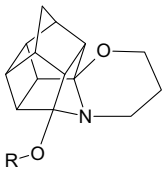
In order to more accurately determine the selectivity of the novel inhibitors, inhibition studies with individual NOS isoforms utilising a larger series of derivatives will be conducted in further investigations. Additional assays on the NMDA receptor, voltage gated Ca^{2+} channel, MAO-B enzyme and blood–brain barrier permeability will furthermore elaborate on potential value of these compounds.

4. Experimental

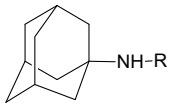
4.1. Chemistry: general procedures

Unless otherwise specified, materials were obtained from commercial suppliers and used without further purifications. All reactions were monitored by thin-layer chromatography on 0.20 mm thick aluminum silica gel sheets (Alugram® SIL G/UV_{254}, Kieselgel 60, Macherey-Nagel, Düren, Germany). Visualisation was achieved using UV light (254 and 366 nm), an ethanol solution of ninhydrin or iodine vapours, with mobile phases prepared on a volume-to-volume basis. Chromatographic purifications were performed on silica gel (0.063–0.2 mm, Merck) except when otherwise stated.

Table 3
NOS enzyme inhibition data and fluorescent properties of compounds



Compounds 5-6



Compounds 7-12

Compound	R (fluorophore)	Mp (°C)	% Yield	Log IC_{50} (μM)	λ_{ex}[a] (nm)	λ_{em}[a] (nm)
5	2-Methylaminobenzoate	213	36	−2.93	368	415
6	1*H*-indazole-3-carboxylate	190	16	−4.22	330	410
7	2-Methylaminobenzamide	209	25	−2.53	366	415
8	1*H*-indazole-3-carboxamide	212	44	−3.52	340	400
9	2,4-Dinitrophenyl-1-amine	300	50	—	396	449
10	1-Cyanoisoindole	160	37	−4.52	358	395
11	1-Thiocyanoisoindole	213	21	−3.88	352	409
12	1-Nitroisoindole	210	25	−3.74	332	393
7-NI[b]		—	—	−4.96	—	—
AG[c]		164	—	−2.72	—	—
I-3-C[d]		266	—	−3.28	360	420

λ_{ex} = excitation λ; λ_{em} = emission λ.
[a] At 10^{-5} M in absolute ethanol at 25 °C.
[b] 7-Nitroindazole.
[c] Aminoguanidine.
[d] Indazole-3-carboxylic acid.

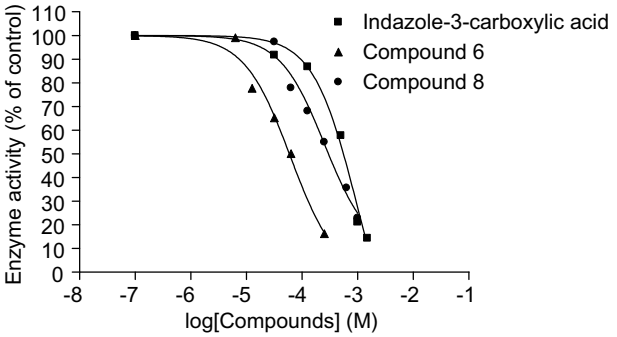


Figure 4. Inhibitory curves of the indazole test compounds, showing the increased activities of the compounds conjugated with the tetradecane (**6**) and adamantane (**8**).

The MS spectra were recorded on an analytical VG 70-70E mass spectrometer using electron ionisation (EI) at 70 eV. Melting points were determined using a Stuart SMP-300 melting point apparatus and capillary tubes. The melting points are uncorrected. IR spectra were recorded on a Nicolet Magna—IR 550 spectrometer. Samples were applied either as film or incorporated in KBr pellets. ^{1}H and ^{13}C spectra were obtained using a Varian Gemini 300 spectrometer at a frequency of 300.075 MHz and 75.462 MHz, respectively. All chemical shifts are reported in parts per million (ppm) relative to the signal from TMS ($\delta = 0$) added to an appropriate deuterated solvent. The following abbreviations are used to describe the multiplicity of the respective signals: s—singlet, br s—broad singlet, d—doublet, dd—doublet of doublets, t—triplet, q—quartet and m—multiplet.

