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INHIBITION OF NITRIC OXIDE SYNTHASE BY BENZOXAZOLONES[1]

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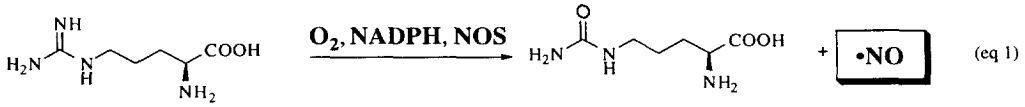
Abstract: A series of benzoxazolones has been synthesized using modifications of literature methods. The synthetic benzoxazolone analogs, along with commercially available analogs, were evaluated as inhibitors of nitric oxide synthases (NOS). Structure-activity relationships are also discussed. © 1997 Elsevier Science Ltd.

The burgeoning importance of nitric oxide (NO), a reactive, inorganic radical gas, as a molecule contributing to a plethora of physiological processes has been revealed by elegant studies from various groups.[2] Several published studies have furnished compelling evidence that links nitric oxide to the pathogenesis of a number of diseases.[3] In mammalian cells, NO is produced (eq 1) by the five-electron oxidation of L-arginine by a P-450 type oxidative enzyme known as nitric oxide synthase (NOS).[4] To date, three isoforms of NOS have been reported. Of these, the neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3) are constitutively expressed and are regulated by Ca^{+2} ion and calmodulin. The third isoform (iNOS or NOS-2), which is induced by exogenous or endogenous inflammatory stimuli, notably endotoxins (LPS) and cytokines (IL-1 etc.), is not regulated by Ca^{+2} ion flux.



(eq 1)

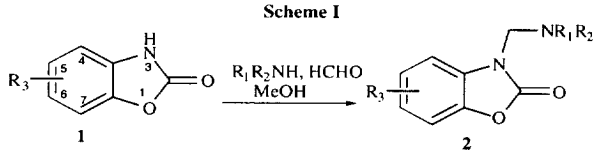
L-Arginine **L-Citrulline** **Nitric Oxide**

The prolonged release of NO mediated by iNOS contributes both to host defense and also to the histopathology associated with acute and chronic inflammation in a wide variety of diseases such as septic shock, inflammatory bowel disease, and arthritis.[5] Thus, selective inhibition of iNOS may offer a new therapy for these diseases. An intense search for selective inhibitors of iNOS has yielded both non-aminoacids based inhibitors, which include aminoguanidines,[6] isothioureas,[7] and amidines,[8] and various aminoacid analogs.[9] We report herein that benzoxazolones are potent inhibitors of the NOS enzymes. A broad based screening of a structurally diverse library initially identified a benzoxazolone (**2a**) with NOS inhibitory activity. We followed up on this observation by establishing a preliminary structure-activity relationship for this class of inhibitors. Initial studies also indicate that these analogs show a modest selectivity for human iNOS over the other isoforms.

Chemistry

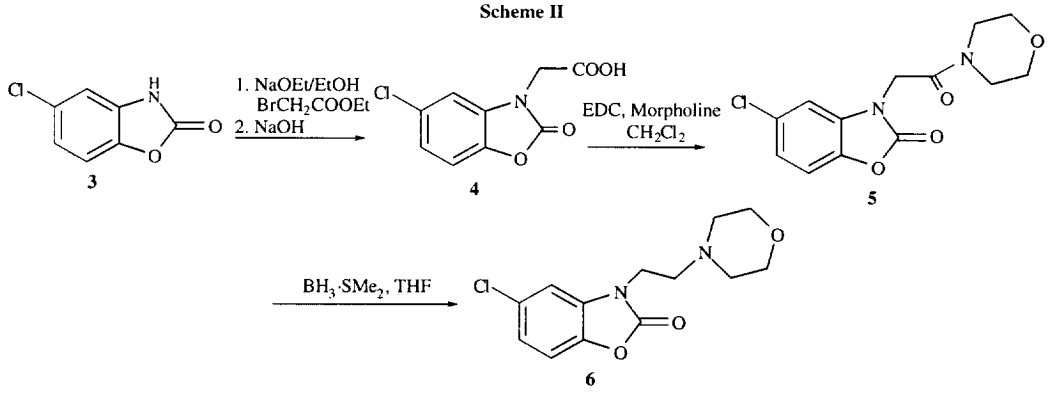
Scheme I shows the preparation of N-substituted benzoxazolones **2**. Mannich reaction[10] of **1** (commercially available) with formalin and secondary amines in methanol at room temperature gave the desired products in good yields.[11,12]

