Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1027–1030

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Thienopyridines: Nitric Oxide Synthase Inhibitors with Potent In Vivo Activity

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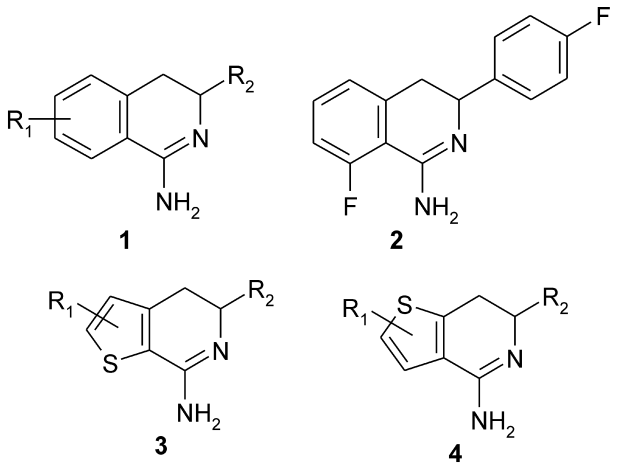
Received 19 December 2000; revised 5 February 2001; accepted 16 February 2001

Abstract—5-Substituted 7-amino-4,5-tetrahydrothieno[2,3-*c*]pyridines and 6-substituted 4-amino-6,7-dihydrothieno[3,2-*c*]pyridines were shown to be exceptionally potent inhibitors of inducible and neuronal nitric oxide synthase. Selectivity and potency could be modulated by variation of the 5- or 6-substituent. Compound **3e** showed potent in vivo inhibition of iNOS.

Nitric oxide synthase is an enzyme that plays a critical and emerging role in many biological processes.[1] Endothelial NOS (eNOS) plays a vital role in the maintenance of vascular homeostasis, neuronal NOS (nNOS) is involved in long-term potentiation and NANC nerve control, and inducible NOS is an immune system regulator and is cytotoxic. Overactivation of nNOS is implicated in neurological diseases such as stroke,[2] while overexpression of iNOS is thought to contribute to inflammatory disease, for example, rheumatoid arthritis.[3] Thus, selective and potent inhibition of either nNOS or iNOS has therapeutic potential.

In the previous paper[4] we showed that 3-substituted-3,4-dihydroisoquinolinamines **1** were nitric oxide synthase inhibitors, and in particular that compound **2** was a highly selective iNOS inhibitor. In a quest for greater potency, we designed new series of compounds **3** and isomeric **4** in which the benzo ring has been replaced by a thieno ring fusion. The rationale for this was the observation that no substitution was allowed on the benzo ring of **1** with the exception of fluorine, which suggests that the active site is sterically constrained in this region. Thus, moving to the smaller ring size should relieve any bad steric interactions that may be present in the six-membered ring.



Chemistry

The compounds were prepared by our previously described lateral lithiation–imine addition/cyclisation reaction (Scheme 1).[5] Using this chemistry a wide variety of aromatic rings could be incorporated adjacent to the amidine by reaction of the appropriate silyl-aldimine with thiophene **5**. In addition, aliphatic groups derived from aldehydes with non-enolisable protons such as cyclopropyl and substituted acetylenes could be synthesised. The same chemistry was utilised for the synthesis of the [3,2] isomers by using protected thiophene **6**.

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0960-894X/01/$ - see front matter
PII: S0960-894X(01)00120-2

The 2-methyl analogue **7** was prepared by lithiation of 2-cyano-3-methylthiophene and cyclisation as before (Scheme 2).

Biology

Compounds were assayed for their ability to inhibit the conversion of [3H]-L-Arg to [3H]-L-citrulline catalysed by: (i) i-NOS from human DLD-1 cells; (ii) n-NOS from rat cerebellum; and (iii) e-NOS from HUVEC cells using minor modifications[6] of methods already described.[7,8]