4.2. Synthesis

The well-described Cookson's diketone, pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**1**), was synthesised according to the published method (Cookson et al., 1964, 1958).[5,24]

4.2.1. 3-Hydroxy-4-aza-8-oxoheptacyclo[9.4.1.0^{2,10}.0^{3,14}.0^{4,9}.0^{9,13}.0^{12,15}]tetradecane

Pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]-undecane-8,11-dione (3 g, 17.24 mmol) was dissolved in 30 ml tetrahydrofuran and cooled down to 5 °C while stirring in an ice bath. 3-amino-1-propanol (1.105 ml, 17.22 mmol) dissolved in 6 ml THF, was added slowly with continued stirring of the reaction mixture at 5 °C. The carbinolamine started precipitating after approximately 15 min, but the reaction was allowed to reach completion for an additional 30 min. Water was removed from the precipitate azeotropically by refluxing it in 60 ml dry benzene using a Dean-Stark apparatus for 1 h or until no more water was collected in the trap. The excess benzene was removed under reduced pressure and the rearranged cage structure, a yellow oil, was crystallised from THF to render the final product as a colourless crystalline solid (Yield: 3 g, 12.987 mmol, 75.33%).

$C_{14}H_{17}NO_2$; Mp: 170–172 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 4.97–3.94 (br s, 1H), 3.85–3.74 (m, 2H), 3.73–3.67 (m, 2H), 3.02–2.53 (3 × m, 8H), 1.80:1.52 (AB-q, 2H, J = 10.58 Hz), 1.75–1.55 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 101.45 (2 × s), 62.69 (t), 54.97 (d), 53.14 (d), 45.73 (d), 44.63 (d), 44.00 (d), 42.92 (d), 42.41 (t), 41.67 (t), 41.48 (d), 41.01 (d), 24.33 (t); MS (EI, 70 eV) m/z: 231 (M^+), 174, 151, 139, 91, 41, 28; IR (KBr) ν_{max}: 3446, 1484, 1346, 1320, 1166 cm^{-1}.

4.2.2. 3-{4-Aza-8-oxo-heptacyclo[0.4.1.0^{2,10}.0^{3,14}.0^{4,9}.^{09,13}.0^{12,15}]tetradecyl}-2-(methylamino)benzoate (5)

N-Methylanthranilic acid (0.390 g, 2.583 mmol) was added to a stirred solution of *N*,*N*′-carbonyldiimidazole (0.421 g, 2.583 mmol) in anhydrous tetrahydrofuran (25 ml). After 24 h 3-Hydroxy-4-aza-8-oxoheptacyclo[9.4.1.0^{2,10}.0^{3,14}.0^{4,9}.0^{9,13}.0^{12,15}]tetradecane (0.6 g, 2.583 mmol) in tetrahydrofuran (10 ml) was added and the mixture was allowed to react for 72 h at room temperature. The precipitate was filtered and washed with cold THF (2× 15 ml) yielding the product as a light yellow powder (Yield: 336 mg, 0.82 mmol, 36%).

$C_{22}H_{21}N_3O_3$; Mp: 213 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 8.14–8.11 (dd, 2H, J = 8.19, 0.89 Hz), 7.70–7.67 (dd, 2H, J = 8.38,

0.94 Hz), 7.42–7.36 (m, 3H), 7.26–7.21 (m, 3H) 3.89–3.09 (2 × m, 4H), 2.95–2.62 (3 × m, 8H), 2.89–2.87 (d, 2H), 1.89–1.12 (2 × m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 166.60 (s), 152.23 (s), 134,75 (d), 131.93 (d), 114.29 (d), 110.68 (d), 109.74 (s), 63.05 (t), 55.52 (2 × d), 43.93 (d), 43.91 (t), 43.88 (t), 29.46 (q), 24.00 (t); MS (EI, 70 eV) *m*/*z*: 364 (M^+), 231, 230, 214, 134, 69, 43; IR (KBr) ν_{max}: 3377, 2957, 2360, 1687, 1520, 1343, 1226, 1180 cm^{-1}.

