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Inhibition of Inducible Nitric Oxide Synthase by Acetamidine Derivatives of Hetero-Substituted Lysine and Homolysine

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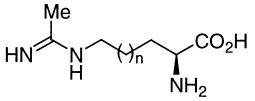
Abstract—The synthesis and in vitro evaluation of the acetamidine derivatives of hetero-substituted lysine and homolysine analogues have identified potent inhibitors of human nitric oxide synthase enzymes, including examples with marked selectivity for the inducible isoform.

There has been extensive scientific interest in the fundamental biochemistry[1] and diverse physiological roles[2] of nitric oxide (NO) since this simple radical gas was first shown to have actions identical to those proposed for endothelium-derived relaxing factor (EDRF).[3] NO is produced by the oxidation of arginine by one of three isoforms of the enzyme nitric oxide synthase (NOS).[4] The calcium-regulated constitutive isoforms, endothelial (eNOS, NOS-3) and neuronal (nNOS, NOS-1) have important roles in, inter alia, regulation of blood pressure and neurotransmission respectively, through production of low-level pulses of NO.[5] Higher, sustained, levels of NO are produced by the calcium-independent inducible isoform (iNOS, NOS-2), which is thought to play a role in host defence mechanisms but is also implicated in a number of diseases such as shock conditions[6] and various inflammatory processes.[7] Despite the identification of various classes of selective iNOS inhibitors, such as amino heterocycles,[8] amidines,[9] and isothioureas,[10] the therapeutic potential of inhibiting this enzyme remains unresolved, largely due to the lack of potent, non-toxic, inhibitors possessing levels of isoform selectivity that preserve the beneficial effects of cNOSs in pathophysiological circumstances.[11]

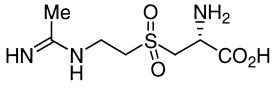
The acetamidine derivatives of L-ornithine (L-*N*-iminoethylornithine, L-NIO)[12] and L-lysine (L-NIL),[13] analogues of the NOS substrate L-arginine, are potent inhibitors of iNOS, with both compounds showing some selectivity over the constitutive isoforms. In this paper we report on the synthesis and SAR of hetero-substituted analogues and homologues of L-NIL, including the sulphone GW273629 and sulphide GW274150, which have similar potency to L-NIL and significantly increased isoform selectivity.

Chemistry

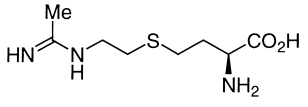
The required orthogonally protected intermediates **1** for the sulphur containing compounds were constructed by



L-NIO n = 1; L-NIL n = 2



GW273629



GW274150

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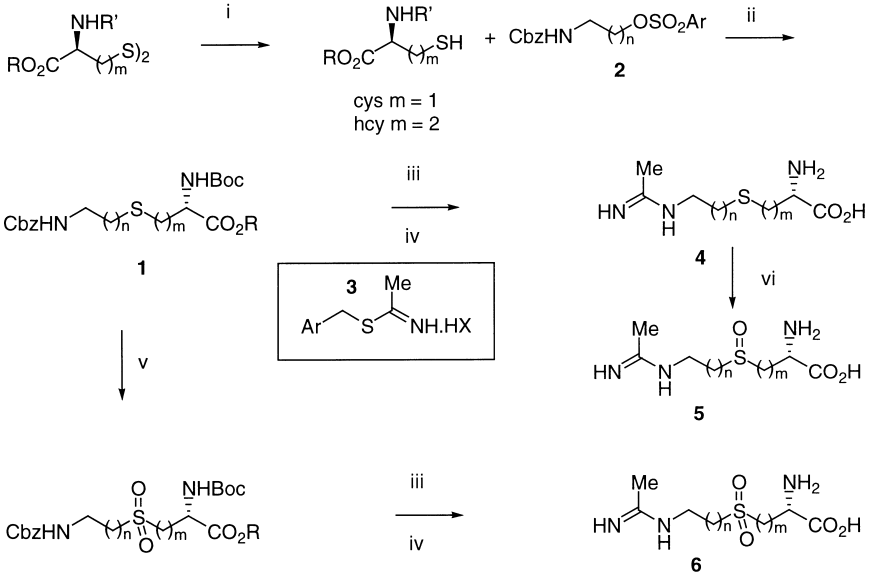
S-alkylation of cysteine or homocysteine derivatives, using aryl sulphonate esters of Cbz ethanolamine or propanolamine **2** (Scheme 1). Appropriate oxidation and/or protecting group manipulation, notably including Cbz removal using transfer hydrogenolysis conditions with the thioethers, produced suitable amine intermediates for further functionalisation. The amines were treated, either as the formate salt or free base, with thioacetimidate reagents **3**[14] to give the acetamidines in essentially quantitative yield, prior to deprotection under acidic conditions to give the target molecules **4**, **5** and **6**.[15] Chiral HPLC/CD analysis confirmed the enantiospecificity of the process in both series - indeed the key 5-thiohomolysine derivative GW274150 was first obtained by separation of a racemate, derived from D/L-homocystine, by preparative chiral HPLC.[16]

