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S-2-Amino-5-azolylpentanoic Acids Related to L-Ornithine as Inhibitors of the Isoforms of Nitric Oxide Synthase (NOS)

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Abstract—*S*-2-Amino-5-(2-aminoimidazol-1-yl)pentanoic acid and *S*-2-amino-5-(2-nitroimidazol-1-yl)pentanoic acid have been used as weakly inhibitory lead compounds in the design of 2-amino-5-azolylpentanoic acids which are more potent in their inhibition of nitric oxide synthases. Treatment of 2-(Boc-amino)-5-bromopentanoic acid *t*-butyl ester with appropriate imidazoles and 1,2,4-triazoles and with tetrazole under basic conditions, followed by acidolytic deprotection, gave many of the required 2-amino-5-azolylpentanoic acids. Tetrazole was alkylated at 1-N and at 2-N in approximately equal amounts whereas the 1,2,4-triazoles reacted principally at 1-N. A nitrile was introduced at the 2-position of the imidazole by reaction of the 2-unsubstituted precursor with 1-cyano-4-dimethylaminopyridine. Of this series of compounds, 2-amino-5-(imidazol-1-yl)pentanoic acid was identified as the most potent member against rat iNOS, rat nNOS and a human-derived cNOS. Examination of the structure–activity relationships for the identity and substitution of the azoles has led to the proposal of a model for the binding of the inhibitors to the binding site for the natural substrate.

Introduction

Nitric oxide (•NO) is the smallest known messenger molecule in biological systems. One site where it acts is the vascular endothelium where it is a vasodilator. It is thus partly responsible for maintaining cardiovascular homeostasis.[1] Nitric oxide is synthesised from L-arginine **1** by the various isoforms of nitric oxide synthase (NOS), yielding L-citrulline **3** as a coproduct. As shown in Scheme 1, the process comprises two separate mono-oxygenation steps involving N^G-hydroxyarginine **2** as an intermediate. Both steps require molecular oxygen (O_2) and NADPH. There are two main groups of isoforms of NOS, the constitutive Ca^{2+}/calmodulin-dependent types (cNOS) and an inducible Ca^{2+}/calmodulin-independent form (iNOS). The cNOS types can be further divided[2] into the neuronal form (nNOS) and the endothelial form (eNOS). Several known inhibitors of the isoforms of the enzyme are analogues of the substrate **1** or of the coproduct **3**; they include N^G-monomethyl-L-arginine (NMMA, **4**)[3], N^G-nitro-L-arginine (NOARG, **5a**),[3,4] N^G-nitro-L-arginine methyl ester (NAME, **5b**), N^δ-iminomethyl-L-ornithine (NIO, **6**) and L-thiocitrulline **7**. The K_i values reported[5] for these inhibitors are given in Table 1 and are comparable with the K_m for the substrate **1**. NOS inhibitors that have particular tissue or isozyme specificities open up a variety of therapeutic possibilities.[6] Recently, NOS inhibitors have been used selectively to modulate tumour blood flow, oxygenation and redox status.[7–9] However, the inhibitors **4** and **5** also have systemic effects related to their effects on production of nitric oxide. Interestingly, Garvey et al.[10] and Thomsen et al.[11] have reported very recently that N-(3-(aminomethyl)benzyl)acetamidine is a highly selective inhibitor of murine iNOS.

Key words: Nitric oxide synthase; *S*-2-amino-5-azolylpentanoic acid; *S*-2-amino-5-(imidazol-1-yl)pentanoic acid; structure–activity relationship.
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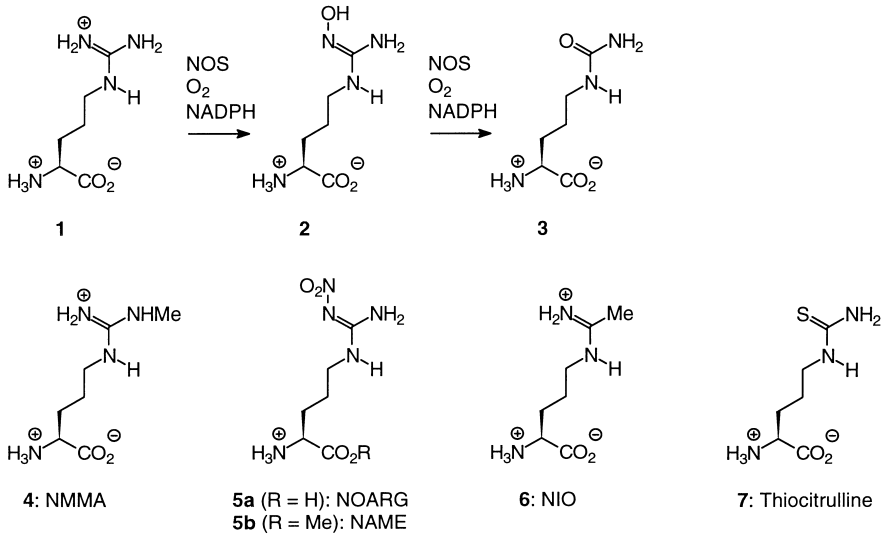


Scheme 1. Steps in the NOS-catalysed oxidation of L-arginine **1** to L-citrulline **3**, via N^G-hydroxyarginine **3** and structures of known inhibitors **4**–**7** of NOS.

Common to many human and experimental solid tumours are regions of hypoxic tissue,[12,13] which have been used as targets for selective bioreductive activation of prodrugs.[14–16] Thus, prodrugs that yield NOS inhibitors following hypoxia-mediated reductive activation could provide a novel strategy for selective vascular shut-down in tumours, while avoiding systemic effects. Such drugs may also be able to potentiate their own activation by further increasing hypoxia. 1-Substituted-2-nitroimidazoles and other nitroheterocycles can undergo bioreductive metabolism to give 2-aminoimidazoles,[17,18] presenting a potential strategy for developing prodrugs that, following hypoxia-mediated bioreduction in tumours, will give species that could inhibit NOS and thereby produce local vascular shut-down. Systemic effects would be avoided through reversal by O_2 of the single-electron reduction step carried out by enzymes such as NADPH: cytochrome P450 reductase.