4.2.3. 3-{4-Aza-8-oxo-heptacyclo[0.4.1.0^{2,10}.0^{3,14}.0^{4,9}.0^{9,13}.0^{12,15}]tetradecyl}-1*H*-indazole-3-carboxylate (6)

1*H*-Indazole-3-carboxylic acid (0.702 g, 4.323 mmol), 3-hydroxy-4-aza-8-oxoheptacyclo-[9.4.1.0^{2,10}.0^{3,14}.0^{4,9}.0^{9,13}.0^{12,15}]-tetradecane (0.702 g, 4.323 mmol) and dimethylaminopyridine (0.09 g, 0.737 mmol) was dissolved in dried dichloromethane (40 ml). The mixture was cooled to 5 °C using an external ice bath. *N*,*N*′-dicyclohexylcarbodiimide (DCC; 1.5 g, 7.27 mmol) was added in molar excess and the mixture was stirred for an additional 5 min at 5 °C. Thereafter the mixture was stirred for 48 h at room temperature. After 48 h the solvents were removed in vacuo and the residue suspended in 50 ml water, extracted with DCM (3× 25 ml) and dried over $MgSO_4$. The solvent was removed and yielded a colourless oil. Resolution of the product mixture was accomplished by column chromatography with Ethyl acetate/DCM/PE, 1:1:1, and with EtOH/THF, 1:1 (R_f = 0.69), yielding the product as a white powder (Yield: 255 mg, 0.68 mmol, 16%).

$C_{22}H_{24}N_2O_2$; Mp: 190 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 7.91–7.90 (dd, 2H, *J* = 9.58, 1.14 Hz), 7.38–7.24 (m, 3H), 6.64–6.61 (dd, 2H, *J* = 8.27, 0.79 Hz), 6.57–6.51 (m, 3H), 3.89–3.094 (2 × m, 4H), 2.95–2.62 (3 × m, 8H), 2.89–2.87 (d, 2H, *J* = 5.99 Hz), 1.81–1.50 (2 × m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 163.47 (s), 140.97 (s), 139.37 (m), 127.06 (m), 123.93 (s), 122.64 (m), 121.66 (d), 110.74 (s), 56.14 (t), 49.96 (2 × d), 49.12 (t), 49.12 (d), 33.89 (s), 30.94 (q), 24.90 (t); MS (EI, 70 eV) *m*/*z*: 375 (M^+), 243, 224, 145, 98, 56, 41, 28; IR (KBr) ν_{max}: 3277, 2931, 2851, 2360, 1625, 1448, 1242 cm^{-1}.

4.2.4. *N*-Adamantan-1-yl-2(methylamino)benzamide (7)

N-Methylanthranilic acid (0.5 g, 3.308 mmol) was added to a stirred solution of *N*,*N*′-carbonyldiimidazole (0.53 g 3.308 mmol) in anhydrous tetrahydrofuran (25 ml). After 24 h amantadine hydrochloride (0.5 g, 3.308 mmol) in tetrahydrofuran (10 ml) was added and the pH was adjusted to 8–9 with triethylamine. The mixture was allowed to react at room temperature. After 74 h the reaction mixture was heated to ensure complete reaction. The precipitate was filtered and washed with cold THF (2× 15 ml) yielding the pure product as a light yellow powder (Yield: 336 mg, 0.82 mmol, 25%).