Scheme I



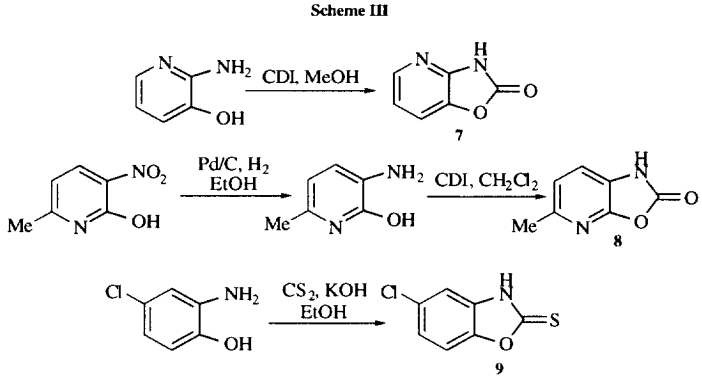
The homologous derivative containing an ethylene spacer, **6**, was synthesized as shown in Scheme II. A two step sequence of reactions involving N-alkylation[13] followed by saponification transformed **3** to the acid (**4**). Conversion of **4** to amide (**5**) followed by reduction using diborane gave **6**.

Scheme II



Scheme III shows the preparation of pyridine analogs **7** and **8**, and benzoxathiazolone **9** from the corresponding aminophenols following published procedures or modifications thereof.[14]

Scheme III



Biological results and Discussion

The compounds synthesized were evaluated for NOS activity by an assay protocol that used recombinant human NOS enzymes.[15,16] In general, the structure-activity profile for the benzoxazolones centered on modification at three critical sites, with the aim of not only increasing the potency but also the selectivity for iNOS. The three critical sites in the following discussion are side chain modification on the oxazolone ring (Table I), substitution on the aryl ring, and finally, the replacement of the heteroatom (Table II). Table I, shows in vitro NOS activity for the benzoxazolones **2** with the side chain modification.

Table I. NOS Inhibition-Effect Of Side Chain Modification on 2 (R_3= 5-Cl)

Compound	R_1	R_2	iNOS IC_{50} (μM)	ecNOS IC_{50} (μM)	nNOS IC_{50} (μM)
2a	$-(CH_2)_2-O-(CH_2)_2-$		0.8	14.7	5.6
2b	$-(CH_2)_2-S-(CH_2)_2-$		3.5	20.3	4.4
2c	$-(CH_2)_5-$		1.5	18.9	4.6
2d	$-(CH_2)_2-N(Me)-(CH_2)_2-$		1.2	15.5	4.4
2e	Me	CH_2Ph	13.4	13.6	11.5
2f	Me	2-Py	74%[a]	8.4	71%[a]
2g	Me	$(CH_2)_2Ph$	15.9	15.9	68
6	—		0%	ND	ND