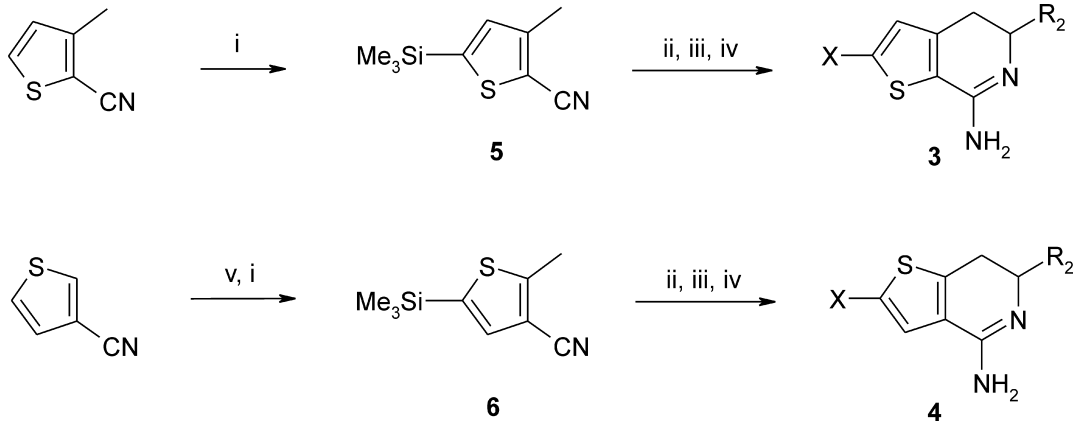
Discussion

The initial compound **3b** with a phenyl ring at the 3-position has moderate potency against all three enzymes. However, dramatic changes occur when this ring is replaced by smaller substituents. For example, bicycles containing five-membered aromatic heterocycles show excellent potency against nNOS in particular. Small aliphatic substituents again result in extremely potent NOS inhibitors. Interestingly, compound **3a** with no 3-substituent is intermediate in activity between phenyl and smaller groups (Table 1).

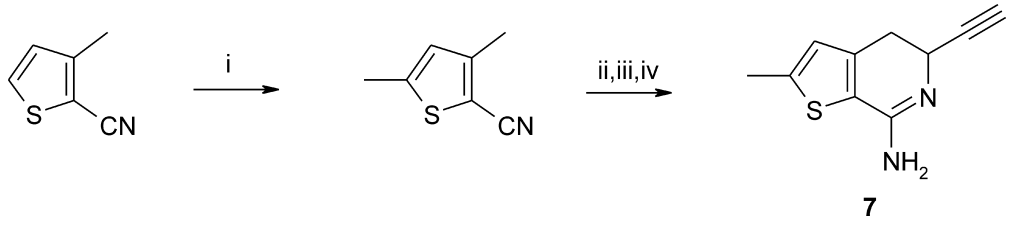
Changing the ring fusion from [2,3] to [3,2] makes little difference to activity, suggesting that there is no interactive role for the thieno ring sulphur (Table 2). Extending the aliphatic group outwards by capping the ethynyl with a methyl group (**3h**) leads to a small loss in potency against iNOS but greater selectivity against eNOS. However, another size constraint seems to be in operation, since addition of a phenyl ring to the ethynyl results in poor activity. Similarly, substitution by a methyl at the 2-position, i.e., on the thieno ring, leads to a ca. 100× drop in activity (compound **7** vs **3g**), underlining the very constricted nature of the active site.

Some of the more potent compounds were examined for their ability to inhibit nitric oxide synthesis in intact DLD-1 cells (Table 3). As noted before,[4] the IC_{50} in these experiments is much higher than in the enzyme assays due in part to the high arginine concentration (~1mM). The compounds show a similar rank order of potency in the intact cell to that seen in the isolated enzyme assays. Compound **3e** is some 80-fold more potent in the cell than the standard inhibitor L-monomethylarginine (L-NMMA, **8**).

Due to good potency in the cell assay, compound **3e** was chosen for further evaluation of its iNOS activity in vivo in a rat LPS model. In this experiment, LPS (0.01 mg/kg) was injected intravenously into anaesthetised rats and compound **3e** was infused for 2 h, between 2 and 4 h after LPS. The inhibition of iNOS was determined from the inhibition of LPS-induced nitrite production measured in blood plasma, taken at 2 and 4 h after LPS, using the Greiss assay. Infusion of compound **3e** resulted in an ED_{50} for nitrite inhibition of 0.4 μmol/kg/15 min (total dose 3.2 μmol/kg). In comparison the compound was some 250-fold more potent than L-NMMA **8** which under the same conditions gave an ED_{50} of 100 μmol/kg/15 min (total dose 800 μmol/kg) (Fig. 1). A similar difference in potency was also found when arterial blood pressure was measured in anaesthetised rats, as a measure of eNOS activity. Compound

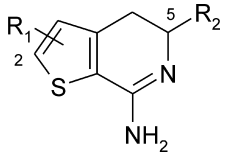


Scheme 1. Reagents and conditions: (i) LDA, THF, −78 °C; TMSCl; (ii) LDA, DMPU, THF, −78 °C; add $R_2CH{=}NTMS$ (prepared from R_2CHO + LiHMDS, THF, 0 °C); (iii) HCl aq; (iv) TBAF, THF; (v) LDA, THF, −78 °C; MeI.



Scheme 2. Reagents and conditions: (i) LDA, THF, −78 °C; MeI; (ii) LDA, DMPU, THF, −78 °C; add TMSC:CCH=NTMS in THF; (iii) HCl aq; (iv) TBAF, THF.