Table 1. Inhibition of the isoforms of NOS by known analogues of L-arginine reported previously

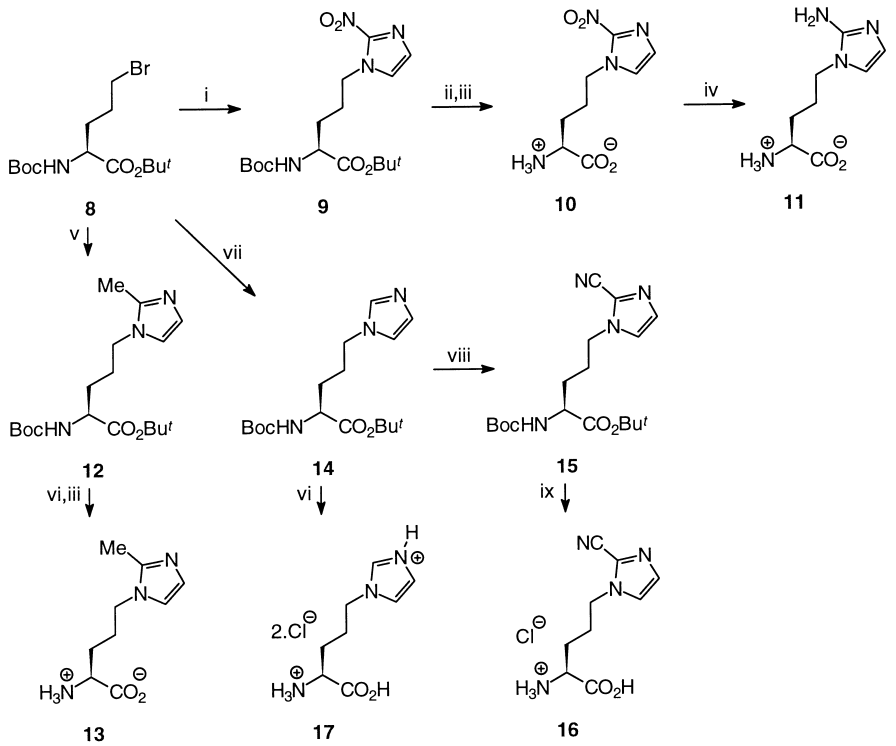
Compound	K_i for inhibition of iNOS (μM) (species)[a]	K_i for inhibition of cNOS (μM) (species)[a]
1 (L-arginine)	2.3[b] (mouse)	1.6[b] (cow)
1 (L-arginine)	19.0[b] (rat)	1.2[b] (rat)
4: (NMMA)	13.0 (mouse)	0.2 (rat)
5a: (NOARG)	4.4 (rat)	0.015 (cow)
6: (NIO)	2.0 (rat)	0.6 (rat)
7: (L-thiocitrulline)	0.06 (rat)	3.6 (rat)

[a] Data taken from Ref. 5.
[b] K_m.

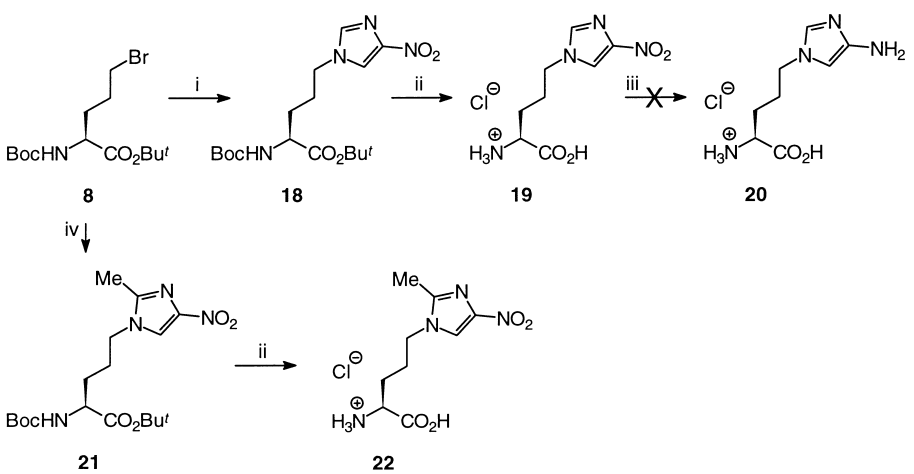
In a preliminary communication,[19] we reported the synthesis and evaluation of a nitroimidazole/aminoimidazole pair of compounds as inhibitors of rat brain nNOS (a cNOS) and rat lung iNOS. The structures of these compounds are shown as **10** and **11**, respectively, in Scheme 2. The latter compound was designed as a structural analogue of the NOS substrate, L-arginine **1**. Inhibition of both isoforms of rat NOS was relatively weak, with IC_{50}s in the low millimolar range, but a potentially exploitable differential was seen between the inhibitory potency of the aminoimidazole **11** and the putative metabolic parent nitroimidazole **10** against both isoforms. Taking these as lead compounds, we describe here the design and synthesis of related substituted and unsubstituted azoles, all carrying the amino- and carboxy-functionalised skeleton of **1** and **3**, the substrate and co-product, respectively, of the NOS-mediated bioreaction. We also report their evaluation as inhibitors of nNOS and iNOS obtained from the brain and lung, respectively, of rats treated with lipopolysaccharide (LPS). Our aim was to increase both the absolute potency of inhibition and the differential between the potential bioreduced metabolites, the aminoheterocycles, and the corresponding parent nitroheterocycles.

Chemical Synthesis

Building on compounds **10** and **11** as leads, we sought to explore the requirements for substituents on the imidazole ring. Schemes 2 and 3 show the synthetic approaches to the imidazoles. As reported previously,[19] BocGluOBu^t was converted in two steps to the bromo



Scheme 2. Synthesis of *S*-2-amino-5-(2-substituted-imidazol-1-yl)pentanoic acids and *S*-2-amino-5-(imidazol-1-yl)pentanoic acid. Reagents: (i) 1-potassio-2-nitroimidazole, DMF, Δ; (ii) aq. HCl, EtOAc; (iii) SiO_2, MeOH, aq. NH_3; (iv) H_2, Pd/C, EtOH; (v) 2-methylimidazole, $NaHCO_3$, THF, Δ; (vi) HCl, THF; (vii) imidazole, DMF, Δ; (viii) BrCN, 4-dimethylaminopyridine, DMF; (ix) HCL, CH_2Cl_2.



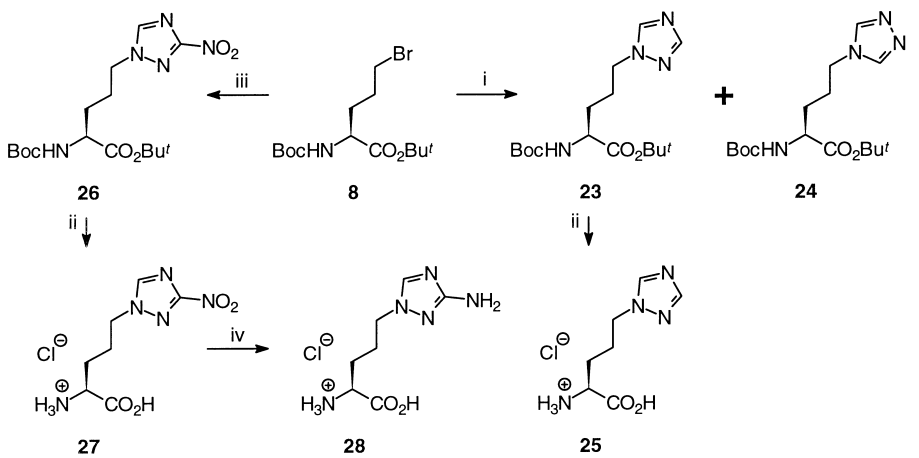
Scheme 3. Synthesis of *S*-2-amino-5-(4-nitroimidazol-1-yl)pentanoic acids. Reagents: (i) 4-nitroimidazole, $NaHCO_3$, THF, Δ; (ii) HCl, THF; (iii) H_2, Pd/C, various conditions; (iv) 2-methyl-4-nitroimidazole, $KOBu^t$, DMF, Δ.