The oxygen-containing compounds were synthesised in one of two ways, depending on substitution. Thus homochiral L-4-oxa lysine (**7**, $n=1$) and L-4-oxa homolysine (**7**, $n=2$) intermediates, with appropriate orthogonal protection, were constructed by reaction of aziridine **8**[17] with protected amino alcohols **9** using the literature route,[18] but with modified protection (Scheme 2). The terminal amine was unmasked by hydrogenolysis, to facilitate the regiospecific amidination reaction, prior to deprotection yielding the target compounds **10**.

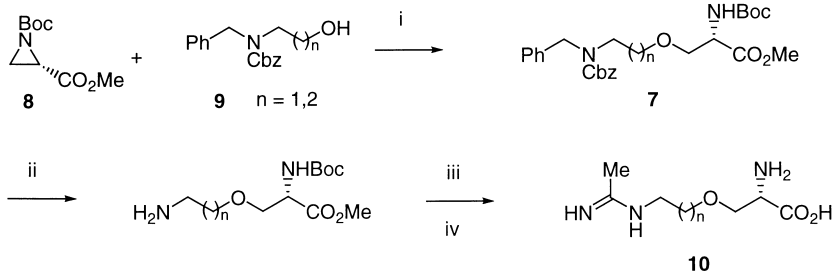
The isomeric 5-oxahomolysine **11** was synthesised in racemic form starting from 2-chloroethyl ether using sequential alkylations with the potassium salts of phthalimide and diethyl phthalimidomalonate (Scheme 3). Following deprotection and decarboxylation, conversion of **11** to the target racemic acetamidine **12** was achieved by reaction with buffered ethyl acetimidate in water, a method latterly superseded by the thioimidate chemistry, vide supra.

Biological Evaluation and Discussion

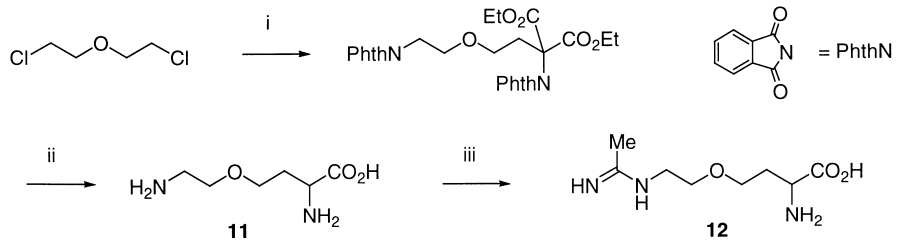
The compounds described were screened to establish iNOS activity and selectivity over the constitutive isoforms using recombinant human NOS enzymes[19] in the oxyhaemoglobin assay.[20] The IC_{50} values are reported



Scheme 1. Synthesis of sulphur-containing compounds. Generic reagents, conditions and typical yields: (i) Na/liquid NH_3 ($R=R'=H$, 90%) or DTT, Et_3N, $CHCl_3$ ($R=t$Bu, $R'=$Boc, 95%); (ii) **2**, in situ reaction ($R=R'=H$), then Boc_2O, NaOH, aq dioxane (80%) or **2**, DBU, PhMe 45 °C ($R=t$Bu, $R'=$Boc, 65%); (iii) HCO_2H, Pd Black, MeOH, Δ, ($R=H$, 85%) or HCO_2NH_4, $Pd(OH)_2$-C, EtOH, Δ ($R=$tBu, 80%); (iv) **3**·HCl or **3**·HBr, EtOH, then HCl, dioxane or HBr AcOH (90%); (v) oxone™ aq MeOH (80%); (vi) H_2O_2, H_2O (40%; 1:1 mixture of sulphoxide diastereomers). N.B. compounds drawn in L-form—racemic homocystine and D-cystine were also employed in this scheme. Final compounds isolated as HBr or HCl salts.



Scheme 2. Synthesis of 4-oxa compounds. Reagents and conditions: (i) BF_3·OEt_2, $CHCl_3$ (40%); (ii) $Pd(OH)_2$-C; HCO_2NH_4, MeOH (95%), Δ; (iii) **3**·HCl, EtOH (90%); (iv) HCl aq MeOH, Δ (90%) to give HCl salt.