$C_{18}H_{24}N_2O$; Mp: 209 °C; ^{1}H NMR (300 MHz, $CDCl_3$): 7.98–7.96 (dd, 2H, *J* = 7.48, 0.821 Hz), 7.49 (br s, 1H, NH), 7.29–7.24 (m, 3H), 6.60–6.52 (m, 3H), 6.59–6.57 (dd, 2H, *J* = 8.43, 1.28 Hz), 2.85 (d, 3H), 2.00 (s, 3H), 1.87– 1.60 (d, 6H), 1.56–1.47 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 151.64 (s), 132.60 (d), 132.42 (d), 117.37 (s) 113.90 (d), 109.984 (d), 51.28 (s), 40.49 (3 × t), 35.65 (3 × t), 29.63 (s), 28.99 (3 × d); MS (EI, 70 eV) *m*/*z*: 284 (M^+), 151, 135, 94, 41, 28; IR (KBr) ν_{max}: 3338, 2924, 1635, 1454, 1172, 848, 755 cm^{-1}.

4.2.5. *N*-Adamantan-1-yl-1*H*-indazole-3-carboxamide (8)

To a solution of 1*H*-indazole-3-carboxylic acid (0.3 g, 1.85 mmol) in DMF (7 ml) was added *N*,*N*′-carbonyldiimidazole (0.33 g, 2.035 mmol). The resulting solution was warmed at 60 °C for 2 h and then cooled to room temperature before adding a solution of amantadine hydrochloride (0.280 g, 1.85 mmol) in DMF (3 ml) and triethylamine (0.77 ml). The resulting solution was reacted for 24 h at room temperature. Thereafter the precipitate was filtered and washed with cold THF (2× 15 ml), yielding the product as a white amorphous solid. (Yield: 288 mg, 0.768 mmol, 41.5%).

$C_{18}H_{21}N_3O$; Mp: 212 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 8.14–8.11 (dd, 2H, *J* = 8.19, 0.89 Hz), 7.70–7.67 (dd, 2H, *J* = 8.38, 0.94 Hz), 7.42–7.36 (m, 3H), 7.26–7.21 (m, 3H), 2.21 (s, 3H), 2.12–2.11 (d, 6H, *J* = 2.96 Hz), 1.76–1.75 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 163.47 (s), 140.97 (s), 139.37 (m), 127.06 (m), 123.93 (s), 122.64 (m), 121.66 (d), 51.28 (s), 40.49 (3 × t), 35.65 (3 × t), 29.63 (s), 28.99 (3 × d); MS (EI, 70 eV) *m*/*z*: 295 (M^+), 238, 135, 91, 43, 28; IR (KBr) ν_{max}: 3459, 2911, 2854, 1672, 1493, 1197, 856 cm^{-1}.

4.2.6. *N*-(2,4-Dinitrophenyl)adamantan-1-amine (9)

1-Fluoro-2,4-dinitrobenzene (0.707 ml, 2.5 mmol), amantadine hydrochloride (0.378 g, 2.5 mmol) and K_2CO_3 (0.691 g, 5 mmol) was dissolved in 50 ml absolute acetonitrile. The pH was adjusted to 8–9 with triethylamine. The reaction mixture was stirred in the dark for 48 h, where after the mixture was filtered, the precipitate extracted with DCM (3 × 25 ml) and dried over $MgSO_4$. The solvent was removed in vacuo rendering the product as a bright yellow amorphous solid (Yield: 403 mg, 1.268 mmol, 50%).

$C_{16}H_{19}N_3O_4$; Mp: 300 °C; ^{1}H NMR (300 MHz, $CDCl_3$): 9.13–9.12 (d, 2H, *J* = 2.77 Hz), 8.18–8.13 (dd, 3H, *J* = 3.40, 1.18 Hz), 7.25–7.23 (d, 2H, *J* = 9.80 Hz), 2.21 (s, 3H), 2.12–2.11 (d, 6H, *J* = 2.96 Hz), 1.76–1.75 (m, 6H), 1.55 (br s, 1H, NH); ^{13} C NMR (75 MHz, $CDCl_3$) δ_C: 147.76 (s), 135.39 (s), 129.17 (d), 124.86 (d), 116.20 (s), 54.28 (s), 42.09 (3 × t), 36.04 (3 × t), 29.80 (3 × d); MS (EI, 70 eV) *m*/*z*: 317 (M^+), 196, 135, 93, 41, 28; IR (KBr) ν_{max}: 3439, 2926, 2855, 2360, 1541, 1338, 1146, 831 cm^{-1}.