compound **8**, essentially by the method of Olsen.[20] This compound formed a suitable electrophilic substrate for introduction of most of the variously substituted imidazoles by nucleophilic substitution. The first group of targets were designed to test some of the steric and electronic requirements of the group in the 2-position of the imidazole in the lead compounds (Scheme 2). The methyl group in **13** maintains the steric bulk of **11** but is not electron-withdrawing, electron-donating or H-bonding (c.f. **10**, **11**). The nitrile of **16** provides an

alternative electron-withdrawing group whereas the 2-substituent is omitted in **17**. Reaction of **8** with 2-methylimidazole in boiling THF gave the expected substitution product **12**. Simultaneous acidolytic deprotection of the Boc and Bu^t ester gave the required 5-(2-methylimidazolyl) amino acid **13**. The reaction of **8** with imidazole required warm DMF as the reaction solvent in forming the protected amino-acid derivative **14**. Introduction of a nitrile at the 2-position of imidazoles has been reported[21] to be effected by cyanogen chloride, via N-cyanoimidazole ylids. This reagent is highly inconvenient but cyanogen bromide gives mixtures of 2-cyano- and 2-bromo-imidazoles.[22] However, modification of the process by allowing the cyanogen bromide to react in situ with 4-dimethylaminopyridine gives 1-cyano-4-dimethylaminopyridinium bromide as the reactive intermediate. This intermediate presents the nitrile carbon as the sole electrophile. Thus the substituted imidazole **14** was converted to the 2-cyano analogue **15** in good yield under mild conditions. Deprotection gave the free amino-acid **16**. Similar deprotection of the intermediate **14** led to **17**, which was used to test the effect on NOS inhibition of removing completely the steric bulk and electronic effects of the imidazole 2-substituent.

In target imidazoles **19**, **20** and **22** (Scheme 3), functionality is introduced into the 4-position of the heterocycles. Particularly, **19** and **20** were designed as a nitroimidazole/aminoimidazole pair analogous to **10** and **11**, respectively, but with a more negative redox potential[23] to investigate the value of the nitro compound **19** as a hypoxia-activated prodrug of a NOS inhibitor. Following the strategy used for all the imidazole-derived amino-acids, the bromo compound **8** was used to alkylate the anions derived from 4-nitroimidazole and 2-methyl-4-nitroimidazole, giving **18** and **21**, respectively, which were deprotected to provide **19** and **22**, respectively. However, reduction of the nitro group of **19** under a variety of conditions gave only impure samples of **20**, in which the 4-aminoimidazole unit was found to be highly unstable.

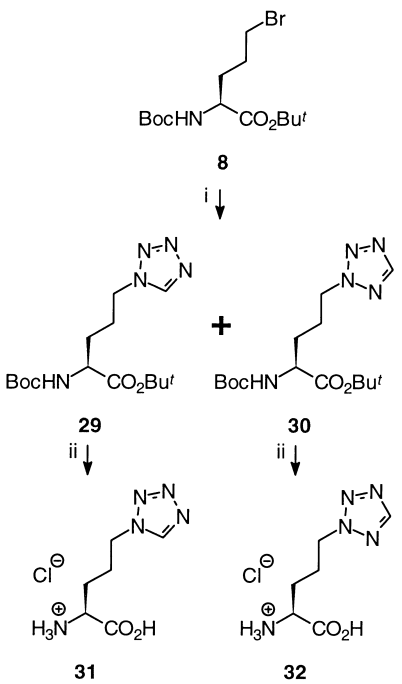
The effect on NOS inhibitory activity of changing the electronic state of the azole was also examined by introducing additional ring nitrogens. 3-Nitro-1,2,4-triazoles, for example, have been reported[24] as having more positive redox potentials than the corresponding nitroimidazoles. Alkylation of 1,2,4-triazole with **8** afforded a mixture of **23** and **24**, arising from alkylation at the triazole 1-nitrogen and the 4-nitrogen, respectively, as shown in Scheme 4. As could be predicted from reported studies[25] of selectivity of alkylation of 1,2,4-triazole, reaction took place principally at 1-N (product ratio **23:24** 36:1). Assignment of the regioisomeric identities of **23** and **24** were made on the basis of the ^{1}H and ^{13}C NMR spectra. In the spectra of **24**, the two ring protons were shown to be magnetically equivalent (δ 8.15, s), as were the corresponding ring carbon atoms (δ 151.9). In contrast, the ring protons (δ 7.95, s and δ 8.18, s) and the ring carbons (δ 142.8 and δ 151.5) were clearly inequivalent in the spectra of **23**, as required by the structure. Acidolytic deprotection of **23** was achieved in high yield, giving the salt **25** of the target triazole-amino-acid. Substituted triazoles **27** and **28** represent another nitroheterocycle/aminoheterocycle pair, in which the nitroheterocycle **27** may act as a bioreducible prodrug for the amine **28**; indeed **27** may be considered as the aza analogue of **19** whereas **28** corresponds to the synthetically elusive 4-aminoimidazole **20**. Here alkylation of 3-nitrotriazole with **8** under basic conditions took place exclusively at 1-N, the lack of nucleophilic reactivity of 2-N and 4-N being



Scheme 4. Synthesis of *S*-2-amino-5-triazolylpentanoic acids. Reagents: (i) 1,2,4-triazole, $NaHCO_3$, DMF, Δ; (ii) HCl, THF; (iii) 3-nitro-1,2,4-triazole, $NaHCO_3$, DMF, Δ; (iv) H_2, Pd/C, EtOH.

attributable both to electronic factors and to the steric effect of the nitro group. The regioisomeric identity of **26** (formed in 60% yield) was demonstrated by the observation of a NOE connectivity between the 5-H of the triazole and the 5-H_2 of the pentanoic acid. Deprotection of the amino-acid functions gave the target nitrotriazole-amino acid salt **27**. In contrast to the analogous 4-substituted imidazole series, reduction of the nitro group was achieved in high yield, affording the 3-aminotriazole **28** as a stable compound.

To extend the series of azole-amino-acids, synthetic approaches to tetrazoles were investigated (Scheme 5). In contrast to the reaction of **8** with the unsubstituted 1,2,4-triazole, treatment of tetrazole with **8** under basic conditions gave a very high yield of an almost equimolar mixture of the regioisomers **29** and **30**. These were readily separable by chromatography. Again, the regioisomeric identities were established by NOE measurements, with through-space connectivity being observed between the tetrazole ring proton and the 5-H_2 of the amino-acid unit in **29** but not in **30**. Deprotection of the carboxylic acid and the α-amino moieties gave the required tetrazole-amino-acids **31** and **32**, respectively. Approximately equimolar mixtures of 1-substituted tetrazoles and 2-substituted tetrazoles have been reported by Finnegan and Henry[26] and by Raap and Howard[27] as arising from alkylations of tetrazole with simple electrophiles.



Scheme 5. Synthesis of *S*-2-amino-5-tetrazolylpentanoic acids. Reagents: (i) tetrazole, $NaHCO_3$, DMF, Δ; (ii) HCl, THF.

Evaluation as Inhibitors of NOS

All the 2-amino-5-azolylpentanoic acids synthesised as described above were evaluated for their inhibitory activity against iNOS and nNOS derived from rat lung and from rat brain, respectively. As described in the Experimental section, the assay was based on the conversion of [^{14}C]-arginine to [^{14}C]-citrulline.