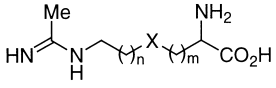
Scheme 3. Synthesis of 5-oxa compound. Reagents: (i) KNPhth, Et_2NH (77%), then $PhthNC(CO_2Et)_2K$, KI, DMF (66%); (ii) 5 N aq NaOH, EtOH, then aq HCl, Δ (34%); MeC(=NH)OEt·HCl, pH 10.5, H_2O (50%).

from measurements taken 15–30 min after the inhibitors were added to the test wells, in order to permit development of the inhibition by these compounds, many of which appear to have time-dependant kinetics.

The SAR shows in Table 1 that the presence of the simple ether or thioether linkage shows a marginal increase in potency in the lysine series ($n=1$, $m=1$) and a more substantial effect in the homologues ($n=1$, $m=2$). The sulphoxides or sulphones are marginally less active in the lysine series and substantially so in the homologues. Where isomers are possible, it is clear that the position of the heteroatom linker is crucial, in that activity is only retained with two methylenes between it and the acetamidine (i.e. $n=1$; but not $n=2$) in all series. Interestingly, whilst not as potent as the natural L-enantiomers, some activity was retained by the homochiral D-isomers we evaluated, which contrasts to the apparent specificity for L-arginine as substrate and L-NMMA as an inhibitor.[21] Determination of the selectivity of these compounds for iNOS versus eNOS and nNOS (Table 2) showed that it was difficult to discern any clear SAR. However, all compounds appeared to show at least low or moderate selectivity for iNOS over both constitutive isoforms, whilst two compounds in particular had high selectivity, the sulphone in the 1,1 series (GW273629) and the sulphide in the 1,2 series (GW274150).

In the interpretation of these results it is difficult to provide rational explanations for either potency or selectivity changes with such conformationally flexible molecules, especially when considering the strong active site homology in the amino acid binding domains

Table 1. IC_{50} values (μM) against human iNOS[a]



Isomer\X	S	O	SO	SO_2	CH_2[b]
$n=1$, $m=1$	0.7 (L) 5.4 (D)	1.2 (L)	8.0 (L)	8.0 (L)	1.6 (L)
$n=1$, $m=2$	1.4 (L) 5.9 (D)	1.4 (DL)	>100 (DL)	>100 (DL)	20.0 (DL)
$n=2$, $m=1$	62.0 (L)	>100 (L)	—	>100 (L)	

[a]Figures shown are averaged figures with an n of at least two separate experiments in all cases, using assay conditions as published,[20] with an arginine concentration of 30 μM.
[b]Comparative figures in our assays using compounds made by the published methods.[13] Data is shown for single enantiomers (L or D) or racemate (DL) as indicated in parentheses; sulphoxides are 1:1 or 1:1:1:1 mixtures of diastereomers.

Table 2. Selectivity data with IC_{50} values (μM) against human NOS isoforms[a,c]

Compound	Isomer	X	Stereo	iNOS	eNOS	e/i	nNOS	n/i
4	$n=1$, $m=1$	S	L	0.7	40	57	13.2	19
ent-4	1,1	S	D	5.4	>100	>18	53	10
5	1,1	SO	L	8.0	155	19	68	9
GW273629	1,1	SO_2	L	8.0	>1000	>125	630	78
10	1,1	O	L	1.2	13.0	11	11.6	10
GW274150	$n=1$, $m=2$	S	L	1.4	466	333	145	104
ent-274150	1,2	S	D	5.9	>100	>17	>100	>17
12	1,2	O	D/L	1.4	72	51	6.0	5
NIL[b]	1,1	CH_2	L	1.6	78	49	37	23
hNIL[b]	2,1 or 1,2	CH_2	D/L	20.0	>100	>5	>100	>5
L-NMMA[b]	$n=0$, $m=1$	CH_2	L	6.6	3.5	0.5	4.9	0.7

[a and b]See equivalent footnotes to Table 1.
[c]Compounds shown in Table 1 but not Table 2 all showed IC_{50}s of >100 μM on eNOS and nNOS.

of iNOS and eNOS.[22] The Asp^{382} to Asn^{368} difference between iNOS and eNOS would seem to be unimportant in imparting selectivity in these molecules,[23] given conservation of the α-amino acid recognition motif. It has also been suggested that imposing conformational rigidity is not a favourable approach to impart selectivity.[24]

Conclusion

We have reported the synthesis and SAR studies that have led to the identification of GW273629 and GW274150, as potent and selective inhibitors of the inducible form of nitric oxide synthase. These compounds, which have very different pharmacokinetic profiles, have been used to help explore the potential role of selective iNOS inhibitors in many disease models, full details of which will be published in due course.[25]

Acknowledgements

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