4.2.7. *N*-(1-Cyano-2*H*-isoindol-2yl)adamantan-1-amine (10)

Amantadine hydrochloride (0.5 g, 3.301 mmol) and NaCN (0.162 g, 3.301 mmol) was dissolved in 20 ml methanol/water. To this was added *o*-phthaldialdehyde (0.442 g, 3.301 mmol) and the pH was adjusted to 8–9 with glacial acetic acid. The reaction mixture was protected from light and stirred at room temperature for 24 h. The mixture was filtered and resolution of the filtrate with flash column chromatography (ethyl acetate/PE 1:1, R_f = 0.65) yielded the product as a white amorphous solid (Yield: 338 mg, 1.223 mmol, 37%).

$C_{19}H_{20}N_2$; Mp: 160 °C; ^{1}H NMR (300 MHz, $CDCl_3$): 7.67–7.61 (2 × m, 6H), 7.48 (s, 1H), 7.24–7.03 (2 × dd, 4H, *J* = 7.84, 1.01 Hz; *J* = 8.64, 0.99 Hz), 2.44–2.43 (d, 6H, *J* = 3.19 Hz) 2.301 (s, 3H), 1.82–1.80 (m, 6H): ^{13} C NMR (75 MHz, $CDCl_3$) δ_C: 133.72 (s), 125.20 (m), 122.61 (s), 122.25 (d), 120.76 (m), 117.67 (d) 60.29 (s), 42.90 (s), 42.85 (3 × t), 35.83 (3 × t), 29.89 (3 × d); MS (EI, 70 eV) *m*/*z*: 276 (M^+), 135, 93, 41, 28; IR (KBr) ν_{max}: 3446, 2910, 2852, 2360, 1624, 1182, 783 cm^{-1}.

4.2.8. *N*-(1-Thiocyano-2*H*-isoindol-2yl)adamantan-1-amine (11)

Amantadine hydrochloride (0.5 g, 3.301 mmol) and NaSCN (0.27 g, 3.301 mmol) was dissolved in 20 ml methanol/water. To this was added *o*-phtaldialdehyde (0.442 g, 3.301 mmol) and the pH was adjusted to 8–9 with glacial acetic acid. The reaction mixture was protected from light and stirred at room temperature for 48 h. The reaction mixture was filtered and resolution of the filtrate with flash column chromatography (ethyl acetate/PE 1:1, R_f = 0.56) yielded the product as an orange amorphous solid (Yield: 210 mg, 0.68 mmol, 21%).

$C_{19}H_{20}N_2S$; Mp: 213 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 7.76 (s, 1H), 7.74–7.41 (2 × dd, 4H, *J* = 9.24, 1.88 Hz; *J* = 13.43, 6.80 Hz), 7.39–7.24 (2 × m, 6H), 2.29–2.28 (d, 6H, *J* = 20.29, 2.89 Hz), 2.13 (s, 3H), 1.79–1.67 (m, 6H); ^{13} C NMR (75 MHz, $CDCl_3$) δ_C: 140.86 (s), 134.69 (m), 130.71 (s), 127.72 (d), 123.11 (m), 122.24 (d), 55.47 (s), 47.43 (s), 40.06 (3 × t), 36.35 (3 × t), 32.62 (3 × d); MS

(EI, 70 eV) *m*/*z*: 307 (M^+), 261, 163, 135, 91, 41, 28; IR (KBr) ν_{max}: 3139, 2910, 2851, 2190, 1421, 1182, 783 cm^{-1}.