As an initial screen, compounds were tested at 1.0 mM against the two isoforms of the rat enzyme; the results are shown in Table 2. IC_{50} values were also obtained for selected compounds. Of the lead compounds from our preliminary study,[19] the 2-aminoimidazole **11** showed weak but significant activity at this concentration. The corresponding 2-nitroimidazole **10** was less active. Since **10** represented a potential hypoxia-selective prodrug for **11**, the IC_{50} values were obtained for the inhibitory activity of the pair of compounds against both rat NOS isoforms. A differential (IC_{50} **10**/IC_{50} **11**) of ca. twofold for the iNOS inhibitory activity and ca. threefold for the nNOS inhibitory activity was noted. Using these as lead compounds, modifications were made to the azole in attempts to increase the inhibitory potency of the series of compounds. Replacement of the electron-withdrawing nitro groups of **10** and the basic amino group of **11** with the electron-neutral but approximately isosteric methyl group in **13** did not significantly change the inhibitory activity against either isoform. Interestingly, introduction of an alternative lipophilic electron-withdrawing group in the 2-position of the imidazole, in the 2-cyanoimidazole **16**, led to an apparent stimulation of the activity of both isoenzymes. Moving the nitro substituent from the 2-position in **10** to the 4-position in **19** led to a striking 30-fold increase in activity (IC_{50} = 178 μM versus IC_{50} = 5300 μM against rat nNOS); the analogous 4-aminoimidazole **20** was too unstable to be isolated. Re-introduction of the 2-methyl group, in the 2,4-disubstituted imidazolepentanoic acid **22** diminished the activity slightly. Again, the corresponding 4-aminoimidazole was not synthetically accessible for comparative evaluation. Since moving the substituent from the 2-position to the 4-position had increased potency so strongly, the effect of removing substitution completely from the imidazole ring was investigated. The simple 2-amino-5-(imidazol-1-yl)pentanoic acid salt **17** confirmed the validity of this approach, in that **17** inhibited the rat iNOS activity with IC_{50} = 32 μM and the rat nNOS activity with IC_{50} = 19 μM. Thus there is a further ca. 10-fold increase in activity on removing the 4-nitro group from **19**. Some structure–activity guidelines can be drawn at this stage from the inhibitory activities of the imidazoles **10**, **11**, **13**, **16**, **17**, **19**, and **22**. Substitution at the 2-position of the imidazole may cause steric crowding as the inhibitors bind to the arginine binding sites of the enzymes and the basic/nucleophilic lone pair

Table 2. Inhibition of the isoforms of NOS by 2-amino-5-azolylpentanoic acids and by known NOS inhibitors

Compound	% inhibition of rat iNOS at 1.0 mM (initial screen)	% inhibition of rat nNOS at 1.0 mM (initial screen)	IC_{50} (μM) versus rat iNOS	IC_{50} (μM) versus rat nNOS	IC_{50} (μM) versus H647 cNOS
5a (NOARG)	—	—	—	3.0	—
5b (NAME)	—	—	—	6.0	—
6 (NIO)	—	—	—	2.0	—
7 (thiocitrulline)	—	—	1.7	5.0	2.0
10	1 ± 0.5	11 ± 1.5	6.6×10^3	5.3×10^3	—
11	32 ± 2	20 ± 1.5	3.2×10^3	1.7×10^3	—
13	18 ± 6	20 ± 7	—	—	—
16	−10 ± 4	−21 ± 5	—	—	—
17	99 ± 2	100 ± 2	32	19	13
19	48 ± 5	78 ± 2	—	178	—
22	40 ± 4	48 ± 1	—	—	—
25	68 ± 4	76 ± 3	—	333	—
27	−23 ± 2	−11 ± 6	—	—	—
28	−24 ± 4	−31 ± 4	—	—	—
31	−3 ± 2	2 ± 4	—	—	—
32	15 ± 6	41 ± 1	—	—	—

at the imidazole 3-N may be important for binding of the inhibitors.

To test the latter hypothesis, 2-aminopentanoic acids with triazoles and tetrazoles at the 5-position were evaluated. These should present progressively less electron density at this nitrogen and should thus be weaker inhibitors. This prediction is borne out, in that the 1,2,4-triazol-1-yl compound **25** was some 17-fold less potent than the corresponding imidazole **17** ($IC_{50} = 333\,\mu M$ versus $IC_{50} = 19\,\mu M$ against rat nNOS). Incorporation of one more ring nitrogen, as in the tetrazol-1-yl and tetrazol-2-yl analogues **31** and **32**, respectively, abolished or severely diminished NOS inhibitory activity.

The effect of introducing the ring nitrogen can also be assessed by comparison of the inhibitory activities of the 4-nitroimidazole **19** and the nitrotriazole **27**. As predicted, the reduction in electron pair availability abolished the inhibition of the enzymes. Indeed, this compound mimicked **16** in appearing to stimulate conversion of arginine to citrulline. In the triazole series, the aminotriazole **28** was synthetically accessible; again a small but significant stimulation of enzymic activity was observed at the standard test concentration, 1.0 mM.

To make our findings and conclusions comparable with those for other inhibitors reported in the literature, values of IC_{50} were measured for the known agents NOARG **5a**, its methyl ester **5b**, NIO **6**, and thiocitrulline **7**.

In our assay, the inhibitory potency of all these agents was found to be in the 1–10 μM range against rat nNOS and rat iNOS (where tested). Thus the optimum inhibitor among the series of 2-amino-5-azolylpentanoic acids, the 2,4-unsubstituted imidazole **17**, was of comparable potency with these currently accepted 'gold standards' for inhibition, being only three to sixfold less potent than thiocitrulline **7** and NOARG **5a** against rat nNOS. This imidazole **17** was then compared with thiocitrulline **7** for inhibition of a cNOS of human origin derived from H647 xenografts, as described in the Experimental section. Again, it proved to be only some sixfold less potent.

The structure–activity concepts gleaned from the wide range in potencies for inhibition of the isoforms of NOS by the 2-amino-5-azolylpentanoic acid analogues of L-ornithine and L-arginine suggest a model for the binding of these agents to the substrate binding site of the enzymes, as shown in Figure 1. In this model, we propose that there may be binding sites for the anionic α-carboxylate and cationic α-ammonium groups of the natural substrate **1** and that this binding presents a side-chain guanidine nitrogen atom to the haem iron for oxidation. This model is consistent with the models proposed by Garvey et al.[28] for binding of isothiourea NOS inhibitors and by Kerwin et al.[29] for binding of **1** and of some known amino-acid NOS inhibitors to the substrate binding site. The α-carboxylate and α-ammonium groups of the inhibitors (exemplified in Fig. 1 by the optimum imidazole **17**) are held similarly. This binding then presents the 3-N of the imidazole as a ligand appropriately located for tight coordination to the haem iron. This model for the inhibitory activity is consistent with the apparent structure–activity relationship in the azole region of the inhibitors, since the