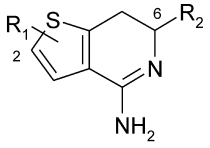
Table 1. Inhibition of NOS enzymes by compound series **3**



	R_1	R_2	iNOS IC_{50} (μM)	nNOS IC_{50} (μM)	eNOS IC_{50} (μM)
3a	H	H	0.73	0.55	0.28
3b	H	Phenyl	8.3	0.14	4.0
3c	H	3-Pyridyl	2.8	0.26	—
3d	H	2-Thienyl	0.15	0.05	1.9
3e	H	2-Furyl	0.089	0.04	0.92
3f	H	Cyclopropyl	0.051	0.011	0.050
3g	H	Ethynyl	0.040	0.006	0.08
3h	H	Propynyl	0.15	0.08	5.0
3i	H	Phenylethynyl	28	4.1	n.s.@100[a]
3j	H	*t*-Butylethynyl	3.6	10	n.s.@100[a]
7	Me	Ethynyl	3.5	0.91	10
	Standard inhibitors:				
8	L-NMMA		0.3	0.1	0.3

[a]Percent inhibition at test concentration n.s. = no significant effect (< 25% inhibition).

Table 2. Inhibition of NOS enzymes by compound series **4**



	R_2	iNOS IC_{50} (μM)	nNOS IC_{50} (μM)	eNOS IC_{50} (μM)
4a	H	1.6	0.1	NT
4b	Phenyl	2.7	0.74	9.5
4c	Ethynyl	0.11	0.025	0.12
4d	Cyclopropyl	0.19	0.018	0.75

Table 3. Inhibitory activity of selected compounds in intact DLD-1 cells

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
8 (L-NMMA)	170	**3g**	5.8
		3h	34
3d	9.2	**4b**	155
3e	2.2	**4c**	15
3f	3.6	**4d**	8.7

3e produced a half maximal increase in mean arterial blood pressure of 40 mmHg at 0.4 μmol/kg/15 min while the dose of L-NMMA required to produce the same effect was 100 μmol/kg/15 min. Thus neither compound **3e** nor L-NMMA are selective over eNOS in vivo.

In conclusion, we have described a series of compounds that have excellent potency against iNOS and nNOS. In vivo compound **3e** is ca. 250-fold more potent than the standard inhibitor L-NMMA. However, selectivity against eNOS is moderate at best, and it does not seem possible to separate nNOS and iNOS activity in this series. This may indicate that the architecture of the active sites of iNOS and nNOS are rather similar, whereas the eNOS site contains enough differences to make the goal of selectivity against this enzyme acheivable. We feel that a more difficult challenge for the design of selective iNOS and nNOS inhibitors will be the separation of activities in these two enzymes. We will continue to communicate our progress towards this goal.

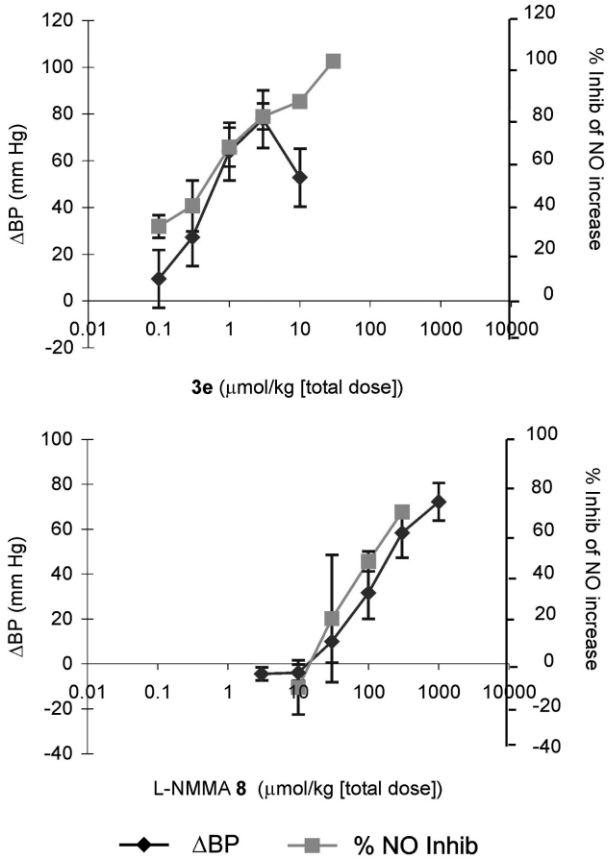


Figure 1. In vivo inhibition of nitrite prodiction in LPS rat by compound **3e** and L-NMMA **8**.

References and Notes

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8. The initial arginine concentration used (3 μM) was chosen as being equal to or lower than measured values of K_m (arginine), which varied from 3 to 15 μM depending on isoform. Assays were performed at 22 °C for 10 min (Type I and III) and 1 h (Type II). Results quoted are a mean of at least two determinations.