4.2.9. *N*-(1-Nitro-2*H*-isoindol-2yl)adamantan-1-amine (12)

Amantadine hydrochloride (0.5 g, 3.301 mmol) and $NaNO_2$ (0.27 g, 3.301 mmol) was dissolved in 20 ml methanol/water. To this was added *o*-phthaldialdehyde (0.442 g, 3.301 mmol) and the pH was adjusted to 8–9 with glacial acetic acid. The reaction mixture was protected from light and stirred at room temperature for 72 h. The reaction mixture was filtered and resolution of the filtrate with flash column chromatography (ethyl acetate/PE 1:1, $R_f = 0.73$) yielded the product as a light yellow amorphous solid (Yield: 245 mg, 0.827 mmol, 25%).

$C_{18}H_{20}N_2O_2$; Mp: 210 °C; ^1H NMR (300 MHz, $CDCl_3$) δ_H: 7.82 (s, 1H), 7.75–7.60 (2 × dd, 2H, $J = 8.32$, 3.26 Hz; $J = 8.24$, 2.96), 7.48–7.24 (2 × m, 2H), 2.27–2.12 (d, 6H, $J = 10.02$ Hz), 2.10 (s, 3H), 1.78–1.67 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 147.76 (s), 135.39 (m), 129.17 (s), 129.17 (d), 124.95 (m), 116.14 (d) 54.23 (s), 42.07 (3 × t), 36.08 (3 × t), 32.46 (3 × d); MS (EI, 70 eV) *m*/*z*: 297 (M^+), 163, 135, 91, 41, 28. IR (KBr) ν_{max}: 3446, 2906, 1666, 1453, 1221, 730 cm^{-1}.

4.3. Biological evaluation

4.3.1. Materials

All chemicals were of analytical grade or spectroscopy grade and was purchased from Sigma–Aldrich (UK) and Merck (St. Louis, MO, USA).

4.3.2. Animals

The study protocol was approved by the Ethics Committee for Research on Experimental Animals of the North-West University (Potchefstroom Campus). Male Sprague–Dawley rats were sacrificed by decapitation and the brain tissue was removed and kept on ice for homogenation. After homogenation, the aliquoted brain homogenate was snap freezed with liquid N_2 and stored at −70 °C.

4.3.3. Methods

Spectrophotometric scans were recorded using a Varian Cary-50® UV–vis spectrophotometer. The slope values were calculated at specific wavelengths and calculated from the inhibition data. All data analysis, calculation and graphs were done using Prism 4.02® (GraphPhad, Sorrento Valley, CA). All data are presented as means ± SEM. Data analysis was carried out using a one-way analysis of variance, followed by the Student–Newman–Keuls multiple range test. The level of significance was accepted at $p < 0.05$.

NOS assay procedure: HEPES buffer (100 mM) was prepared by dissolving the HEPES in double-distilled water and brought to pH 7.4 by the addition of 4 N NaOH at 37 °C. This can be stored for several weeks at 4 °C. The extraction buffer was prepared by dissolving sucrose (320 mM), HEPES (20 mM), and ethylenediaminetetra-acetic acid (1 mM) in double-distilled water and adjusting its pH to 7.4 at room temperature by addition of 10% HCl.[20] The following constituents were then added to the final concentrations indicated: 0.1 mM D/L dithiotherol (DTT), 0.5 μM leupeptin, soybean-trypsin inhibitor (10 μg/ml) and aprotin (2 μg/ml). The extraction buffer was then made up to its final volume with distilled water and distributed into aliquots (typically 50 ml per aliquot) and stored at −20 °C until required.

Phenylmethylsulfonyl fluoride (PMSF; 10 mg/ml) is unstable in aqueous solution and is not included in the buffer at this stage, but prepared as a solution in absolute ethanol, stored at −20 °C, and added to the extraction buffer during the extraction procedure. The composition of the extraction buffer is designed to permit extraction of NOS from tissues without breaking intracellular organelles and minimising proteolysis.[25]

Extractions and storage of tissue samples prior to the assay were carried out at 0–4 °C to avoid loss of enzyme activity. Fresh rat brain was weighed in 50 ml pre-cooled Falcon tubes and placed on ice. After rinsing with ice cold extraction buffer, a measured volume of extraction buffer (5 ml/g tissue) was added to the tissue. The sample was then homogenised with a mechanical homogeniser while the temperature was maintained at 4 °C. After 10 s of homogenisation the PMSF (10 μM/ml of extraction buffer) was added to the mixture and it was homogenised for a further 30 s. The homogenate was then centrifuged at 12,000*g* for 10 min. Once the supernatant was collected, it was divided into 2 ml aliquots which were assayed immediately or snap freezed and stored at −70 °C.