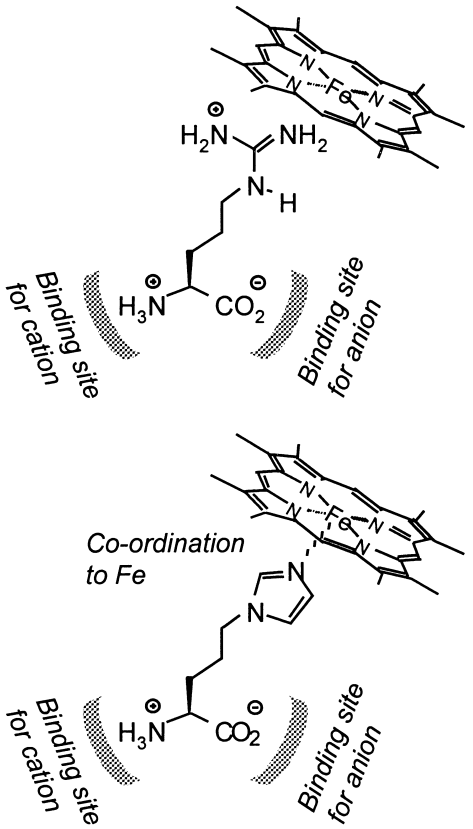


Figure 1. Models for binding of the substrate **1** and the optimum 2-amino-5-azolylpentanoic acid inhibitor **17** to the substrate binding site of NOS.

strength of this coordination would be expected to depend on the electron-density at this nitrogen. In this model, steric crowding would also diminish the interaction of the nitrogen lone-pair of electrons with the iron; this is borne out by the reduction in inhibitory activity when substituents are attached at the 2-position or at the 4-position of the imidazole or at the corresponding 3-position on the 1,2,4-triazol-1-yl compounds.

The origin of the stimulation of enzymic activity by compounds **16**, **27**, and **28** is not clear. However, one may speculate that there may be another binding site, remote from the catalytically active site of the enzymes. If so, then binding of these analogues to this allosteric site may change the protein conformation at the catalytic site, thus increasing the turnover of the natural substrate.

Conclusion

In this paper, we have described our use of the weak NOS inhibitors **10** and **11** as lead compounds to design further 2-amino-5-azolylpentanoic acids which are more potent in their inhibitory activity. Synthetic routes have been developed for the preparation of seven 2-amino-5-(imidazol-1-yl)pentanoic acids, three 2-amino-5-(1,2,4-triazol-1-yl)pentanoic acids and two 2-amino-5-tetrazolylpentanoic acids, using a protected 2-amino-5-bromopentanoic acid **8** as a late common intermediate. Of this series of compounds, 2-amino-5-(imidazol-1-yl)-pentanoic acid **17** was identified as the most potent member against rat iNOS, rat nNOS and a human-derived cNOS. The inhibitory potency of **17** is similar but slightly weaker than those of the known inhibitors NOARG **5a**, NAME **5b**, NIO **6** and thiocitrulline **7**. Examination of the structure–activity relationships for the identity and substitution of the azoles has led to the proposal of a model (Fig. 1) for the binding of the inhibitors to the binding site for the natural substrate **1**. Interestingly, **17** would be predicted to be a weak inhibitor, using a previously reported model[29] for binding, in contrast to its evident potency. The results of our continuing work to use this model for the design of yet more potent NOS inhibitors and to refine the model further will be reported in due course.

Experimental

General methods

NMR spectra were recorded on samples in $CDCl_3$, unless otherwise stated. IR spectra were recorded on KBr discs, unless otherwise stated. Mass spectra were obtained by chemical ionisation (CI) or fast atom bombardment (FAB) techniques in the positive ion mode, unless otherwise stated. The stationary phase for chromatography was silica gel. Melting points are uncorrected. Solutions in organic solvents were dried with $MgSO_4$. Solvents were evaporated under reduced pressure. All chiral amino acids are of L configuration, unless otherwise stated. The brine was saturated. THF refers to tetrahydrofuran, DMF refers to dimethylformamide. Compounds **8–11** were prepared as previously described by us.[19]

1,1-Dimethylethyl *S*-2-(1,1-dimethylethoxycarbonylamino)-5-(2-methylimidazol-1-yl)pentanoate (12). 2-Methylimidazole (376 mg, 3.0 mmol) was stirred with **8**[20] (650 mg, 1.8 mmol) and $NaHCO_3$ (155 mg, 1.8 mmol) in THF (10 mL) at 45 °C for 16 h. Evaporation and chromatography (CH_2Cl_2 /MeOH, 10/1) gave **12** (290 mg, 44%) as a colourless oil: ^{1}H NMR δ 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.61–1.85 (4H, m, 3,4-H_4), 2.38 (3H, s, imidazole-Me), 3.90 (2H, d, J = 7 Hz, 5-H_2), 4.23 (1H, m, 2-H), 5.13 (1H, d, J = 7 Hz, NH), 6.83 (1H, s, imidazole-H), 7.29 (1H, s, imidazole-H); ^{13}C NMR δ 26.34, 28.20, 28.24, 30.02, 46.18, 52.96, 79.85,

82.21, 119.03, 126.84, 130.82, 155.38, 171.29; MS (CI) m/z 354 (M + H), 298, 242.

***S*-2-Amino-5-(2-methylimidazol-1-yl)pentanoic acid (13).** Compound **12** (150 mg, 420 μmol) in THF (5 mL) was saturated with HCl at 0 °C and was stirred at 20 °C for 30° min. Evaporation and chromatography (CH_2Cl_2/MeOH/35% aq NH_3, 10/10/5) gave **13** (25 mg, 30%) as a highly hygroscopic white solid: Found: C, 50.60; H, 7.47; N, 19.80. $C_9H_{15}N_3O_2 \cdot H_2O$ requires C, 50.22; H, 7.96; N, 19.52%; ^{1}H NMR (D_2O) δ 1.60–1.82 (4H, m, 3,4-H_4), 2.26 (3H, s, imidazole-Me), 3.56 (1H, m, 2-H), 3.85 (2H, m, 5-H_2), 6.84 (1H, s, imidazole-H), 6.99 (1H, s, imidazole-H); MS (FAB) m/z 198.1247 (M + H) ($C_8H_{15}N_4O_2$ requires 198.1243).

1,1-Dimethylethyl *S*-2-(1,1-dimethylethoxycarbonylamino)-5-(imidazol-1-yl)pentanoate (14). Imidazole (180 mg, 2.8 mmol) was stirred with **8**[20] (500 mg, 1.4 mmol) in DMF (10 mL) for 2 h at 60°C. Evaporation and chromatography (CH_2Cl_2/MeOH, 19/1) gave **14** (420 mg, 87%) as a yellow viscous oil: ^{1}H NMR δ 1.45 (18H, s, 2×Bu^t), 1.61–1.82 (4H, m, 3,4-H_4), 4.06 (2H, m, 5-H_2), 4.18 (1H, m, 2-H), 5.18 (1H, d, J = 7 Hz, NH), 6.91 (imidazole-H), 7.06 (imidazole-H), 7.48 (imidazole-H); ^{13}C NMR δ 27.70, 27.86, 28.24, 30.04, 46.24, 52.95, 79.87, 86.66, 118.68, 129.54, 137.19, 155.38, 171.28; MS (FAB) m/z 340 (M + H), 284 (M − $Me_2C{=}CH_2$), 228 (M − 2×$Me_2C{=}CH_2$).