ND = Not Determined, a = Inhibition at 50 μM

It is clear from Table I that compound **2a**, bearing the morpholino residue, displayed the best activity and some selectivity. By contrast, the thiomorpholine analog **2b** was less active, which indicates that the larger size of the ring may be detrimental to the overall activity. The fact that both **2c** and **2d** have similar potency to **2a** lends additional support to the importance of size of the substituent. The compounds bearing acyclic residues as exemplified by structures **2e**, **2f**, and **2g** showed loss of activity thus underscoring the importance of both the size as well as the shape of the auxiliary unit attached to the benzoxazolone template. The sensitivity of the *enzymes* to these inhibitors is best illustrated by compound **6**, which bears a two carbon linker between the morpholino and benzoxazolone nitrogen and was totally inactive. Perhaps, an explanation as to why compound such as **6** is inactive may lie in the fact that the derivatives of **2** might be converted to the parent benzoxazolone **1** during the assay (by a retro-Mannich reaction), an event impossible for **6**. Thus the difference in activity of analogs **2** is a reflection of the different rate at which the parent benoxazolone **1** is formed. In order to test this hypothesis and other related issues we evaluated the parent benzoxazolone **1** and its derivatives for NOS activity and these results are displayed in Table II.

Table I I. NOS Inhibition-Effect Of Substitution on 1

Compound	R_3	iNOS IC_{50} (μM)	ecNOS IC_{50} (μM)	nNOS IC_{50} (μM)
3	—	14.1	8.7	50
1a	5-F	>50	>50	>50
1b	5-CN	14.5	50	50
1c	5-Me	9.9	10.6	50
1d	4-Me	>50	>50	>50
1e	6-Me	>50	>50	13.1
7	—	>50	ND	ND
8	—	7.4	>50	50
9	5-Cl	>50	>50	>50

ND = Not Determined

Table II indicates that, among the substituted benzoxazolone analogs, the methyl analog **1c** was the best, followed by the 5-chloro derivative **3**. Results for the regioisomeric methyl analogs (**1c-1e**) revealed that the 5-Me (**1c**) isomer was the best for NOS activity. Replacement of the aryl ring by the pyridine ring system dramatically reduces the overall activity. Although **7** was inactive, **8** had modest activity and also displayed better selectivity for the iNOS enzyme. Finally, departure from **3** to oxathiazolone **9** led to loss of all NOS activity. It is obvious from these data that the retro-Mannich reactions of derivatives of **2** during the assay is not the reason for NOS activity of these compounds simply because the parent benzoxazolones are less active than **2** (compare **2a**, **2b**, **2c**, **2d**, with **3**)[17]. A detailed study from these laboratories has indicated that **3** is a type I inhibitor and it competes with L-arginine at the active site of the all three NOS isoforms[18]. By contrast it was recently pointed out that **3** was a substrate for one of the mammalian cytochrome P-450[19] (CYP2E1) isozymes.

Results disclosed herein for NOS inhibition by substituted benzoxazolones (Tables I and II) indicates modest selectivity for the i-NOS isozymes and should be contrasted from 7-nitro indazole,[20] which has been shown to be selective for the nNOS isozyme.

In conclusion, we have reported the syntheses and evaluation of a number of benzoxazolones and have shown that they constitute a novel class of non-amino acid based NOS inhibitors.

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References and Notes

1. Preliminary results of this work were presented at the 212th ACS national meeting, Orlando, FL, 1996 (Abstract # 183).

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11. When the reaction was complete (12-24 h) the product precipitated out and was simply filtered, dried and used for the biological studies.

12. All new compounds were characterized by full (NMR, MS, IR) spectroscopic data.

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16. The NOS inhibitory activity of the compound was determined by comparing the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline in presence of the inhibitor with control. The assay mixture (pH 7.5) containing 1 μM of ^{3}H-L-Arginine, cofactors and the inhibitor or aq. DMSO (control) was incubated for 30 min at room temperature. The reaction was quenched by adding a slurry of Dowex 50W-X8 resin to complex and remove the unreacted substrate. The concetration of the ^{3}H-L-citrulline product in the supernant fluid was determined on a scintillation counter. For each inhibitor, the percentage inhibition was determined at 10 different concentrations. Each observation was performed in duplicate and the replicate values typically varied < 20%. IC_{50} values were determined from the average of the duplicate observations using SIGMAPLOT. Under the assay conditions the production of L-citrulline was linear with time for the duration of the experiment.

17. In a separate experiment the compound **2a** was dissolved in dimethyl-d_6-sulfoxide and its NMR spectra recorded over the period of time indicated absence retro-Mannich product (**3**).

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