The oxyhaemoglobin solution was prepared by carefully dissolving the haemoglobin crystals (25 mg) in 1000 μl of cold HEPES buffer[21] and subsequent reduction with excess sodium dithionate (0.958 mg). The solution immediately changed from brownish red (mixture of oxyHb and metHb) to a dark red (deoxyhaemoglobin) colour after the reductant was added. Oxygenation was carried out by blowing 100% oxygen over the surface while the solution was gently swirled for 15 min. The gradual colour change from dark red to bright red was indicative of the oxygenation of haemoglobin. Desalting and purification was performed by passing the resulting oxyHb solution through a Sephadex G-25 column. The oxyHb is eluted as a single bright-red band. The front and back edges were discarded.

The concentration of oxyHb was calculated by methods described by Feelisch[26] and Hevel.[27,28] Ten microlitres of the oxyHb stock solution was added to 2990 μl of HEPES buffer in a cuvette and the absolute absorbance was determined in triplicate at 415 nm against a blank buffer. The concentration of oxyHb (C_{oxyHb}) was calculated with the following Eq. (1) using a molar extinction coefficient $E_{414(oxyHb)}$ of 131.0 mM^{-1} cm^{-1}.

$$C_{oxyHb} = \frac{A_{415\ nm} \times 300\ (\text{dilution factor})}{E_{415(oxyHb)}} \quad (1)$$

Using the above equation, the final calculated oxyHb concentration was found to be 0.76 mM. The stock solution was then aliquoted in 200 μl units, snap freezed with liquid N_2 and stored at −70 °C.

Calcium chloride solution ($CaCl_2$; 12.5 mM), L-arginine (1 mM) and NADPH (5 mM) were prepared in HEPES buffer.

The test compounds were dissolved in HEPES buffer, methanol, tetrahydrofuran or DMSO, the concentration of the organic solvents did not exceed 2% of the final incubation concentrations. This gave a series of concentrations in the micromolar range. 7-Nitroindazole was dissolved in methanol and aminoguanidine was dissolved in the HEPES buffer. These two compounds were used as the reference compounds in final concentrations ranging from 10 μM to 10 mM.

Oxyhaemoglobin, $CaCl_2$, L-arginine and the test compound was then diluted in the HEPES buffer to give final concentrations 250 μM $CaCl_2$ and 1 mM L-arginine.

The reaction mixture was prewarmed for 3 min to the required assay temperature of 37 °C and the reaction was started by the addition of NADPH and the tissue extract (100 μl) (in the form of rat brain homogenate) with a final NADPH concentration of 100 μM. After establishing the baseline, continuous scans with a scan rate of 600 nm/min every 10 s were recorded between 390 and 430 nm. The conversion of oxyHb to metHb was monitored over a period of 10 min.

4.4. Fluorescence spectrometry

A Cary Eclipse® fluorescence spectrometer was used for fluorescence measurements. The fluorescent compounds were measured at a concentration of 10^{-5} M in absolute ethanol at room temper-

ature. Emission spectra were recorded at the excitation maximal wavelength.

Acknowledgements

We are grateful to the National Research Foundation for financial support.