1,1-Dimethylethyl 5-(2-cyanoimidazol-1-yl)-2-(1,1-dimethylethoxycarbonylamino)pentanoate (15). Cyanogen bromide (Caution: highly toxic) (313 mg, 2.9 mmol) was added to 4-dimethylaminopyridine (350 mg, 2.9 mmol) in DMF (10 mL) at −10 °C. The mixture was brought to 10 °C during 5 min and **14** (400 mg, 1.2 mmol) was added. The mixture was stirred at 40 °C for 16 h, quenched with aq $NaHCO_3$ (0.1 M, 30 mL) and extracted with EtOAc (3×30 mL). Drying, evaporation and chromatography (CH_2Cl_2/MeOH, 19/1) gave **15** (320 mg, 73%) as a pale-yellow solid: mp 117–119 °C; IR (KBr disc) ν 3400, 3280, 2170, 1725, 1695 cm^{-1}; ^{1}H NMR δ 1.37 (9H, s, Bu^t), 1.39 (9H, s, Bu^t), 1.51–1.90 (4H, m, 3,4-H_4), 4.10 (1H, m, 2-H), 4.19 (2H, m, 5-H_2), 5.18 (1H, d, J = 7.0 Hz, NH), 7.94 (2H, s, imidazole 4,5-H_2); ^{13}C NMR δ 26.58, 27.54, 28.36, 29.82, 46.95, 52.73, 79.80, 82.40, 110.85, 121.57, 131.61, 155.36, 162.10, 170.91; MS (FAB) m/z 365.2191 (M + H) ($C_{18}H_{29}N_4O_4$ requires 365.2189).

***S*-2-Amino-5-(2-cyanoimidazol-1-yl)pentanoate hydrochloride (16).** Compound **15** was treated with HCl, as for the synthesis of **17** except that the solvent was CH_2Cl_2, to give **16** (53%) as a highly hygroscopic white solid: ^{1}H NMR (D_2O) δ 1.76–2.10 (4H, m, 3,4-H_4), 3.71 (1H, t, J = 7 Hz, 2-H), 4.30 (2H, t, J = 6 Hz, 5-H_2), 7.32 (1H, d, J = 3 Hz, imidazole-H), 7.50 (1H, d, J = 3 Hz, imidazole-H); ^{13}C NMR δ 28.51, 30.05, 48.00, 56.73, 113.64, 130.48, 133.96, 176.37, 176.59; MS (FAB) m/z 209.1035 (M + H) ($C_9H_{13}N_4O_2$ requires 209.1039).

***S*-2-Amino-5-(imidazol-1-yl)pentanoic acid dihydrochloride (17).** Compound **14** (520 mg, 1.5 mmol) in THF (25 mL) was cooled to 0 °C and was saturated with HCl. The mixture was stirred at 20 °C for 30 min. The precipitate was collected by filtration under N_2, washed (THF) and dried to give **17** (300 mg, 81%) as a hygroscopic white solid: mp 213–215 °C; ^{1}H NMR (D_2O) δ 1.47 (18H, s, 2×Bu^t), 1.97–2.10 (4H, m, 3,4-H_4), 4.95 (2H, m, 5-H_2), 5.15 (1H, m, 2-H), 7.56 (2H, s, imidazole 4,5-H_2), 7.80 (1H, s, imidazole 2-H); ^{13}C NMR (D_2O) δ 26.21, 29.31, 47.12, 54.23, 127.27, 128.14, 136.23, 175.12; MS (FAB) m/z 184.1059 (M + H), ($C_8H_{14}N_3O_2$ requires 184.1087).

1,1-Dimethylethyl *S*-2-(1,1-dimethylethoxycarbonylamino)-5-(4-nitroimidazol-1-yl)pentanoate (18). 4-Nitroimidazole (381 mg, 3.4 mmol) was stirred with **8**[20] (591 mg, 1.7 mmol) and $NaHCO_3$ (141 mg, 1.7 mmol) in THF (10 mL) at 55 °C for 16 h. Evaporation and chromatography (CH_2Cl_2/MeOH, 19/1) gave **18** (293 mg, 46%) as a colourless oil: ^{1}H NMR δ 1.44 (9 H, s, Bu^t), 1.46 (9 H, s, Bu^t), 1.65–2.04 (4H, m, 3,4-H_4), 4.07–4.22 (3H, m, 2,5-H_3), 5.30 (1H, d, J = 7 Hz, NH), 7.52 (1H, s, imidazole-H), 7.88 (1H, s, imidazole-H); ^{13}C NMR (D_2O) δ 26.79, 27.96, 28.30, 29.66, 47.47, 52.74, 80.08, 82.58, 132.74, 136.11, 148.08, 155.64, 171.06; MS (FAB) m/z 385 (M + H).

***S*-2-Amino-5-(4-nitroimidazol-1-yl)pentanoic acid hydrochloride (19).** Compound **18** was treated with HCl, as for the synthesis of **17**, to give **19** (81%) as a highly hygroscopic white solid: ^{1}H NMR ($(CD_3)_2SO$) δ 1.61–1.98 (4H, m, 3,4-H_4), 3.92 (1H, m, 2-H), 4.14 (2H, t, J = 6 Hz, 5-H_2), 8.32 (1H, s, imidazole-H), 8.40 (3H, br, N^+H_3), 8.45 (1H, s, imidazole-H); ^{13}C NMR ($(CD_3)_2SO$) δ 25.68, 26.58, 46.40, 51.14, 121.40, 137.36, 146.92, 170.75; MS (FAB) m/z 229.0925 (M + H) ($C_8H_{13}N_4O_4$ requires 229.0937).

1,1-Dimethylethyl *S*-2-(1,1-dimethylethoxycarbonylamino)-5-(2-methyl-4-nitroimidazol-1-yl)pentanoate (21). Compound **8**[20] (1.00 g, 2.8 mmol) was stirred with KOBu^t (318 mg, 2.8 mmol) and 2-methyl-4-nitroimidazole (725 mg, 5.7 mmol) in DMF (25 mL) at −3 °C for 20 min and then at 80 °C for 16 h. Evaporation and chromatography (CH_2Cl_2/MeOH, 19/1) gave **21** (380 mg, 34%) as a yellow viscous oil; ^{1}H NMR δ 1.45 (9H, s, Bu^t) 1.46 (9H, s, Bu^t), 1.60–1.93 (4H, m, 3,4-H_4), 2.44 (3H, s, imidazole 2-Me) 3.80–4.15 (2H, m, 5-H_2), 4.25 (1H, m, 2-H), 5.17 (1H, d, J = 7 Hz, NH), 7.73 (1H, s, imidazole 5-H); ^{13}C NMR δ 26.13, 28.31, 28.93, 30.36,

46.29, 52.63, 80.23, 82.77, 119.49, 136.11, 145.54, 155.61, 171.05; MS (FAB) *m/z* 399 (M + H).

***S*-2-Amino-5-(2-methyl-4-nitroimidazol-1-yl)pentanoic acid hydrochloride (22).** Compound **21** was treated with HCl, as for the synthesis of **17**, to give **22** (81%) as a highly hygroscopic white solid: ^{1}H NMR (D_2O) δ 2.10–2.30 (4H, m, 3,4-H_4), 2.51 (3H, s, Me), 4.10–4.26 (2H, m, 5-H_2), 4.36 (1H, t, J = 6 Hz, 2-H), 7.80 (1H, s, imidazole 5-H); MS (FAB) *m/z* 243.1088 (M + H) ($C_9H_{15}N_4O_4$ requires 243.1093).

1,1-Dimethylethyl *S*-2-(1,1-dimethylethoxycarbonylamino)-5-(1,2,4-triazol-1-yl)pentanoate (23) and 1,1-dimethylethyl *S*-2-(1,1-dimethylethoxycarbonylamino)-5-(1,2,4-triazol-4-yl)pentanoate (24). 1,2,4-Triazole (392 mg, 5.7 mmol) was stirred with **8**[20] (1.0 g, 2.8 mmol) and $NaHCO_3$ (239 mg, 2.8 mmol) in DMF (10 mL) at 65 °C for 2 °h. Evaporation and chromatography (CH_2Cl_2 /MeOH, 19/1) gave **23** (700 mg, 72%) as a pale-yellow viscous oil: ^{1}H NMR δ 1.42 (9H, s, Bu^t), 1.43 (9H, s, Bu^t), 1.50–2.00 (4H, m, 3,4-H_4), 4.02–4.28 (3H, m, 2,5-H_3), 5.09 (1H, m, NH), 7.95 (1H, s, triazole 3-H), 8.18 (1H, s, triazole 5-H); ^{13}C NMR δ 25.52, 28.28, 29.65, 29.87, 48.96, 52.95, 79.89, 82.36, 142.82, 151.49, 155.40, 171.22; MS (FAB) *m/z* 341 (M + H), 285 (M − $Me_2C{=}CH_2$), 229 (M − 2 × $Me_2C{=}CH_2$). Further elution gave **24** (17 mg, 2%) as a pale-yellow viscous oil: ^{1}H NMR δ 1.38 (9H, s, Bu^t), 1.39 (9H, s, Bu^t), 1.73–1.97 (4H, m, 3,4-H_4), 4.02–4.28 (3H, m, 2,5-H_3), 5.17 (1H, d, J = 7 Hz, NH), 8.15 (2H, s, triazole 3,5-H_2); ^{13}C NMR δ 26.71, 28.08, 29.27, 29.78, 48.78, 52.60, 80.04, 82.56, 151.89, 155.51, 171.20; MS (FAB) *m/z* 703 (2M + Na), 681 (2M + H), 363 (M + Na), 341 (M + H), 285 (M − $Me_2C{=}CH_2$), 229 (M − 2 × $Me_2C{=}CH_2$).

***S*-2-Amino-5-(1,2,4-triazol-1-yl)pentanoic acid hydrochloride (25).** Compound **23** was treated with HCl, as for the synthesis of **17**, to give **25** (81%) as a hygroscopic white solid: mp 216–218°C; ^{1}H NMR (D_2O) δ 1.90–2.25 (4H, m, 3,4-H_4), 4.12 (1H, t, J = 6 Hz, 2-H), 4.52 (2H, t, J = 6 Hz, 5-H_2), 8.69 (1H, s, triazole 3-H), 9.48 (1H, s, triazole 5-H); ^{13}C NMR (D_2O) δ 25.01, 27.84, 51.25, 53.21, 142.58, 145.90, 172.56; MS (FAB) *m/z* 185.1042 (M + H), ($C_7H_{11}N_4O_2$ requires 185.1039).

1,1-Dimethylethyl *S*-N-(1,1-dimethylethoxycarbonylamino)-5-(3-nitro-1,2,4-triazol-1-yl)pentanoate (26). Compound **8**[20] (700 mg, 2.0 mmol) was stirred with 3-nitro-1,2,4-triazole (342 mg, 4.0 mmol) and $NaHCO_3$ (167 mg, 2.0 mmol) in DMF (10 mL) at 71 °C for 6 h. Evaporation and chromatography (EtOAc/hexane, 1/1) gave **26** (460 mg, 60%) as a colourless oil: ^{1}H NMR δ 1.44 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.61–1.81 (2H, m, 4-H_2), 1.93–2.13 (2H, m, 3-H_2), 4.05–4.10 (1H, m, 2-H), 4.15–4.50 (2H, m, 5-H_2), 5.20 (1H, d, J = 7 Hz, NH), 8.24 (1H, s, triazole 3-H); ^{13}C NMR δ 25.42, 27.96, 28.31, 29.90, 50.46, 52.62, 80.18, 82.77, 144.95, 155.59, 170.98, 171.18; MS (FAB) *m/z* 386.2051 (M + H) ($C_{16}H_{24}N_5O_6$ requires 386.2040).

***S*-2-Amino-5-(3-nitro-1,2,4-triazol-1-yl)pentanoic acid hydrochloride (27).** Compound **26** was treated with HCl, as for the synthesis of **17**, to give **27** (63%) as a highly hygroscopic white solid: ^{1}H NMR (D_2O) δ 1.97–2.25 (4H, m, 3,4-H_4), 4.12 (1H, t, J = 6 Hz, 2-H), 4.48 (2H, t, J = 7 Hz, 5-H_2), 8.67 (1H, s, triazole 2-H); ^{13}C NMR (D_2O) δ 25.34, 27.46, 51.12, 53.32, 147.47, 162.57, 172.67; MS (FAB) *m/z* 230.0888 (M + H) ($C_7H_{12}N_5O_4$ requires 230.0889).

***S*-2-Amino-5-(3-amino-1,2,4-triazol-1-yl)pentanoic acid hydrochloride (28).** Compound **27** (150 mg, 590 μmol) in EtOH (15 mL) was treated with H_2 in the presence of Pd/C (10%, 50 mg) for 2 h. Filtration (Celite®), evaporation and recrystallisation (MeOH/Et_2O) gave **28** (80 mg, 62%) as a highly hygroscopic white solid: ^{1}H NMR (D_2O) δ 2.10–2.31 (4H, m, 3,4-H_4), 3.87 (1H, t, J = 6 Hz, 2-H), 4.28 (2H, t, J = 7 Hz, 5-H_2), 8.28 (1H, s, triazole 2-H); ^{13}C NMR (D_2O) δ 26.10, 28.16, 52.54, 55.21, 145.61, 163.13, 174.32; MS (FAB) *m/z* 200.1141 (M + H) ($C_7H_{12}N_5O_4$ requires 200.1149).