References and notes

1. Mizoguci, K.; Yokoo, H.; Yoshida, M.; Tanaka, K.; Tanaka, M.. *Brain Res.* **1994**, *662*, 255.
2. Danysz, W.; Parsons, W. G.; Kornhuber, J.; Schmidt, W. J.; Quack, G. *Neurosci. Biobehav. Rev.* **1997**, *21*, 455.
3. Parsons, C. G.; Danysz, W.; Quack, G. *Neuropharmacology* **1999**, *38*, 735.
4. Oliver, D. W.; Dekker, T. G.; Snyckers, F. O. *Eur. J. Med. Chem.* **1991**, *26*, 375.
5. Cookson, R. C.; Grundwell, E.; Hill, R. R.; Hudec, J. *J. Chem. Soc.* **1964**, 3062.
6. Kerwin, J. F.; Lancanster, J. R.; Feldman, P. L. *J. Med. Chem.* **1995**, *38*, 4343.
7. Dawson, V. L.; Dawson, T. M.; London, E. D.; Bredt, D. S.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6368.
8. Martin, I. N.; Woodward, J. J.; Winter, M. B.; Beeson, T. W.; Marletta, M. A. *J. Am. Chem. Soc.* **2007**, *129*, 12563.
9. Stuehr, D. J. *Biochim. Biophys. Acta* **1999**, *1411*, 217.
10. Knowles, R. G.; Moncada, S. J. *Biochemistry* **1994**, *12*, 275.
11. Moncada, S. J.; Palmer, R. M.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.
12. Moore, P. K.; Wallace, P.; Gaffen, Z.; Hart, S. L.; Baddebege, R. C. *Br. J. Pharmacol.* **1993**, *110*, 219.
13. Handy, R. L. C.; Wallace, P.; Gaffen, Z. A.; Whitehead, K. J.; Moore, P. K. *Br. J. Pharmacol.* **1995**, *116*, 2349.
14. Reif, A.; Frohlich, L. G.; Kotsonis, P.; Frey, A.; Bommel, H. M.; Wink, D. A.; Pfleiderer, W.; Schmidt, H. H. H. W. *J. Biol. Chem.* **1999**, *274*, 24921.
15. Huang, H.; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Silverman, R. B. *J. Med. Chem.* **1999**, *42*, 3147.
16. Malan, S. F.; Van Marle, A.; Menge, W. M.; Zuliana, V.; Hoffman, M.; Timmerman, H.; Leurs, R. *Bioorg. Med. Chem.* **2004**, *12*, 6495.
17. Dean, E. S.; Klein, G.; Renaudet, O.; Reymond, J. *Bioorg. Med. Chem.* **2003**, *13*, 1653.
18. Bagshaw, C. R.; Cherny, D. *Biochem. Soc. Trans.* **2006**, *34*, 979.
19. Handy, R. L. C.; Wallace, P.; Gaffen, Z. A.; Whitehead, K. J.; Moore, P. K. *Br. J. Pharmacol.* **1995**, *116*, 2349.
20. Salter, M.; Knowles, R. G. Methods in molecular biology. In: *Nitric Oxide Protocols*; Titheradge, M. A., Ed.; Humana Press: Totowa, NJ, 1996; Vol. 100, pp 61–65.
21. Corbett, J. A.; McDaniel, L. M. *Methods Enzymol.* **1996**, *10*, 21.
22. Geldenhuys, J. W.; Terre'Blanche, G.; Van der Schyf, C. J.; Malan, S. F. *Eur. J. Pharmacol.* **2003**, *1–2*, 73.
23. Geldenhuys, J. W.; Malan, S. F.; Bloomquist, J. R.; Van der Schyf, C. J. *Bioorg. Med. Chem.* **2007**, *15*, 2007.
24. Cookson, R. C.; Grundwell, E.; Hudec, J. *Chem. Ind.* **1958**, *39*, 1003.
25. Dawson, J.; Knowles, R. G. *Mol. Biotechnol.* **1999**, *12*, 275.
26. Feelish, M.; Kubitzek, D.; Werringloer, J. The oxyhemoglobin assay. In *Methods in Nitric Oxide Research*; Feelish, M., Stamler, J. S., Eds.; Wiley & Sons Ltd.: London, 1996; pp 455–478.
27. Hevel, J. M.; Marletta, M. A. *Methods. Enzymol* **1994**, *233*, 250.
28. Hevel, J. M.; White, K. A. *J. Biol. Chem.* **1991**, *266*, 22789.