1,1-Dimethylethyl *S*-2-(1,1-dimethylethoxycarbonylamino)-5-(tetrazol-1-yl)pentanoate (29) and 1,1-dimethylethyl *S*-2-(1,1-dimethylethoxycarbonylamino)-5-(tetrazol-2-yl)pentanoate (30). Compound **8**[20] (700 mg, 2.0 mmol) was stirred at 65 °C with tetrazole (348 mg, 5.0 mmol) and $NaHCO_3$ (167 mg, 2.0 mmol) in DMF (10 mL) for 2 h. Evaporation and chromatography (CH_2Cl_2/MeOH, 19/1) gave **30** (370 mg, 54%) as a pale-yellow viscous oil: ^{1}H NMR δ 1.43 (9H, s, Bu^t), 1.44 (9H, s, Bu^t), 1.60 (1H, m) and 1.81 (1H, m) and 2.05 (2H, m) (4H, m, 3,4-H_4), 4.22 (1H, brq, J = 7 Hz, 2-H), 4.60 (2H, t, J = 7.0 Hz, 5-H_2), 5.10 (1H, d, J = 6.7 Hz, NH), 8.50 (1H, s, tetrazole-H); ^{13}C NMR δ 25.46, 28.24, 28.90, 30.16, 52.79, 53.41, 80.24, 82.72, 153.19, 155.66, 171.48; MS (FAB) *m/z* 705 (2M + Na), 683 (2M + H), 364 (M + Na), 342 (M + H), 286 (M − $Me_2C{=}CH_2$), 230 (M − 2 × $Me_2C{=}CH_2$). Further elution gave **29** (280 mg, 41%) as a pale-yellow viscous oil: ^{1}H NMR δ 1.38 (9H, s, Bu^t), 1.39 (9H, s, Bu^t), 1.60 (1H, m) and 1.81 (1H, m) and 2.05 (2H, m) (4H, m, 3,4-H_4), 4.16 (1H, brq, J = 7 Hz, 2-H), 4.42 (1H, dt, J = 13, 6.8 Hz, 5-H), 4.45 (1H, dt, J = 13, 6.8 Hz, 5-H), 5.10 (1H, d, J = 7 Hz, NH), 8.58 (1H, s, tetrazole-H); ^{13}C NMR δ 26.07, 28.26, 28.61, 30.31, 47.72, 52.95, 80.47, 83.05, 142.87, 155.86, 171.28; MS (FAB) *m/z* 342 (M + H), 286 (M − $Me_2C{=}CH_2$), 230 (M − 2 × $Me_2C{=}CH_2$).

***S*-2-Amino-5-(tetrazol-1-yl)pentanoic acid hydrochloride (31).** Compound **29** was treated with HCl, as for the

synthesis of **17**, to give **31** (74%) as a white solid: mp 210–212 °C; ^{1}H NMR (D_2O) δ 1.85–2.10 (2H, m, 4-H_2), 2.19–2.32 (2H, m, 3-H_2), 4.11 (1H, t, J = 7 Hz, 2-H), 4.77–4.87 (2H, m, 5-H_2), 8.81 (1H, s, tetrazole-H). ^{13}C NMR (D_2O) δ 25.12, 27.57, 53.23, 53.26, 153.87, 172.65; MS (FAB) m/z 371 (2M + H), 187.1008 (M + H) ($^{13}C^{12}C_5H_{12}N_5O_2$ requires 187.1026), 186.0932 (M + H) ($C_6H_{12}N_5O_2$ requires 186.0989), 116 (M − tetrazole).

***S*-2-Amino-5-(tetrazol-2-yl)pentanoic acid hydrochloride (32).** Compound **30** was treated with HCl, as for the synthesis of **17**, to give **32** (70%) as a white solid: mp 193–195 °C; ^{1}H NMR (D_2O) δ 1.85–2.32 (4H, m, 3,4-H_4), 4.11 (1H, t, J = 6 Hz, 2-H), 4.77–4.87 (2H, m, 5-H_2), 8.81 (1H, s, tetrazole-H); ^{1}H NMR ($(CD_3)_2SO$) δ 1.60–2.05 (4H, m, 3,4-H_4), 3.94 (1H, brq, J = 5 Hz, 2-H), 4.53 (2H, t, J = 6.6 Hz, 5-H_2), 8.47 (3H, br, N^+H_3), 9.47 (1H, s, tetrazole-H); ^{13}C NMR (D_2O) δ 25.12, 27.57, 53.23, 53.26, 153.87, 172.65; m/z (FAB positive ion) 186.0989 (M + H) ($C_6H_{12}N_5O_2$ requires 186.0989).

NOS inhibition studies

Male Wistar rats (200–300 g), fed ad libitum, were injected intraperitoneally with 4 mg Kg^{-1} CCl_3CO_2H-extracted lipopolysaccharide. After 6 h, the rats were killed. The brain and lungs were removed and used directly or snap frozen in liquid N_2 for subsequent preparation of extracts for measurements of NOS activity.

Pieces of H647 tumour measuring approximately 2 mm^3 from a donor nude mouse were implanted subcutaneously on the lower back of fully anaesthetised recipient nude mice. The recipients were then placed on to a warming plate and covered with Vet Bed to recover. Once recovery was ascertained, the animals were then re-caged and supplied with diet and water, while observing for any ill effects. Tumours were allowed to grow until they reached 600–800 mm^3 in size (ca. eight weeks from implantation). The animals were killed and the tumours were excised and either used directly or snap-frozen in liquid N_2 for subsequent preparation of extracts for measurements of NO synthase activity.

Freshly excised or frozen rat brains/lungs and H647 tumours were homogenised (Ultra-Turrax T25 homogeniser) in four volumes of ice-cold buffer containing HEPES (10 mM, pH 7.4), sucrose (320 mM), EDTA (0.1 mM), dithiothreitol (0.5 mM), leupeptin (10 μg mL^{-1}), soybean trypsin inhibitor (10 μg mL^{-1}) and aprotinin (2 μg mL^{-1}). The preparations were sonicated and then centrifuged using the TLA-100.3 Fixed Angle Rotor in a Beckman TL-100 Tabletop Ultracentrifuge at 15000 rpm for 30 min at 4 °C. The resultant pellet was discarded and the post-mitochondrial supernatant (cytosol + microsomes) was treated with cation exchange resin (Dowex-50W 50X8-400) to remove endogenous **1**. The supernatant was incubated with the resin for 5 min and centrifuged. This process was repeated twice, after which the cytosol was taken to be free of endogenous **1**. Small aliquots in cryotubes were stored at −70 °C until required for protein determination and for the measurement of NOS activity.

For the assays, rat brain extract, rat lung extract or H647 tumour extract (50 μL containing 200 μg protein) was added to 10 mL plastic tubes pre-warmed to 37 °C, containing buffer (pH 7.4, 100 μL) which comprised HEPES (20 mM), L-valine (50 mM), NADPH (125 μM), **3** (100 μM), **1** (10 μM) and L-[*U*-^{14}C]-**1** (50 μCi mL^{-1}), tetrahydrobiopterin (10 μM), calmodulin (400 U mL^{-1}), dithiothreitol (375 mM), bovine serum albumin (75 mg mL^{-1}) and $CaCl_2$ (0.25 mM). Samples were incubated for 10 min at 37 °C in the presence or absence of the candidate inhibitors. The reaction was terminated by removal of substrate through addition of a 50% slurry (1.5 mL) of cation exchange resin (Dowex-50W 50X8-400, Na^+ form) in water. Water (5 mL) was added to the resin-incubate mix, which was left to settle for 20 min. An aliquot (4 mL) of supernatant was removed and the L-[*U*-^{14}C]-**3** was measured by liquid-scintillation counting (Beckman LS380). The activity of the Ca^{2+}-dependent NOS was determined from the difference between the L-[*U*-^{14}C]-**3** generated from samples with and without ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) (1 mM). The results are shown in Table 2 as the mean of triplicate experiments ± standard deviation.

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